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(74) Agents: HAGER, Alicia, J. et al.; Morrison & Foerster
LLP, 755 Page Mill Road, Palo Alto, CA 94304-1018 (US).

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(71) Applicant (for all designated States except US): CERUS CORPORATION [US/US]; 2411 Stanwell Drive, Concord, CA 94520 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): DUBENSKY, Thomas, W., Jr. [US/US]; 15 King Avenue, Piedmont, CA 94611 (US). SKOBLE, Justin [US/US]; 2423 Roosevelt Avenue, Berkeley, CA 94703 (US). LAUER, Peter, M. [US/US]; 1003 Solano Avenue, Albany, CA 94706 (US). COOK, David, N. [US/US]; 1975 Marion Court, Lafayette, CA 94549 (US).

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(54) Title: ENGINEERED LISTERIA AND METHODS OF USE THEREOF

(57) Abstract: The invention provides a bacterium containing a polynucleotide comprising a nucleic acid encoding a heterologous antigen, as well as fusion protein partners. Also provided are vectors for mediating site-specific recombination and vectors comprising removable antibiotic resistance genes.



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ENGINEERED LISTERIA AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application No. 60/788,558, filed on March 30, 2006, U.S. Provisional Application No. 60/784,915, filed on March 21, 2006, and U.S. Provisional Application No. 60/778,471, filed on March 1, 2006, the contents of each of which are hereby incorporated by reference herein in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made, in part, with U.S. government support under National Cancer Institute NHI 1 K23CA104160-01. The government may have certain rights in the invention.

FIELD OF THE INVENTION

[0003] The invention provides engineered *Listeria* bacteria, useful for stimulating the immune system and treating cancers and infections. Also provided are polynucleotides, fusion protein partners, and integration vectors useful for modifying *Listeria* and other bacterial species.

BACKGROUND OF THE INVENTION

[0004] Cancers and infections can be treated by administering reagents that modulate the immune system. These reagents include vaccines, cytokines, antibodies, and small molecules, such as CpG oligodeoxynucleotides and imidazoquinolines (see, e.g., Becker (2005) *Virus Genes* 30:251-266; Schetter and Vollmer (2004) *Curr. Opin. Drug Devel.* 7:204-210; Majewski, *et al.* (2005) *Int. J. Dermatol.* 44:14-19), Hofmann, *et al.* (2005) *J. Clin. Virol.* 32:86-91; Huber, *et al.* (2005) *Infection* 33:25-29; Carter (2001) *Nature Revs. Cancer* 1:118-129; Dechant and Valaerius (2001) *Crit. Revs. Oncol.* 39:69-77; O'Connor, *et al.* (2004) *Neurology* 62:2038-2043). Vaccines, including classical vaccines (inactivated whole organisms, extracts, or antigens), dendritic cell (DC) vaccines, and nucleic acid-based vaccines, are all useful for treating cancers and infections (see, e.g., Robinson and Amara (2005) *Nat. Med. Suppl.* 11:S25-S32; Plotkin (2005) *Nat. Med. Suppl.* 11:S5-S11; Pashine, *et al.* (2005) *Nat. Med. Suppl.* 11:S63-S68; Larche and Wraith (2005) *Nat. Med. Suppl.* 11:S69-S76). Another reagent useful for modulating the immune system is *Listeria monocytogenes*.

(*L. monocytogenes*), and this reagent has proven to be successful in treating cancers and tumors (see, e.g., Brockstedt, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:13832-13837; Brockstedt, *et al.* (2005) *Nat. Med.* 11:853-860); Starks, *et al.* (2004) *J. Immunol.* 173:420-427; Shen, *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:3987-3991).

[0005] Recombinant *Listeria* strains have been developed as vaccines against viruses and tumors (see, e.g., Starks, *et al.* (2004) *J. Immunol.* 173:420-427; Gunn, *et al.* (2001) *J. Immunol.* 167:6471-6479; Ikonomidis, *et al.* (1994) *J. Exp. Med.* 180:2209-2218; Mata, *et al.* (2001) *Vaccine* 19:1435-1445; Mata and Paterson (1999) *J. Immunol.* 163:1449-1456; Mata, *et al.* (1998) *J. Immunol.* 161:2985-2993; Friedman, *et al.* (2000) *J. Virol.* 74:9987-9993; Soussi, *et al.* (2002) *Vaccine* 20:2702-2712; Saklani-Jusforgues, *et al.* (2003) *Infect. Immun.* 71:1083-1090; Soussi, *et al.* (2000) *Infect. Immunity* 68:1498-1506; Tvinnereim, *et al.* (2002) *Infect. Immunity* 70:153-162; Rayevskaya, *et al.* (2002) *J. Virol.* 76:918-922; Frankel, *et al.* (1995) *J. Immunol.* 55:4775-4782; Jensen, *et al.* (1997) *J. Virol.* 71:8467-8474; Jensen, *et al.* (1997) *Immunol. Rev.* 158:147-157; Lin, *et al.* (2002) *Int. J. Cancer* 102:629-637; Peters, *et al.* (2003) *FEMS Immunol. Med. Microbiol.* 35:243-253; Peters, *et al.* (2003) *J. Immunol.* 170:5176-5187; Paterson (2003) *Immunol. Res.* 27:451-462; Paterson and Johnson (2004) *Expert Rev. Vaccines* 3:S119-S134; Ochsenbein, *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:9293-9298; Hess, *et al.* (2000) *Adv. Immunol.* 75:1-88).

[0006] *L. monocytogenes* has a natural tropism for the liver and spleen and, to some extent, other tissues such as the small intestines (see, e.g., Dussurget, *et al.* (2004) *Ann. Rev. Microbiol.* 58:587-610; Gouin, *et al.* (2005) *Curr. Opin. Microbiol.* 8:35-45; Cossart (2002) *Int. J. Med. Microbiol.* 291:401-409; Vazquez-Boland, *et al.* (2001) *Clin. Microbiol. Rev.* 14:584-640; Schluter, *et al.* (1999) *Immunobiol.* 201:188-195). Where the bacterium resides in the intestines, passage to the bloodstream is mediated by listerial proteins, such as ActA and internalin A (see, e.g., Manohar, *et al.* (2001) *Infection Immunity* 69:3542-3549; Lecuit, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:6152-6157; Lecuit and Cossart (2002) *Trends Mol. Med.* 8:537-542). Once the bacterium enters a host cell, the life cycle of *L. monocytogenes* involves escape from the phagolysosome and to the cytosol. This life cycle contrasts with that of *Mycobacterium*, which remains inside the phagolysosome (see, e.g., Clemens, *et al.* (2002) *Infection Immunity* 70:5800-5807; Schluter, *et al.* (1998) *Infect. Immunity* 66:5930-5938; Gutierrez, *et al.* (2004) *Cell* 119:753-766). *L. monocytogenes*' escape from the phagolysosome is mediated by listerial proteins, such as listeriolysin (LLO), PI-PLC, and PC-PLC (see Portnoy, *et al.* (2002) *J. Cell Biol.* 158:409-414).

[0007] Vaccines for treating cancers or infections are often ineffective because of a lack of appropriate reagents. The present invention fulfills this need by providing polynucleotides, fusion protein partners, plasmids and bacterial vaccines, useful for enhancing the expression or immune processing of antigens, and for increasing survival to cancers and infections.

SUMMARY OF THE INVENTION

[0008] The present invention is based, in part, on the recognition that administering an attenuated *Listeria* to a mammal bearing a tumor results in enhanced survival, where the *Listeria* was engineered to contain a nucleic acid encoding an ActA-based fusion protein linked to a tumor antigen.

[0009] In one aspect, the invention provides a polynucleotide comprising a promoter operably linked to a nucleic acid sequence encoding a fusion protein, wherein the fusion protein comprises (a) modified ActA and (b) a heterologous antigen. In some embodiments, the promoter is a bacterial promoter (e.g., a *Listerial* promoter). In some embodiments, the promoter is an ActA promoter. In some embodiments, the modified ActA comprises at least the first 59 amino acids of ActA. In some embodiments, the modified ActA comprises more than the first 59 amino acids of ActA. In some embodiments, the modified ActA comprises less than the first 380 amino acids or less than the first 265 amino acids. In some embodiments, the modified ActA comprises more than the first 59 amino acids of ActA, and less than the first 380 amino acids of ActA. For example, in some embodiments, the modified ActA comprises at least about the first 59 amino acids of ActA, but less than about the first 265 amino acids of ActA. In some embodiments, the modified ActA comprises more than about the first 59 amino acids of ActA, but less than about the first 265 amino acids of ActA. In other embodiments, the modified ActA comprises more than the first 59 amino acids of ActA, and less than the first 380 amino acids of ActA. In still further embodiments, the modified ActA comprises at least the first 85 amino acids of ActA and less than the first 125 amino acids of ActA. In some embodiments, the modified ActA comprises amino acids 1-100 of ActA. In some embodiments, the modified ActA consists of amino acids 1-100 of ActA. The heterologous antigen may be non-*Listerial*. In some embodiments, the heterologous antigen is from, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is immunologically cross-reactive with, or shares at least one epitope with, the cancer, tumor, or infectious agent. In some embodiments, the heterologous antigen is a tumor antigen or is derived from a tumor antigen. In some embodiments, the heterologous antigen is, or is derived from, mesothelin. For

example, in some embodiments, the heterologous antigen is, or is derived from, human mesothelin. In some embodiments, the *Listeria* is hMeso26 or hMeso38 (see Table 11 of Example VII, below). In some embodiments, the heterologous antigen comprises an EphA2 antigenic peptide. In some embodiments, the heterologous antigen does not comprise an EphA2 antigenic peptide. In some embodiments, the nucleic acid sequence encoding the fusion protein is codon-optimized for expression in *Listeria*. The invention provides plasmids and cells comprising the polynucleotide. The invention further provides a *Listeria* bacterium (e.g., *Listeria monocytogenes*) comprising the polynucleotide, as well as vaccines comprising the *Listeria*. The *Listeria* bacterium may be attenuated (e.g., an *actA* deletion mutant or an *actA* insertion mutant). In certain embodiments, the bacterium may comprise an attenuating mutation in *actA* and/or *inlB*. In some embodiments, the *Listeria* comprises the polynucleotide in its genome. In some embodiments, the polynucleotide has been integrated into a virulence gene in the *Listerial* genome. In some embodiments, a polynucleotide (or nucleic acid) has been integrated into a virulence gene in the genome of the *Listeria*, wherein the integration of the polynucleotide (a) disrupts expression of the virulence gene and/or (b) disrupts a coding sequence of the virulence gene. In some embodiments, the virulence gene is *prfA*-dependent. In other embodiments, the virulence gene is *prfA*-independent. In some embodiments, the nucleic acid or the polynucleotide has been integrated into the genome of the *Listeria* at the *actA* locus and/or *inlB* locus. In some embodiments, the *Listeria* comprises a plasmid comprising the polynucleotide. The invention further provides immunogenic and pharmaceutical compositions comprising the *Listeria*. The invention also provides methods for stimulating immune responses to the heterologous antigen in a mammal (e.g., a human), comprising administering an effective amount of the *Listeria* (or an effective amount of a composition comprising the *Listeria*) to the mammal. For instance, the invention also provides methods for stimulating immune responses to an antigen from, or derived from, a cancer or infectious agent, comprising administering an effective amount of the *Listeria* (or a composition comprising the *Listeria*) to a mammal having the cancer or infectious agent, wherein the heterologous antigen shares at least one epitope with or is immunologically cross-reactive with the antigen from, or derived from, the cancer or infectious agent. In some embodiments, inclusion of the modified Act A sequence in the fusion protein enhances the immunogenicity of the *Listeria* comprising the polynucleotide (e.g., relative to the immunogenicity of *Listeria* comprising a polynucleotide encoding a fusion protein comprising the heterologous antigen and a non-ActA signal sequence and/or leader sequence, instead of the modified ActA). In some embodiments, inclusion of the modified Act A

sequence in the fusion protein enhances expression and/or secretion of the heterologous antigen in *Listeria* (e.g., relative to the expression and/or secretion in *Listeria* of the heterologous antigen fused to a non-ActA signal sequence and/or leader sequence instead of the modified ActA).

[0010] In another aspect, the invention provides a polynucleotide comprising a first nucleic acid encoding a modified ActA (e.g., actA-N-100), operably linked and in frame with, a second nucleic acid encoding a heterologous antigen. In some embodiments, the modified ActA comprises at least the first 59 amino acids of ActA, but less than about the first 265 amino acids of ActA. In some embodiments, the modified ActA comprises more than the first 59 amino acids of ActA, but less than about the first 265 amino acids of ActA. In some embodiments, the first nucleic acid encodes amino acids 1-100 of ActA. In some embodiments, the polynucleotide is genomic. For instance, the polynucleotide may be integrated into the *actA* or *inlB* gene. In some alternative embodiments, the polynucleotide is plasmid-based. In some embodiments, the polynucleotide is operably linked with one or more of the following: (a) *actA* promoter; or (b) a bacterial promoter that is not *actA* promoter. In some embodiments, the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is immunologically cross-reactive with, or shares at least one epitope with, the cancer, tumor, or infectious agent. In some embodiments, the heterologous antigen is, or is derived from, mesothelin (e.g., human mesothelin). The invention further provides a *Listeria* bacterium e.g., *Listeria monocytogenes*) comprising the polynucleotide, as well as vaccines comprising the *Listeria*. In some embodiments, the *Listeria* is hMeso26 or hMeso38 (see Table 11 of Example VII, below). The invention also provides methods for stimulating immune responses to an antigen from, or derived from, a cancer (e.g., a tumor or pre-cancerous cell) or infectious agent (e.g., a virus, pathogenic bacterium, or parasitic organism), comprising administering the *Listeria* to a mammal having the cancer or infectious agent, wherein the heterologous antigen shares at least one epitope with or is immunologically cross-reactive with the antigen from, or derived from, the cancer or infectious agent. In some embodiments of the methods, the stimulating is relative to immune response without administering the *Listeria*. In some embodiments of the methods, the heterologous antigen is from, or is derived from, the cancer cell, tumor, or infectious agent.

[0011] In another aspect, the invention provides a polynucleotide comprising a first nucleic acid encoding a modified actA, wherein the modified actA comprises (a) amino acids 1-59 of actA, (b) an inactivating mutation in, deletion of, or truncation prior to, at least one domain

for actA-mediated regulation of the host cell cytoskeleton, wherein the first nucleic acid is operably linked and in frame with a second nucleic acid encoding a heterologous antigen. In some embodiments the modified ActA comprises more than the first 59 amino acids of ActA. In some embodiments, the domain is the cofilin homology region (KKRR (SEQ ID NO:23)). In some embodiments, the domain is the phospholipid core binding domain (KVFKKIKDAGKWVRDKI (SEQ ID NO:20)). In some embodiments, the at least one domain comprises all four proline-rich domains (FPPPP (SEQ ID NO:21), FPPPP (SEQ ID NO:21), FPPPP (SEQ ID NO:21), FPIIP (SEQ ID NO:22)) of ActA. In some embodiments, the modified actA is actA-N100. In some embodiments, the polynucleotide is genomic. In some embodiments, the polynucleotide is not genomic. In some embodiments, the polynucleotide is operably linked with one or more of the following: (a) actA promoter; or (b) a bacterial (e.g., listerial) promoter that is not actA promoter. The invention further provides a *Listeria* bacterium (e.g., *Listeria monocytogenes*) comprising the polynucleotide, as well as vaccines comprising the *Listeria*. In some embodiments, the *Listeria* comprises an attenuating mutation in *actA* and/or *inlB*. In some embodiments, the *Listeria* is hMeso26 or hMeso38 (see Table 11 of Example VII, below). The invention also provides methods for stimulating immune responses to an antigen from, or derived from, a cancer or infectious agent, comprising administering the *Listeria* to a mammal having the cancer or infectious agent, wherein the heterologous antigen shares at least one epitope with or is immunologically cross-reactive with the antigen from, or derived from, the cancer or infectious agent. In some embodiments, the stimulating is relative to immune response without administering the *Listeria*. In some embodiments, the cancer comprises a tumor or pre-cancerous cell. In some embodiments, the infectious agent comprises a virus, pathogenic bacterium, or parasitic organism. In some embodiments, the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is immunologically cross-reactive with, or shares at least one epitope with, the cancer, tumor, or infectious agent. In some embodiments, the heterologous antigen is, or is derived from, mesothelin. For instance, in some embodiments, the heterologous antigen is, or is derived from, human mesothelin. In some embodiments, inclusion of the modified Act A sequence in the polynucleotide enhances expression and/or secretion of the heterologous antigen in *Listeria*. In some embodiments, inclusion of the modified Act A sequence in the polynucleotide enhances the immunogenicity of vaccine compositions comprising the *Listeria*.

[0012] In still another aspect, the invention provides a plasmid comprising a first nucleic acid encoding a phage integrase, a second nucleic acid encoding a phage attachment site (attPP' site), and a third nucleic acid encoding a heterologous antigen or regulatory nucleic acid, wherein the plasmid is useful for mediating site-specific integration of the nucleic acid encoding the heterologous antigen at a bacterial attachment site (attBB' site) in a bacterial genome that is compatible with the attPP' site of the plasmid. In some embodiments, each of the nucleic acids is derivable from *L. innocua* 0071, each of the nucleic acids is derivable from *L. innocua* 1765, each of the nucleic acids is derivable from *L. innocua* 2601, or each of the nucleic acids is derivable from *L. monocytogenes* f6854_2703. In some embodiments, the first nucleic acid encodes a phiC31 integrase. In some embodiments, the plasmid is the polynucleotide sequence of pINT; or a polynucleotide hybridizable under stringent conditions to a polynucleotide encoding pINT, wherein the polynucleotide that is hybridizable is capable of mediating site specific integration at the same bacterial attachment site (attBB') in a bacterial genome as that used by pINT. In some embodiments, the bacterial genome is of a *Listeria*, *Bacillus anthracis*, or *Francisella tularensis*. In some embodiments, the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the regulatory nucleic acid is a bacterial attachment site (attBB'). In some embodiments, the plasmid further comprises a fourth nucleic acid encoding a first lox site, a fifth nucleic acid encoding a second lox site, and a sixth nucleic acid encoding a selection marker, wherein the first lox site and second lox site are operably linked with the sixth nucleic acid, and wherein the operably linked lox sites are useful for mediating Cre recombinase catalyzed excision of the sixth nucleic acid. In some embodiments, the first lox site is a loxP site and the second lox site is a loxP site. In some embodiments, the plasmid further comprises a non compatible bacterial attachment site (attBB'), wherein the non compatible attBB' site is not compatible with the phage attachment site (attPP'). In some embodiments, the plasmid further comprises a first promoter operably linked with the first nucleic acid, and a second promoter operably linked with the third nucleic acid. The invention further provides a method of modifying a bacterial genome, comprising transfecting the bacterium with the plasmid, and allowing integrase-catalyzed integration of the third nucleic acid into the bacterial genome under conditions suitable for integration. In some embodiments of the method, the bacterium is *Listeria*, *Bacillus anthracis*, or *Francisella tularensis*.

[0013] The invention further provides a plasmid comprising: (a) a first nucleic acid encoding a first region of homology to a bacterial genome, (b) a second nucleic acid

encoding a second region of homology to the bacterial genome, and (c) a third nucleic acid comprising a bacterial attachment site (attBB'), wherein the third nucleic acid is flanked by the first and second nucleic acids, wherein the first nucleic acid and second nucleic acid are operably linked with each other and able to mediate homologous integration of the third nucleic acid into the bacterial genome. In some embodiments, the bacterial attachment site (attBB') comprises the attBB' of: *listerial* tRNAArg-attBB'; *listerial* comK attBB'; *Listeria innocua* 0071; *Listeria innocua* 1231; *Listeria innocua* 1765; *Listeria innocua* 2610; or *Listeria monocytogenes* f6854_2703; or phiC31. In some embodiments, the genome is of a *Listeria*, *Bacillus anthracis*, or *Francisella tularensis*. In some embodiments, the third nucleic acid encodes a selection marker flanked by a first lox site and a second lox site, wherein the lox sites are recognized as substrates by Cre recombinase and allow Cre recombinase catalyzed excision of the third nucleic acid, and wherein the selection marker is useful for detecting integration of the third nucleic acid into the bacterial genome. In some embodiments, the first lox site is a loxP site, and the second lox site is a loxP site. In some embodiments, the third nucleic acid comprises an antibiotic resistance gene. In some embodiments, the first nucleic acid is homologous to a first region of a virulence factor gene and the second nucleic acid is homologous to a second region of the virulence factor gene, wherein the first and second regions of the virulence factor gene are distinct from each other and do not overlap each other. In some embodiments, the first region of the virulence factor gene covalently contacts or abuts the second region of the virulence factor gene. In other embodiments, the first region of the virulence factor gene is not in covalent contact with, and does not covalently about, the second region of the virulence factor gene. The invention further provides bacteria modified by integration of the plasmid. In some embodiments, the integration is in a region of the genome that is necessary for mediating growth or spread. In other embodiments, the integration is in a region of the genome that is not necessary for mediating growth or spread.

[0014] In yet another aspect, the invention provides a bacterium wherein the genome comprises a polynucleotide containing two operably linked heterologous recombinase binding sites flanking a first nucleic acid, wherein the two sites are: (a) two lox sites; or (b) two Frt sites, and wherein the nucleic acid flanked by the two lox sites is excisable by Cre recombinase, and wherein the nucleic acid flanked by the two Frt sites is excisable by FLP recombinase. In some embodiments, the two lox sites are both loxP sites. In some embodiments, the first nucleic acid encodes a selection marker or a heterologous antigen. In some embodiments, the first nucleic acid encodes an antibiotic resistance gene. In some

embodiments, the bacterium is *Listeria*, *Bacillus anthracis*, or *Francisella tularensis*. In some embodiments, the polynucleotide further comprises a second nucleic acid, wherein the second nucleic acid is not flanked by, and is not operably linked with, the first and second heterologous recombinase binding site. In some embodiments, the second nucleic acid encodes one or both of: heterologous antigen; or a bacterial attachment site (attBB'). In some embodiments, the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent. The invention further provides a method of excising the first nucleic acid from the bacterial genome, comprising contacting the genome with Cre recombinase or FLP recombinase, and allowing the recombinase to catalyze excision of the first nucleic acid, under conditions allowing or facilitating excision: (a) wherein the first nucleic acid is flanked by lox sites and the recombinase is Cre recombinase; or (b) wherein the first nucleic acid is flanked by Frt sites and the recombinase is FLP recombinase. In some embodiments, the recombinase is transiently expressed in the bacterium.

[0015] In another aspect, the invention provides *Listeria* (e.g., *Listeria monocytogenes*) in which the genome comprises a polynucleotide comprising a nucleic acid encoding a heterologous antigen. In some embodiments, the nucleic acid encoding the heterologous antigen has been integrated into the genome by site-specific recombination or homologous recombination. In some embodiments, the site of integration into the genome is the tRNA^{Arg} locus. In some embodiments, the presence of the nucleic acid in the genome attenuates the *Listeria*. In some embodiments, the nucleic acid encoding the heterologous antigen has been integrated into the locus of a virulence gene. In some embodiments, the nucleic acid encoding the heterologous antigen has been integrated into the *actA* locus. In some embodiments, the nucleic acid encoding the heterologous antigen has been integrated into the *inlB* locus. In some embodiments, the genome of the *Listeria* comprises a first nucleic acid encoding a heterologous antigen that has been integrated into a first locus (e.g., the *actA* locus) and a second nucleic acid encoding a second heterologous antigen that has been integrated into a second locus (e.g., the *inlB* locus). The first and second heterologous antigens may be identical to each other or different. In some embodiments, the first and second heterologous antigens differ from each other, but are derived from the same tumor antigen or infectious agent antigen. In some embodiments, the first and second heterologous antigens are each a different fragment of an antigen derived from a cancer cell, tumor, or infectious agent. In some embodiments, the integrated nucleic acid encodes a fusion protein comprising a modified ActA and the heterologous antigen. In some embodiments, at least

two, at least three, at least four, at least five, at least six, or at least seven nucleic acid sequences encoding heterologous antigens have been integrated into the *Listeria* genome.

[0016] In another aspect, the invention provides a *Listeria* bacterium comprising a genome, wherein the genome comprises a polynucleotide comprising a nucleic acid encoding a heterologous antigen, wherein the nucleic acid has been integrated into a virulence gene in the genome. In some embodiments, the *Listeria* is attenuated by disruption of expression of the virulence gene or disruption of a coding sequence of the virulence gene. In some embodiments, integration of the polynucleotide (a) disrupts expression of the virulence gene; or (b) disrupts a coding sequence of the virulence gene. In some embodiments, all or part of the virulence gene has been deleted. In some embodiments, none of the virulence gene has been deleted. In some embodiments, the integration attenuates the *Listeria*. In some embodiments, the virulence gene is *prfA*-dependent. In other embodiments, the virulence gene is *prfA*-independent. In some embodiments, the virulence gene is necessary for mediated growth or spread of the bacterium. In some embodiments, the virulence gene is not necessary for growth and spread of the bacterium. In some embodiments, the virulence gene is *actA* or *inlB*. In some embodiments, the *Listeria* bacterium is *Listeria monocytogenes*. In some embodiments, the heterologous antigen is from, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is mesothelin (e.g., human mesothelin), or derived from mesothelin. In some embodiments, the nucleic acid encodes a fusion protein comprising a modified *actA* and the heterologous antigen. In some embodiments, the bacterium comprises a second nucleic acid encoding a second heterologous antigen that has been integrated into a second virulence gene. The invention provides vaccines comprising the *Listeria* bacterium. The invention further provides a method for stimulating an immune response to the heterologous antigen in a mammal, comprising administering an effective amount of the *Listeria* bacterium, or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal.

[0017] In still another aspect, the invention provides a method of producing a *Listeria* bacterium (e.g., an attenuated bacterium), comprising integrating a polynucleotide into a virulence gene in the genome of the *Listeria* bacterium, wherein the polynucleotide comprises a nucleic acid encoding a heterologous antigen. In some embodiments, the *Listeria* is attenuated by disruption of expression of the virulence gene or disruption of a coding sequence of the virulence gene. In some embodiments, the integration of the polynucleotide (a) disrupts expression of the virulence gene or (b) disrupts a coding sequence of the virulence gene. In some embodiments, the integration of the polynucleotide results in both

(a) and (b). In some embodiments the method produces a *Listeria* bacterium for use in a vaccine. In some embodiments, the polynucleotide is integrated into the virulence gene by homologous recombination. In some embodiments, the polynucleotide is integrated via site-specific recombination. In some embodiments, all or part of the virulence gene is deleted during integration of the polynucleotide. In other embodiments, none of the virulence gene is deleted during the integration. In some embodiments, the virulence gene is *actA* or *inlB*. In some embodiments, the heterologous antigen is from, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is mesothelin (e.g., human mesothelin), or derived from mesothelin. In some embodiments, the nucleic acid encodes a fusion protein comprising a modified ActA and the heterologous antigen. The invention further provides a *Listeria* bacterium produced by the method, and vaccine compositions comprising the bacterium. The invention also provides a *Listeria* bacterium having the properties of a *Listeria* bacterium produced by the method, as well as vaccines comprising the bacterium. Methods for stimulating an immune response to the heterologous antigen in a mammal, comprising administering an effective amount of the *Listeria* bacterium, or an effective amount of a composition comprising the *Listeria* bacterium, are also provided.

[0018] In an additional aspect, the invention provides a *Listeria* bacterium comprising a genome, wherein the genome comprises a polynucleotide comprising a nucleic acid encoding a heterologous antigen, wherein the nucleic acid has been integrated into a gene necessary for mediating growth or spread. In some embodiments, integration of the polynucleotide attenuates the *Listeria* for growth or spread. In some embodiments, part or all of the gene has been deleted. In some embodiments, none of the gene has been deleted. In some embodiments, the gene is *actA*. In some embodiments, the *Listeria* bacterium is *Listeria monocytogenes*. In some embodiments, the heterologous antigen is from, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is mesothelin (e.g., human mesothelin), or derived from mesothelin. In some embodiments, the nucleic acid encodes a fusion protein comprising a modified ActA and the heterologous antigen. The invention provides vaccines comprising the *Listeria* bacterium. The invention further provides a method for stimulating an immune response to the heterologous antigen in a mammal, comprising administering an effective amount of the *Listeria* bacterium, or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal.

[0019] In still another aspect, the invention provides a method of producing a *Listeria* bacterium (e.g., an attenuated bacterium), comprising integrating a polynucleotide into a gene

in the genome of the *Listeria* bacterium that is necessary for mediating growth or spread, wherein the polynucleotide comprises a nucleic acid encoding a heterologous antigen. In some embodiments, the integration of the polynucleotide attenuates the *Listeria* for growth or spread. In some embodiments the method produces a *Listeria* bacterium for use in a vaccine. In some embodiments, the polynucleotide is integrated into the gene by homologous recombination. In some embodiments, the polynucleotide is integrated via site-specific recombination. In some embodiments, all or part of the gene necessary for mediating growth or spread is deleted during integration of the polynucleotide. In other embodiments, none of the gene is deleted during the integration. In some embodiments, the gene necessary for mediating growth or spread is *actA*. In some embodiments, the heterologous antigen is from, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is mesothelin (e.g., human mesothelin), or derived from mesothelin. In some embodiments, the nucleic acid encodes a fusion protein comprising the heterologous antigen and a modified ActA. The invention further provides a *Listeria* bacterium produced by the method, and vaccine compositions comprising the bacterium. The invention also provides a *Listeria* bacterium having the properties of a *Listeria* bacterium produced by the method, as well as vaccines comprising the bacterium. Methods for stimulating an immune response to the heterologous antigen in a mammal, comprising administering an effective amount of the *Listeria* bacterium, or an effective amount of a composition comprising the *Listeria* bacterium, are also provided.

[0020] In an additional aspect, the invention provides a *Listeria* bacterium comprising a genome, wherein the genome comprises a polynucleotide comprising a nucleic acid encoding a heterologous antigen, wherein the nucleic acid has been integrated into a virulence gene in the genome and the bacterium is attenuated by disruption of expression of the virulence gene or disruption of a coding sequence of the virulence gene. In some embodiments, all or part of the virulence gene has been deleted. In some embodiments, none of the virulence gene has been deleted. In some embodiments, the virulence gene is *actA* or *inlB*. In some embodiments, the *Listeria* is *Listeria monocytogenes*. In some embodiments, the heterologous antigen is from, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, bacterium further comprises a second nucleic acid encoding a second heterologous antigen that has been integrated into a second virulence gene. In some embodiments, the nucleic acid encodes a fusion protein comprising the heterologous antigen and a modified ActA. Vaccines comprising the *Listeria* bacterium are further provided, as are methods for for stimulating an immune response to the heterologous antigen in a

mammal, comprising administering an effective amount of the *Listeria* bacterium or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal.

[0021] In still another aspect, the invention provides a method of producing a *Listeria* bacterium for use in a vaccine, comprising: integrating a polynucleotide into a virulence gene in the genome of the *Listeria* bacterium, wherein the polynucleotide comprises a nucleic acid encoding a heterologous antigen and wherein the bacterium is attenuated by disruption of the expression of the virulence gene or disruption of a coding sequence of the virulence gene. In some embodiments, the polynucleotide is integrated into the virulence gene by homologous recombination. In some embodiments, all or part of the virulence gene is deleted during integration of the polynucleotide into the virulence gene. In some embodiments, the virulence gene is *actA* or *inlB*. The invention further provides a *Listeria* bacterium produced by the method.

[0022] In another aspect, the invention provides a method of producing a *Listeria* bacterium for use in a vaccine, comprising integrating a polynucleotide into a virulence gene in the genome of the *Listeria* bacterium, wherein the polynucleotide comprises a nucleic acid encoding a heterologous antigen and wherein the *Listeria* bacterium is or has been attenuated by mutation of the virulence gene. The invention further provides a *Listeria* bacterium produced by this method.

[0023] In some embodiments of each of the aforementioned aspects, as well as other aspects described herein, the *Listeria* is attenuated with respect to cell-to-cell spread and/or entry into nonphagocytic cells. For instance, in some embodiments, the *Listeria* comprises an attenuating mutation in *actA* and/or *inlB*.

[0024] In some embodiments of each of the aforementioned aspects, as well as other aspects described herein, the polynucleotide and/or the nucleic acid encoding the fusion protein comprising the heterologous antigen has been integrated into a virulence gene in the genome.

[0025] In some embodiments of each of the aforementioned aspects, as well as other aspects described herein, the heterologous antigen is prostate stem cell antigen (PSCA) (e.g., human PSCA), or an antigen derived from PSCA.

[0026] In some embodiments of each of the aforementioned aspects, as well as other aspects described herein, the heterologous antigen is mesothelin, or an antigen derived from mesothelin.

[0027] In some embodiments of each of the aforementioned aspects, as well as other aspects described herein, the polynucleotide comprises two nucleic acid sequences, each of

which encodes the fusion protein comprising a heterologous antigen. In further embodiments, the polynucleotide comprises three nucleic acids, each of which encodes a fusion protein comprising a heterologous antigen. In some embodiments, the polynucleotide comprises four or more nucleic acids, each of which encodes a fusion protein comprising a heterologous antigen.

[0028] In another aspect, the invention provides a *Listeria* bacterium comprising a genome, wherein the genome comprises: (a) a first polynucleotide comprising a first nucleic acid encoding a first heterologous antigen, wherein the first polynucleotide has been integrated into a virulence gene in the genome and wherein the polynucleotide comprises a nucleic acid encoding a heterologous antigen; and (b) a second polynucleotide comprising a second nucleic acid encoding a second heterologous antigen, wherein the second polynucleotide has also been integrated into the genome. In some embodiments, the bacterium is attenuated by disruption of the expression of the virulence gene or by disruption of a coding sequence of the virulence gene. In some embodiments, the integration of the first polynucleotide disrupts expression of the virulence gene or disrupts a coding sequence of the virulence gene. In some embodiments, all or part of the virulence gene has been deleted. In some other embodiments, none of the virulence gene has been deleted. In some embodiments, the virulence gene is *actA* or *inlB*. In some embodiments, the *Listeria* is *Listeria monocytogenes*. In some embodiments, the first and second heterologous antigens are from, or are derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the first and/or second heterologous antigen is mesothelin, an antigen derived from mesothelin, PSCA, or an antigen derived from PSCA (e.g., the first heterologous antigen may be mesothelin, and the second heterologous antigen may be PSCA). In some embodiments, the second polynucleotide has been integrated into a virulence gene in the genome and the *Listeria* may be attenuated by disruption of the expression of the virulence gene or disruption of a coding sequence of the virulence gene. In some embodiments, the second polynucleotide has been integrated into *actA* or *inlB*. In some embodiments, the second polynucleotide has been integrated into a region of the genome which does not comprise a virulence gene (e.g., at the tRNA^{Arg} locus). In some embodiments, the first and/or second nucleic acid encodes a fusion protein comprising a modified ActA (e.g., ActA-N100) and the first or second heterologous antigen. In some embodiments, the first and/or second nucleic acid encodes a fusion protein comprising a signal sequence and the first or second heterologous antigen, wherein the signal sequence is an ActA signal sequence, p60 signal sequence, an LLO signal sequence, a BaPa signal sequence, a Llusp45 signal sequence, or a PhoD signal sequence. Vaccines comprising

the *Listeria* bacterium are further provided. Methods of stimulating an immune response to the heterologous antigen in a mammal, comprising administering an effective amount of the *Listeria* bacterium, or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal are also provided. In some embodiments, the genome of the *Listeria* further comprises a third polynucleotide comprising a third nucleic acid encoding a third heterologous antigen, wherein the third polynucleotide has also been integrated into the genome.

[0029] In some embodiments of each of the aforementioned aspects, as well as other aspects described herein, the polynucleotides comprising the nucleic acid(s) encoding a heterologous antigen(s) or the fusion protein(s) comprising a heterologous antigen that are integrated into a region of the genome further comprise a promoter and/or other regulatory sequences necessary for expression of the nucleic acids once integrated. In some alternative embodiments, the polynucleotides comprising the nucleic acids encoding the heterologous antigen or the fusion protein comprising the heterologous antigen are integrated into a region of the genome such that the nucleic acid is operably linked to a promoter and/or secretory signal already present in the *Listeria* genome. In some embodiments, the polynucleotides that are inserted in virulence genes are inserted in coding regions. In some other embodiments, the polynucleotides are inserted into non-coding regions of the virulence genes.

[0030] In some embodiments of each of the aforementioned aspects, as well as other aspects described herein, polynucleotides integrated into the *Listeria* genome are integrated via homologous recombination. In some alternative embodiments, the polynucleotides are integrated into the *Listeria* genome via site-specific integration.

[0031] In some embodiments, the invention provides a *Listeria* bacterium containing a polynucleotide comprising a first nucleic acid encoding a fusion protein partner, operably linked and in frame with and a second nucleic acid encoding human mesothelin, or a derivative thereof. The first nucleic acid can encode, e.g., LLO62 (non-codon optimized); LLO26 (codon optimized); LLO441 (non-codon optimized); LLO441 (codon optimized); full length LLO (non-codon optimized); full length LLO (codon optimized); BaPA secretory sequence; *B. subtilis* phoD secretory sequence (Bs phoD SS); p60 (non-codon optimized); p60 (codon optimized); actA (non-codon optimized); actA (codon optimized); actA-N100 (non-codon optimized); actA-N100 (codon optimized); actA (A30R). The second nucleic acid can encode full length human mesothelin; human mesothelin deleted in its signal sequence; human mesothelin deleted in its GPI anchor; or human mesothelin deleted in both

the signal sequence and the GPI anchor, where codon-optimized and non-codon optimized versions of mesothelin are provided. In another aspect, the present invention provides the above polynucleotide integrated at the position of the *inlB* gene, *actA* gene, *hly* gene, where integration can be mediated by homologous recombination, and where integration can optionally be with operable linking with the promoter of the *inlB*, *actA*, or *hly* gene. In yet another aspect, the invention provides listerial embodiments where the above polynucleotide is integrated into the listerial genome by way of site-specific integration, e.g., at the tRNA^{Arg} site. Each of the individual embodiments disclosed herein, optionally, encompasses a *Listeria* comprising a constitutively active *pfrA* gene (*prfA**). The listerial constructs are not limited to polynucleotides operably linked with an *actA* promoter or *hly* promoter. What is also encompassed is operable linkages with other bacterial promoters, synthetic promoters, bacteriovirus promoters, and combinations of two or more promoters.

[0032] In some embodiments, the heterologous antigen encoded by a nucleic acid in the polynucleotides, *Listeria* bacteria, and/or vaccines described above, or elsewhere herein, comprises an EphA2 antigenic peptide. In some embodiments, the heterologous antigen encoded by a nucleic acid in the polynucleotides, *Listeria* bacteria, and/or vaccines comprises full-length EphA2 or an antigenic fragment, analog or derivative thereof. In some embodiments, the heterologous antigen encoded by a nucleic acid in the polynucleotides, *Listeria* bacteria, and/or vaccines described above, or elsewhere herein, does not comprise an EphA2 antigenic peptide. In some embodiments, the heterologous antigen encoded by a nucleic acid in the polynucleotides, *Listeria* bacteria, and/or vaccines, does not comprise full-length EphA2 or an antigenic fragment, analog or derivative thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] Figure 1 discloses pINT, a 6055 bp plasmid. Once pINT is integrated in a listerial genome, the *Listeria* can be isolated by erythromycin resistance (ErmC), followed by treatment with Cre recombinase to remove a region of the plasmid encoding the antibiotic resistance genes (CAT and ErmC).

[0034] Figure 2 shows pKSV7, a 7096 plasmid that mediates homologous recombination.

[0035] Figure 3 shows steps, or intermediates, occurring with pKSV7-mediated homologous recombination into a bacterial genome.

[0036] Figure 4 discloses a method for preparing an insert bearing homologous arms, where the insert bearing the homologous arms is placed into pKSV7. The loxP-flanked region is bracketed by the homologous arms. After integration into a bacterial genome,

transient exposure to Cre recombinase catalyzes removal of the antibiotic resistance gene. Integration occurs with deletion of part of the genome, corresponding to the region between areas matching the homologous arms.

[0037] Figure 5 shows an alternate method for preparing an insert bearing homologous arms, where the insert bearing homologous arms is placed into pKSV7. The loxP-flanked region resides outside the homologous arms. After integration into a bacterial genome, transient exposure to Cre recombinase catalyzes removal of the antibiotic resistance gene (or other selection marker). Integration occurs with deletion of part of the genome, corresponding to the region between areas matching the homologous arms.

[0038] Figure 6 discloses the preparation of an insert bearing homologous arms, where the insert bearing homologous arms is placed into pKSV7. The loxP-flanked region resides in between the homologous arms. In vectors prepared according to this figure, integration is not followed by deletion of any corresponding region of the genome.

[0039] Figure 7 is a schematic disclosing some of the mesothelin constructs of the present invention, including, e.g., any promoters, secretory sequences, fusion protein partners, and so on.

[0040] Figure 8 is a gel showing expression of mesothelin from various listerial constructs.

[0041] Figure 9 is a gel showing expression of mesothelin from a number of listerial constructs.

[0042] Figures 10-12 show expression of interferon-gamma (IFN γ) from spot forming cell (SFC) assays, and compare immune responses where mice had been vaccinated with various numbers (colony forming units; c.f.u.) of engineered *L. monocytogenes*.

[0043] Figures 13 disclose numbers of tumor metastases on the surfaces of livers, after treating tumor-bearing mice with various preparations of recombinant *L. monocytogenes*. Figure 13 reveals the raw data (photographs of fixed livers).

[0044] Figure 14 also disclose numbers of tumor metastases on the surfaces of livers, after treatment of tumor-bearing mice with various preparations of recombinant *L. monocytogenes*.

[0045] Figure 15A-G further disclose numbers of tumor metastases on the surfaces of livers, after treating tumor-bearing mice with recombinant *L. monocytogenes*.

[0046] Figure 16 demonstrates increased survival to tumors by tumor-bearing mice with treatment with various preparations of recombinant *L. monocytogenes*.

[0047] Figure 17 illustrates mesothelin constructs and secretion of mesothelin by various preparations of recombinant *L. monocytogenes*.

[0048] Figure 18 discloses secretion of mesothelin and immune responses stimulated by various preparations of recombinant *L. monocytogenes*.

[0049] Figure 19 shows secretion of mesothelin and immune responses stimulated by various preparations of recombinant *L. monocytogenes*.

[0050] Figure 20 further reveals mesothelin expression and immune responses stimulated by various preparations of recombinant *L. monocytogenes*.

[0051] Figure 21 additionally illustrates secretion of mesothelin and immune responses stimulated by various preparations of recombinant *L. monocytogenes*.

[0052] Figure 22 demonstrates mesothelin expression and immune responses stimulated by various preparations of recombinant *L. monocytogenes*.

[0053] Figure 23 discloses immune responses stimulated by vaccination with various preparations of recombinant *Listeria*.

[0054] Figure 24 further discloses secretion of mesothelin and immune responses stimulated by various preparations of recombinant *L. monocytogenes*.

[0055] Figure 25 reveals immune responses stimulated after vaccination with a number of preparations of recombinant *Listeria*.

[0056] Figure 26 additionally discloses secretion of mesothelin and immune responses stimulated by various preparations of recombinant *L. monocytogenes*. hMeso6: *L. monocytogenes* Δ actA Δ inlB encoding actA promoter; actA-N100-hMeso Δ SS Δ GPI; integrated at *actA* locus. hMeso25: *L. monocytogenes* Δ actA Δ inlB encoding actA promoter; actA-N100-hMeso Δ SS Δ GPI; integrated at *inlB* locus.

[0057] Figure 27 further demonstrates secretion of mesothelin and immune responses stimulated by various preparations of recombinant *L. monocytogenes*.

[0058] Figure 28 shows photographs of fixed lungs.

[0059] Figure 29 shows a histogram of data from the photographs of fixed lung.

[0060] Figure 30 reveals the effectiveness of various preparations of recombinant *Listeria* in improving survival of tumor-bearing mice.

[0061] Figure 31 discloses secretion of mesothelin and immune responses stimulated by various preparations of recombinant *L. monocytogenes*.

[0062] Figure 32 compares mesothelin expression from various preparations of recombinant *Listeria*.

[0063] Figure 33 depicts mesothelin secretion and immune responses stimulated after vaccination with recombinant *L. monocytogenes*.

[0064] Figure 34 demonstrates immune response stimulated after vaccination with the preparations and doses of recombinant *Listeria*.

[0065] Figures 35A and 35B disclose numbers of tumor metastases on livers, after treatment of tumor-bearing mice with various preparations of recombinant *L. monocytogenes*. Figure 35A illustrates raw data (photographs of fixed livers).

[0066] Figure 36 demonstrates the effectiveness of various preparations of recombinant *Listeria* in improving survival of tumor-bearing mice.

[0067] Figure 37 discloses immune response after vaccination with various preparations of recombinant *Listeria*, and compares CD4⁺ T cell and CD8⁺ T cell responses.

[0068] Figure 38 reveals survival of tumor-bearing mice to the tumors after vaccination with various preparations of recombinant *Listeria*.

[0069] Figure 39 further illustrates survival of tumor-bearing mice to the tumors after vaccination with various preparations of recombinant *Listeria*.

[0070] Figure 40 discloses alignment of a phage integrase of the present invention with a another phage integrase (U153 int: SEQ ID NO:1; lin 1231: SEQ ID NO:2).

[0071] Figure 41 discloses alignment of yet another phage integrase of the present invention another phage integrase (PSA int: SEQ ID NO:3; lin 0071: SEQ ID NO:4).

[0072] Figure 42 shows alignment of still another phage integrase of the present invention with a different phage integrase (PSA int: SEQ ID NO:5; lin 1765: SEQ ID NO:6).

[0073] Figure 43 discloses alignment of a further phage integrase of the present invention with another phage integrase (PSA int: SEQ ID NO:7; lin 2601: SEQ ID NO:8).

[0074] Figure 44 provides an alignment of an additional phage integrase of the present invention with a nucleic acid encoding another phage integrase (PSA int: SEQ ID NO:119; Imof6854_2703: SEQ ID NO:120).

[0075] Figure 45A-B provides the human EphA2 cDNA sequence (CDS: nt 138 to 3068) (SEQ ID NO:152) published as SEQ ID NO:1 in U.S. Patent Publication No. 2005/0281783 A1, incorporated by reference herein in its entirety.

[0076] Figure 46A-B provides the human EphA2 polypeptide sequence (SEQ ID NO: 153) published as SEQ ID NO:2 in U.S. Patent Publication No. 2005/0281783 A1, incorporated by reference herein in its entirety.

[0077] Figure 47A and 47B discloses pINT-ActAN100-BamHI-SpeI-MfeI-SIINF EKL, a 6594 bp plasmid based on pINT (Fig. 1) and containing an ActA promoter, actA-N100 and a SIINF EKL sequence. Figure 47B shows the cloning region of pINT-ActAN100-BamHI-

SpeI-MfeI-SIINFEEKL. (Nucleic acid sequence is SEQ ID NO:150, Peptide sequence is SEQ ID NO:151)

[0078] Figure 48 discloses the schematic configuration of PSCA molecular constructs with Kyte-Doolittle overlay.

[0079] Figure 49A shows the results of dendritic cell presentation of peptides to the reporter cell line B3Z. Figure 49B is a Western blot analysis of expression and secretion of ActA-N100-PSCA fusion proteins in J774 cells.

[0080] Figure 50 demonstrates immune response stimulated after vaccination with recombinant *Listeria monocytogenes* expressing ActA-N100-PSCA fusion proteins.

[0081] Figure 51A and 51B disclose bivalent vaccine strains expressing two different human tumor antigens. Figure 51A discloses fusion molecules encoded by integrated constructs. Figure 51B discloses the expression and secretion of ActA fusion proteins following infection of J774 cells with monovalent or bivalent *Listeria monocytogenes* vaccines.

[0082] Figure 52 demonstrates immune response stimulated after vaccination with monovalent or divalent recombinant *Listeria monocytogenes* vaccines.

DETAILED DESCRIPTION

[0083] As used herein, including the appended claims, the singular forms of words such as “a,” “an,” and “the,” include their corresponding plural references unless the context clearly dictates otherwise. All references cited herein are incorporated by reference to the same extent as if each individual publication, sequences accessed by a GenBank Accession No., patent application, patent, Sequence Listing, nucleotide or oligo- or polypeptide sequence in the Sequence Listing, as well as figures and drawings in said publications and patent documents, was specifically and individually indicated to be incorporated by reference. The term “present invention” refers to certain embodiments of the present invention, or to some embodiments of the present invention. Unless stated otherwise, the term “present invention” does not necessarily refer to all embodiments of the invention.

I. DEFINITIONS.

[0084] Abbreviations used to indicate a mutation in a gene, or a mutation in a bacterium comprising the gene, are as follows. By way of example, the abbreviation “*L. monocytogenes* Δ ActA” means that part, or all, of the ActA gene was deleted. The delta symbol (Δ) means

deletion. An abbreviation including a superscripted minus sign (*Listeria* ActA⁻) means that the ActA gene was mutated, e.g., by way of a deletion, point mutation, or frameshift mutation, but not limited to these types of mutations. Exponentials are abbreviated, where, for example, “3e7” means 3×10^7 .

[0085] “Administration” as it applies to a human, mammal, mammalian subject, animal, veterinary subject, placebo subject, research subject, experimental subject, cell, tissue, organ, or biological fluid, refers without limitation to contact of an exogenous ligand, reagent, placebo, small molecule, pharmaceutical agent, therapeutic agent, diagnostic agent, or composition to the subject, cell, tissue, organ, or biological fluid, and the like.

“Administration” can refer, e.g., to therapeutic, pharmacokinetic, diagnostic, research, placebo, and experimental methods. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell.

“Administration” also encompasses *in vitro* and *ex vivo* treatments, e.g., of a cell, by a reagent, diagnostic, binding composition, or by another cell.

[0086] An “agonist,” as it relates to a ligand and receptor, comprises a molecule, combination of molecules, a complex, or a combination of reagents, that stimulates the receptor. For example, an agonist of granulocyte-macrophage colony stimulating factor (GM-CSF) can encompass GM-CSF, a mutein or derivative of GM-CSF, a peptide mimetic of GM-CSF, a small molecule that mimics the biological function of GM-CSF, or an antibody that stimulates GM-CSF receptor. An antagonist, as it relates to a ligand and receptor, comprises a molecule, combination of molecules, or a complex, that inhibits, counteracts, downregulates, and/or desensitizes the receptor. “Antagonist” encompasses any reagent that inhibits a constitutive activity of the receptor. A constitutive activity is one that is manifest in the absence of a ligand/receptor interaction. “Antagonist” also encompasses any reagent that inhibits or prevents a stimulated (or regulated) activity of a receptor. By way of example, an antagonist of GM-CSF receptor includes, without implying any limitation, an antibody that binds to the ligand (GM-CSF) and prevents it from binding to the receptor, or an antibody that binds to the receptor and prevents the ligand from binding to the receptor, or where the antibody locks the receptor in an inactive conformation.

[0087] As used herein, an “analog” in the context of an EphA2 polypeptide (or a fragment of an EphA2 polypeptide) refers to a proteinaceous agent (e.g., a peptide, polypeptide or protein) that possesses a similar or identical function as the EphA2 polypeptide (or fragment of an EphA2 polypeptide), but does not necessarily comprise a similar or identical amino acid sequence or structure of the EphA2 polypeptide (or fragment). An analog of an EphA2

polypeptide that has a similar amino acid sequence to an EphA2 polypeptide refers to a proteinaceous agent that satisfies at least one of the following: (a) a proteinaceous agent having an amino acid sequence that is at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% identical to the amino acid sequence of an EphA2 polypeptide; (b) a proteinaceous agent encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence encoding an EphA2 polypeptide of at least 20 amino acid residues, at least 30 amino acid residues, at least 40 amino acid residues, at least 50 amino acid residues, at least 60 amino acid residues, at least 70 amino acid residues, at least 80 amino acid residues, at least 90 amino acid residues, at least 100 amino acid residues, at least 125 amino acid residues, or at least 150 amino acid residues; and (c) a proteinaceous agent encoded by a nucleotide sequence that is at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% identical to the nucleotide sequence encoding an EphA2 polypeptide. A proteinaceous agent with similar structure to an EphA2 polypeptide refers to a proteinaceous agent that has a similar secondary, tertiary or quaternary structure of the EphA2 polypeptide. The structure of a proteinaceous agent can be determined by methods known to those skilled in the art, including but not limited to, X-ray crystallography, nuclear magnetic resonance, and crystallographic electron microscopy. In some embodiments, the proteinaceous agent has EphA2 activity.

[0088] "Antigen presenting cells" (APCs) are cells of the immune system used for presenting antigen to T cells. APCs include dendritic cells, monocytes, macrophages, marginal zone Kupffer cells, microglia, Langerhans cells, T cells, and B cells (see, e.g., Rodriguez-Pinto and Moreno (2005) *Eur. J. Immunol.* 35:1097-1105). Dendritic cells occur in at least two lineages. The first lineage encompasses pre-DC1, myeloid DC1, and mature DC1. The second lineage encompasses CD34⁺⁺CD45RA⁻ early progenitor multipotent cells, CD34⁺⁺CD45RA⁺ cells, CD34⁺⁺CD45RA⁺⁺CD4⁺ IL-3Ralpha⁺⁺ pro-DC2 cells, CD4⁺CD11c⁻ plasmacytoid pre-DC2 cells, lymphoid human DC2 plasmacytoid-derived DC2s, and mature DC2s (see, e.g., Gilliet and Liu (2002) *J. Exp. Med.* 195:695-704; Bauer, *et al.* (2001) *J. Immunol.* 166:5000-5007; Arpinati, *et al.* (2000) *Blood* 95:2484-2490; Kadowaki, *et al.* (2001) *J. Exp. Med.* 194:863-869; Liu (2002) *Human Immunology* 63:1067-1071; McKenna, *et al.* (2005) *J. Virol.* 79:17-27; O'Neill, *et al.* (2004) *Blood* 104:2235-2246; Rossi and

Young (2005) *J. Immunol.* 175:1373-1381; Banchereau and Palucka (2005) *Nat. Rev. Immunol.* 5:296-306).

[0089] “Attenuation” and “attenuated” encompasses a bacterium, virus, parasite, infectious organism, prion, tumor cell, gene in the infectious organism, and the like, that is modified to reduce toxicity to a host. The host can be a human or animal host, or an organ, tissue, or cell. The bacterium, to give a non-limiting example, can be attenuated to reduce binding to a host cell, to reduce spread from one host cell to another host cell, to reduce extracellular growth, or to reduce intracellular growth in a host cell. Attenuation can be assessed by measuring, e.g., an indicum or indicia of toxicity, the LD₅₀, the rate of clearance from an organ, or the competitive index (see, e.g., Auerbuch, *et al.* (2001) *Infect. Immunity* 69:5953-5957).

Generally, an attenuation results an increase in the LD₅₀ and/or an increase in the rate of clearance by at least 25%; more generally by at least 50%; most generally by at least 100% (2-fold); normally by at least 5-fold; more normally by at least 10-fold; most normally by at least 50-fold; often by at least 100-fold; more often by at least 500-fold; and most often by at least 1000-fold; usually by at least 5000-fold; more usually by at least 10,000-fold; and most usually by at least 50,000-fold; and most often by at least 100,000-fold.

[0090] “Attenuated gene” encompasses a gene that mediates toxicity, pathology, or virulence, to a host, growth within the host, or survival within the host, where the gene is mutated in a way that mitigates, reduces, or eliminates the toxicity, pathology, or virulence. The reduction or elimination can be assessed by comparing the virulence or toxicity mediated by the mutated gene with that mediated by the non-mutated (or parent) gene. “Mutated gene” encompasses deletions, point mutations, and frameshift mutations in regulatory regions of the gene, coding regions of the gene, non-coding regions of the gene, or any combination thereof.

[0091] “Cancerous condition” and “cancerous disorder” encompass, without implying any limitation, a cancer, a tumor, metastasis, angiogenesis of a tumor, and precancerous disorders such as dysplasias.

[0092] “Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, a conservatively modified variant refers to nucleic acids encoding identical amino acid sequences, or amino acid sequences that have one or more conservative substitutions. An example of a conservative substitution is the exchange of an amino acid in one of the following groups for another amino acid of the same group (U.S. Pat. No. 5,767,063 issued to Lee, *et al.*; Kyte and Doolittle (1982) *J. Mol. Biol.* 157:105-132).

(1) Hydrophobic: Norleucine, Ile, Val, Leu, Phe, Cys, Met;

- (2) Neutral hydrophilic: Cys, Ser, Thr;
- (3) Acidic: Asp, Glu;
- (4) Basic: Asn, Gln, His, Lys, Arg;
- (5) Residues that influence chain orientation: Gly, Pro;
- (6) Aromatic: Trp, Tyr, Phe; and
- (7) Small amino acids: Gly, Ala, Ser.

[0093] A “derivative” in the context of an EphA2 polypeptide or a fragment of an EphA2 polypeptide refers to a proteinaceous agent that comprises an amino acid sequence of an EphA2 polypeptide or a fragment of an EphA2 polypeptide that has been altered by the introduction of amino acid residue substitutions, deletions or additions (i.e., mutations). The term “derivative” in the context of EphA2 proteinaceous agents also refers to an EphA2 polypeptide or a fragment of an EphA2 polypeptide which has been modified, i.e. by the covalent attachment of any type of molecule to the polypeptide. For example, but not by way of limitation, an EphA2 polypeptide or a fragment of an EphA2 polypeptide may be modified, e.g., by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, etc. A derivative of an EphA2 polypeptide or a fragment of an EphA2 polypeptide may be modified by chemical modifications using techniques known to those of skill in the art, including, but not limited to, specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, etc. Further, a derivative of an EphA2 polypeptide or a fragment of an EphA2 polypeptide may contain one or more non-classical amino acids. In one embodiment, a polypeptide derivative possesses a similar or identical function as an EphA2 polypeptide or a fragment of an EphA2 polypeptide described herein. In another embodiment, a derivative of EphA2 polypeptide or a fragment of an EphA2 polypeptide has an altered activity when compared to an unaltered polypeptide. For example, a derivative of an EphA2 polypeptide or fragment thereof can differ in phosphorylation relative to an EphA2 polypeptide or fragment thereof.

[0094] “Effective amount” encompasses, without limitation, an amount that can ameliorate, reverse, mitigate, prevent, or diagnose a symptom or sign of a medical condition or disorder. Unless dictated otherwise, explicitly or by context, an “effective amount” is not limited to a minimal amount sufficient to ameliorate a condition.

[0095] “EphA2 antigenic peptides” (sometimes referred to as “EphA2 antigenic polypeptides”), are defined and described in U.S. Patent Publication No. 2005/0281783 A1, which is hereby incorporated by reference herein in its entirety, including all sequences

contained therein. EphA2 is a 130 kDa receptor tyrosine kinase expressed in adult epithelia (Zantek et al. (1999) *Cell Growth & Differentiation* 10:629; Lindberg et al. (1990) *Molecular & Cellular Biology* 10:6316). An "EphA2 antigenic peptide" or an "EphA2 antigenic polypeptide" refers to an EphA2 polypeptide, or a fragment, analog or derivative thereof comprising one or more B cell epitopes or T cell epitopes of EphA2. The EphA2 polypeptide may be from any species. For example the EphA2 polypeptide may be a human EphA2 polypeptide. The term "EphA2 polypeptide" includes the mature, processed form of EphA2, as well as immature forms of EphA2. In some embodiments, the EphA2 polypeptide is the sequence shown in Figure 46A-B (SEQ ID NO:2 of U.S. Patent Publication No.

2005/0281783 A1). Examples of the nucleotide sequence of human EphA2 can be found in the GenBank database (see, e.g., Accession Nos. BC037166, M59371 and M36395).

Examples of the amino acid sequence of human EphA2 can also be found in the GenBank database (see, e.g., Accession Nos. NP_004422, AAH37166, and AAA53375). Additional examples of amino acid sequences of EphA2 include those listed as GenBank Accession Nos. NP_034269 (mouse), AAH06954 (mouse), XP_345597 (rat), and BAB63910 (chicken).

[0096] An "extracellular fluid" encompasses, e.g., serum, plasma, blood, interstitial fluid, cerebrospinal fluid, secreted fluids, lymph, bile, sweat, fecal matter, and urine. An "extracellular fluid" can comprise a colloid or a suspension, e.g., whole blood or coagulated blood.

[0097] The term "fragments" in the context of EphA2 polypeptides include an EphA2 antigenic peptide or polypeptide comprising an amino acid sequence of at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino acid residues, at least 70 contiguous amino acid residues, at least 80 contiguous amino acid residues, at least 90 contiguous amino acid residues, at least 100 contiguous amino acid residues, at least 125 contiguous amino acid residues, at least 150 contiguous amino acid residues, at least 175 contiguous amino acid residues, at least 200 contiguous amino acid residues, or at least 250 contiguous amino acid residues of the amino acid sequence of an EphA2 polypeptide.

[0098] "Gene" refers to a nucleic acid sequence encoding an oligopeptide or polypeptide. The oligopeptide or polypeptide can be biologically active, antigenically active, biologically inactive, or antigenically inactive, and the like. The term gene encompasses, e.g., the sum of the open reading frames (ORFs) encoding a specific oligopeptide or polypeptide; the sum of

the ORFs plus the nucleic acids encoding introns; the sum of the ORFs and the operably linked promoter(s); the sum of the ORFs and the operably linked promoter(s) and any introns; the sum of the ORFs and the operably linked promoter(s), intron(s), and promoter(s), and other regulatory elements, such as enhancer(s). In certain embodiments, "gene" encompasses any sequences required in cis for regulating expression of the gene. The term gene can also refer to a nucleic acid that encodes a peptide encompassing an antigen or an antigenically active fragment of a peptide, oligopeptide, polypeptide, or protein. The term gene does not necessarily imply that the encoded peptide or protein has any biological activity, or even that the peptide or protein is antigenically active. A nucleic acid sequence encoding a non-expressable sequence is generally considered a pseudogene. The term gene also encompasses nucleic acid sequences encoding a ribonucleic acid such as rRNA, tRNA, or a ribozyme.

[0099] "Growth" of a *Listeria* bacterium encompasses, without limitation, functions of bacterial physiology and genes relating to colonization, replication, increase in listerial protein content, increase in listerial lipid content. Unless specified otherwise explicitly or by context, growth of a *Listeria* encompasses growth of the bacterium outside a host cell, and also growth inside a host cell. Growth related genes include, without implying any limitation, those that mediate energy production (e.g., glycolysis, Krebs cycle, cytochromes), anabolism and/or catabolism of amino acids, sugars, lipids, minerals, purines, and pyrimidines, nutrient transport, transcription, translation, and/or replication. In some embodiments, "growth" of a *Listeria* bacterium refers to intracellular growth of the *Listeria* bacterium, that is, growth inside a host cell such as a mammalian cell. While intracellular growth of a *Listeria* bacterium can be measured by light microscopy or colony forming unit (CFU) assays, growth is not to be limited by any technique of measurement. Biochemical parameters such as the quantity of a listerial antigen, listerial nucleic acid sequence, or lipid specific to the *Listeria* bacterium, can be used to assess growth. In some embodiments, a gene that mediates growth is one that specifically mediates intracellular growth. In some embodiments, a gene that specifically mediates intracellular growth encompasses, but is not limited to, a gene where inactivation of the gene reduces the rate of intracellular growth but does not detectably, substantially, or appreciably, reduce the rate of extracellular growth (e.g., growth in broth), or a gene where inactivation of the gene reduces the rate of intracellular growth to a greater extent than it reduces the rate of extracellular growth. To provide a non-limiting example, in some embodiments, a gene where inactivation reduces the rate of intracellular growth to a greater extent than extracellular growth encompasses the situation

where inactivation reduces intracellular growth to less than 50% the normal or maximal value, but reduces extracellular growth to only 1-5%, 5-10%, or 10-15% the maximal value. The invention, in certain aspects, encompasses a *Listeria* attenuated in intracellular growth but not attenuated in extracellular growth, a *Listeria* not attenuated in intracellular growth and not attenuated in extracellular growth, as well as a *Listeria* not attenuated in intracellular growth but attenuated in extracellular growth.

[0100] “Immune condition” or “immune disorder” encompasses a disorder, condition, syndrome, or disease resulting from ineffective, inappropriate, or pathological response of the immune system, e.g., to a persistent infection or to a persistent cancer (see, e.g., Jacobson, *et al.* (1997) Clin. Immunol. Immunopathol. 84:223-243). “Immune condition” or “immune disorder” encompasses, e.g., pathological inflammation, an inflammatory disorder, and an autoimmune disorder or disease. “Immune condition” or “immune disorder” also can refer to infections, persistent infections, cancer, tumors, precancerous disorders, cancers that resist irradiation by the immune system, and angiogenesis of tumors. “Immune condition” or “immune disorder” also encompasses cancers induced by an infective agent, including the non-limiting examples of cancers induced by hepatitis B virus, hepatitis C virus, simian virus 40 (SV40), Epstein-Barr virus, papillomaviruses, polyomaviruses, Kaposi’s sarcoma herpesvirus, human T-cell leukemia virus, and *Helicobacter pylori* (see, e.g., Young and Rickinson (2004) Nat. Rev. Cancer 4:757-768; Pagano, *et al.* (2004) Semin. Cancer Biol. 14:453-471; Li, *et al.* (2005) Cell Res. 15:262-271).

[0101] A composition that is “labeled” is detectable, either directly or indirectly, by spectroscopic, photochemical, biochemical, immunochemical, isotopic, or chemical methods. For example, useful labels include ^{32}P , ^{33}P , ^{35}S , ^{14}C , ^3H , ^{125}I , stable isotopes, epitope tags, fluorescent dyes, electron-dense reagents, substrates, or enzymes, e.g., as used in enzyme-linked immunoassays, or fluorettes (see, e.g., Rozinov and Nolan (1998) Chem. Biol. 5:713-728).

[0102] “Ligand” refers to a small molecule, peptide, polypeptide, or membrane associated or membrane-bound molecule, that is an agonist or antagonist of a receptor. “Ligand” also encompasses a binding agent that is not an agonist or antagonist, and has no agonist or antagonist properties. By convention, where a ligand is membrane-bound on a first cell, the receptor usually occurs on a second cell. The second cell may have the same identity (the same name), or it may have a different identity (a different name), as the first cell. A ligand or receptor may be entirely intracellular, that is, it may reside in the cytosol, nucleus, or in some other intracellular compartment. The ligand or receptor may change its location, e.g.,

from an intracellular compartment to the outer face of the plasma membrane. The complex of a ligand and receptor is termed a “ligand receptor complex.” Where a ligand and receptor are involved in a signaling pathway, the ligand occurs at an upstream position and the receptor occurs at a downstream position of the signaling pathway.

[0103] “Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single stranded, double-stranded form, or multi-stranded form. Non-limiting examples of a nucleic acid are a, e.g., cDNA, mRNA, oligonucleotide, and polynucleotide. A particular nucleic acid sequence can also implicitly encompass “allelic variants” and “splice variants.”

[0104] “Operably linked” in the context of a promoter and a nucleic acid encoding a mRNA means that the promoter can be used to initiate transcription of that nucleic acid.

[0105] The terms “percent sequence identity” and “% sequence identity” refer to the percentage of sequence similarity found by a comparison or alignment of two or more amino acid or nucleic acid sequences. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. An algorithm for calculating percent identity is the Smith-Waterman homology search algorithm (see, e.g., Kann and Goldstein (2002) *Proteins* 48:367-376; Arslan, *et al.* (2001) *Bioinformatics* 17:327-337).

[0106] “Precancerous condition” encompasses, without limitation, dysplasias, preneoplastic nodules; macroregenerative nodules (MRN); low-grade dysplastic nodules (LG-DN); high-grade dysplastic nodules (HG-DN); biliary epithelial dysplasia; foci of altered hepatocytes (FAH); nodules of altered hepatocytes (NAH); chromosomal imbalances; aberrant activation of telomerase; re-expression of the catalytic subunit of telomerase; expression of endothelial cell markers such as CD31, CD34, and BNH9 (see, e.g., Terracciano and Tornillo (2003) *Pathologica* 95:71-82; Su and Bannasch (2003) *Toxicol. Pathol.* 31:126-133; Rocken and Carl-McGrath (2001) *Dig. Dis.* 19:269-278; Kotoula, *et al.* (2002) *Liver* 22:57-69; Frachon, *et al.* (2001) *J. Hepatol.* 34:850-857; Shimonishi, *et al.* (2000) *J. Hepatobiliary Pancreat. Surg.* 7:542-550; Nakanuma, *et al.* (2003) *J. Hepatobiliary Pancreat. Surg.* 10:265-281). Methods for diagnosing cancer and dysplasia are disclosed (see, e.g., Riegler (1996) *Semin. Gastrointest. Dis.* 7:74-87; Benvegnu, *et al.* (1992) *Liver* 12:80-83; Giannini, *et al.* (1987) *Hepatogastroenterol.* 34:95-97; Anthony (1976) *Cancer Res.* 36:2579-2583).

[0107] By “purified” and “isolated” is meant, when referring to a polypeptide, that the polypeptide is present in the substantial absence of the other biological macromolecules with which it is associated in nature. The term “purified” as used herein means that an identified polypeptide often accounts for at least 50%, more often accounts for at least 60%, typically accounts for at least 70%, more typically accounts for at least 75%, most typically accounts for at least 80%, usually accounts for at least 85%, more usually accounts for at least 90%, most usually accounts for at least 95%, and conventionally accounts for at least 98% by weight, or greater, of the polypeptides present. The weights of water, buffers, salts, detergents, reductants, protease inhibitors, stabilizers (including an added protein such as albumin), and excipients, and molecules having a molecular weight of less than 1000, are generally not used in the determination of polypeptide purity. See, e.g., discussion of purity in U.S. Pat. No. 6,090,611 issued to Covacci, *et al.*

[0108] “Peptide” refers to a short sequence of amino acids, where the amino acids are connected to each other by peptide bonds. A peptide may occur free or bound to another moiety, such as a macromolecule, lipid, oligo- or polysaccharide, and/or a polypeptide. Where a peptide is incorporated into a polypeptide chain, the term “peptide” may still be used to refer specifically to the short sequence of amino acids. A “peptide” may be connected to another moiety by way of a peptide bond or some other type of linkage. A peptide is at least two amino acids in length and generally less than about 25 amino acids in length, where the maximal length is a function of custom or context. The terms “peptide” and “oligopeptide” may be used interchangeably.

[0109] “Protein” generally refers to the sequence of amino acids comprising a polypeptide chain. Protein may also refer to a three dimensional structure of the polypeptide. “Denatured protein” refers to a partially denatured polypeptide, having some residual three dimensional structure or, alternatively, to an essentially random three dimensional structure, i.e., totally denatured. The invention encompasses reagents of, and methods using, polypeptide variants, e.g., involving glycosylation, phosphorylation, sulfation, disulfide bond formation, deamidation, isomerization, cleavage points in signal or leader sequence processing, covalent and non-covalently bound cofactors, oxidized variants, and the like. The formation of disulfide linked proteins is described (see, e.g., Woycechowsky and Raines (2000) *Curr. Opin. Chem. Biol.* 4:533-539; Creighton, *et al.* (1995) *Trends Biotechnol.* 13:18-23).

[0110] “Recombinant” when used with reference, e.g., to a nucleic acid, cell, animal, virus, plasmid, vector, or the like, indicates modification by the introduction of an exogenous, non-native nucleic acid, alteration of a native nucleic acid, or by derivation in whole or in part

from a recombinant nucleic acid, cell, virus, plasmid, or vector. Recombinant protein refers to a protein derived, e.g., from a recombinant nucleic acid, virus, plasmid, vector, or the like. "Recombinant bacterium" encompasses a bacterium where the genome is engineered by recombinant methods, e.g., by way of a mutation, deletion, insertion, and/or a rearrangement. "Recombinant bacterium" also encompasses a bacterium modified to include a recombinant extra-genomic nucleic acid, e.g., a plasmid or a second chromosome, or a bacterium where an existing extra-genomic nucleic acid is altered.

[0111] "Sample" refers to a sample from a human, animal, placebo, or research sample, e.g., a cell, tissue, organ, fluid, gas, aerosol, slurry, colloid, or coagulated material. The "sample" may be tested *in vivo*, e.g., without removal from the human or animal, or it may be tested *in vitro*. The sample may be tested after processing, e.g., by histological methods. "Sample" also refers, e.g., to a cell comprising a fluid or tissue sample or a cell separated from a fluid or tissue sample. "Sample" may also refer to a cell, tissue, organ, or fluid that is freshly taken from a human or animal, or to a cell, tissue, organ, or fluid that is processed or stored.

[0112] A "selectable marker" encompasses a nucleic acid that allows one to select for or against a cell that contains the selectable marker. Examples of selectable markers include, without limitation, e.g.: (1) A nucleic acid encoding a product providing resistance to an otherwise toxic compound (e.g., an antibiotic), or encoding susceptibility to an otherwise harmless compound (e.g., sucrose); (2) A nucleic acid encoding a product that is otherwise lacking in the recipient cell (e.g., tRNA genes, auxotrophic markers); (3) A nucleic acid encoding a product that suppresses an activity of a gene product; (4) A nucleic acid that encodes a product that can be readily identified (e.g., phenotypic markers such as beta-galactosidase, green fluorescent protein (GFP), cell surface proteins, an epitope tag, a FLAG tag); (5) A nucleic acid that can be identified by hybridization techniques, for example, PCR or molecular beacons.

[0113] "Specifically" or "selectively" binds, when referring to a ligand/receptor, nucleic acid/complementary nucleic acid, antibody/antigen, or other binding pair (e.g., a cytokine to a cytokine receptor) indicates a binding reaction which is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated conditions, a specified ligand binds to a particular receptor and does not bind in a significant amount to other proteins present in the sample. Specific binding can also mean, e.g., that the binding compound, nucleic acid ligand, antibody, or binding composition derived from the antigen-binding site of an antibody, of the contemplated method binds to its target with an affinity that is often at least 25% greater, more often at least 50% greater, most

often at least 100% (2-fold) greater, normally at least ten times greater, more normally at least 20-times greater, and most normally at least 100-times greater than the affinity with any other binding compound.

[0114] In a typical embodiment an antibody will have an affinity that is greater than about 10^9 liters/mol, as determined, e.g., by Scatchard analysis (Munsen, *et al.* (1980) *Analyt. Biochem.* 107:220-239). It is recognized by the skilled artisan that some binding compounds can specifically bind to more than one target, e.g., an antibody specifically binds to its antigen, to lectins by way of the antibody's oligosaccharide, and/or to an Fc receptor by way of the antibody's Fc region.

[0115] "Spread" of a bacterium encompasses "cell to cell spread," that is, transmission of the bacterium from a first host cell to a second host cell, as mediated, for example, by a vesicle. Functions relating to spread include, but are not limited to, e.g., formation of an actin tail, formation of a pseudopod-like extension, and formation of a double-membraned vacuole.

[0116] The "target site" of a recombinase is the nucleic acid sequence or region that is recognized, bound, and/or acted upon by the recombinase (see, e.g., U.S. Pat. No. 6,379,943 issued to Graham, *et al.*; Smith and Thorpe (2002) *Mol. Microbiol.* 44:299-307; Groth and Calos (2004) *J. Mol. Biol.* 335:667-678; Nunes-Duby, *et al.* (1998) *Nucleic Acids Res.* 26:391-406).

[0117] "Therapeutically effective amount" is defined as an amount of a reagent or pharmaceutical composition that is sufficient to show a patient benefit, i.e., to cause a decrease, prevention, or amelioration of the symptoms of the condition being treated. When the agent or pharmaceutical composition comprises a diagnostic agent, a "diagnostically effective amount" is defined as an amount that is sufficient to produce a signal, image, or other diagnostic parameter. Effective amounts of the pharmaceutical formulation will vary according to factors such as the degree of susceptibility of the individual, the age, gender, and weight of the individual, and idiosyncratic responses of the individual (see, e.g., U.S. Pat. No. 5,888,530 issued to Netti, *et al.*).

[0118] "Treatment" or "treating" (with respect to a condition or a disease) is an approach for obtaining beneficial or desired results including and preferably clinical results. For purposes of this invention, beneficial or desired results with respect to a disease include, but are not limited to, one or more of the following: improving a condition associated with a disease, curing a disease, lessening severity of a disease, delaying progression of a disease, alleviating one or more symptoms associated with a disease, increasing the quality of life of one suffering from a disease, and/or prolonging survival. Likewise, for purposes of this

invention, beneficial or desired results with respect to a condition include, but are not limited to, one or more of the following: improving a condition, curing a condition, lessening severity of a condition, delaying progression of a condition, alleviating one or more symptoms associated with a condition, increasing the quality of life of one suffering from a condition, and/or prolonging survival. For instance, in some embodiments where the compositions described herein are used for treatment of cancer, the beneficial or desired results include, but are not limited to, one or more of the following: reducing the proliferation of (or destroying) neoplastic or cancerous cells, reducing metastasis of neoplastic cells found in cancers, shrinking the size of a tumor, decreasing symptoms resulting from the cancer, increasing the quality of life of those suffering from the cancer, decreasing the dose of other medications required to treat the disease, delaying the progression of the cancer, and/or prolonging survival of patients having cancer. Depending on the context, "treatment" of a subject can imply that the subject is in need of treatment, e.g., in the situation where the subject comprises a disorder expected to be ameliorated by administration of a reagent.

[0119] "Vaccine" encompasses preventative vaccines. Vaccine also encompasses therapeutic vaccines, e.g., a vaccine administered to a mammal that comprises a condition or disorder associated with the antigen or epitope provided by the vaccine.

II. GENERAL.

[0120] The present invention provides reagents and methods useful for the treatment and diagnosis of cancer, tumors, precancerous disorders, and infections. Provided are nucleic acids, *Listeria* bacteria, and vaccines comprising a *Listeria* bacterium. The invention encompasses listerial cells that have been modified *in vitro*, including during storage, or *in vivo*, including products of bacterial cell division and products of bacterial deterioration.

[0121] Provided are nucleic acids encoding at least one heterologous antigen (heterologous to the *Listeria* bacterium). The heterologous antigen can be derived from a tumor, cancer cell, or and/or infective agent, e.g., a virus, bacterium, or protozoan. The heterologous antigen can also be a listerial antigen, for example, where the antigen is expressed in greater amounts than that which naturally occurs within the *Listeria* bacterium, where the listerial antigen is operably linked with a non-native regulatory sequence, or where the listerial antigen is modified to be attenuated or to increase its antigenicity.

[0122] Where a *Listeria* contains a nucleic acid encoding a heterologous antigen, the term "heterologous" encompasses, but is not necessarily limited to, an antigen from, or derived

from: (1) A non-listerial organism; (2) An antigen of synthetic origin; (3) An antigen of listerial origin where the nucleic acid is integrated at a position in the listerial genome that is different from that found in the wild type; and (4) An antigen of listerial origin, but where the nucleic acid is operably linked with a regulatory sequence not normally used in a wild type *Listeria*. The preceding commentary also applies to the term "heterologous antigen," when used, for example, in the context of a viral vector. Here, heterologous antigen encompasses antigens that are not from, and not derived from, that viral vector, as well as, for example, antigens from the viral vector that are controlled by a non-native nucleic acid regulatory sequence.

[0123] Provided are reagents and methods for stimulating the mammalian immune system, for reducing the number and/or size of tumors, for reducing metastasis, and for reducing titer of an infectious organism. The present invention also provides reagents and methods for improving survival of a cell, tissue, organ, or mammal, to a cancer or infection. The present invention also provides reagents and methods for improving survival of a cell (*in vivo* or *in vitro*), a tissue (*in vivo* or *in vitro*), an organ (*in vivo* or *in vitro*), an organism, a mammal, a veterinary subject, a research subject, or a human subject, to a cancer, tumor, or infection. What is encompassed is administration that is *in vivo* or *in vitro*, survival of the cell, tissue, or organ *in vitro* or *in vivo*, or any combination thereof. Any combination includes, e.g., administration that is *in vivo* where subsequent survival is *in vitro*, or administration that is *in vitro* and where subsequent survival is *in vivo*.

[0124] Provided is a *Listeria* comprising a polynucleotide encoding at least one heterologous antigen wherein the one polynucleotide is genomic. Also encompassed is a *Listeria* comprising a polynucleotide encoding at least one heterologous antigen, wherein the polynucleotide is genomic and not residing on a plasmid within the *Listeria*. Moreover, encompassed is a *Listeria* comprising a polynucleotide encoding at least one heterologous antigen, wherein the polynucleotide resides on a plasmid within the *Listeria*. Furthermore, what is provided is a *Listeria* comprising a polynucleotide encoding at least one heterologous antigen, where the polynucleotide resides on a plasmid and does not occur integrated in the genome. In another aspect, the present invention provides a *Listeria* comprising a polynucleotide encoding at least one heterologous antigen, where the polynucleotide is integrated in the genome and also separately resides in a plasmid.

[0125] The mouse is an accepted model for human immune response. In detail, mouse T cells are a model for human T cells, mouse dendritic cells (DCs) are a model for human DCs, mouse NK cells are a model for human NK cells, mouse NKT cells are a model for

human NKT cells, mouse innate response is an accepted model for human innate response, and so on. Model studies are disclosed, for example, for CD8⁺ T cells, central memory T cells, and effector memory T cells (see, e.g., Walzer, *et al.* (2002) *J. Immunol.* 168:2704-2711); the two subsets of NK cells (see, e.g., Chakir, *et al.* (2000) *J. Immunol.* 165:4985-4993; Smith, *et al.* (2000) *J. Exp. Med.* 191:1341-1354; Ehrlich, *et al.* (2005) *J. Immunol.* 174:1922-1931; Peritt, *et al.* (1998) *J. Immunol.* 161:5821-5824); NKT cells (see, e.g., Couedel, *et al.* (1998) *Eur. J. Immunol.* 28:4391-4397; Sakamoto, *et al.* (1999) *J. Allergy Clin. Immunol.* 103:S445-S451; Saikh, *et al.* (2003) *J. Infect. Dis.* 188:1562-1570; Emoto, *et al.* (1997) *Infection Immunity* 65:5003-5009; Taniguchi, *et al.* (2003) *Annu. Rev. Immunol.* 21:483-513; Sidobre, *et al.* (2004) *Proc. Natl. Acad. Sci.* 101:12254-12259); monocytes/macrophages (Sunderkotter, *et al.* (2004) *J. Immunol.* 172:4410-4417); the two lineages of DCs (Boonstra, *et al.* (2003) *J. Exp. Med.* 197:101-109; Donnenberg, *et al.* (2001) *Transplantation* 72:1946-1951; Becker (2003) *Virus Genes* 26:119-130; Carine, *et al.* (2003) *J. Immunol.* 171:6466-6477; Penna, *et al.* (2002) *J. Immunol.* 169:6673-6676; Alferink, *et al.* (2003) *J. Exp. Med.* 197:585-599).

[0126] Mouse innate response, including the Toll-Like Receptors (TLRs), is a model for human innate immune response, as disclosed (see, e.g., Janssens and Beyaert (2003) *Clinical Microb. Revs.* 16:637-646). Mouse neutrophils are an accepted model for human neutrophils (see, e.g., Kobayashi, *et al.* (2003) *Proc. Natl. Acad. Sci. USA* 100:10948-10953; Torres, *et al.* (2004) 72:2131-2139; Sibelius, *et al.* (1999) *Infection Immunity* 67:1125-1130; Tvinnereim, *et al.* (2004) *J. Immunol.* 173:1994-2002). Murine immune response to *Listeria* is an accepted model for human response to *Listeria* (see, e.g., Kolb-Maurer, *et al.* (2000) *Infection Immunity* 68:3680-3688; Brzoza, *et al.* (2004) *J. Immunol.* 173:2641-2651; Esplugues, *et al.* (2005) *Blood* Feb. 3 (epub ahead of print); Paschen, *et al.* (2000) *Eur. J. Immunol.* 30:3447-3456; Way and Wilson (2004) *J. Immunol.* 173:5918-5922; Ouadrhiri, *et al.* (1999) *J. Infectious Diseases* 180:1195-1204; Neighbors, *et al.* (2001) *J. Exp. Med.* 194:343-354; Calorini, *et al.* (2002) *Clin. Exp. Metastasis* 19:259-264; Andersson, *et al.* (1998) *J. Immunol.* 161:5600-5606; Flo, *et al.* (2000) *J. Immunol.* 164:2064-2069; Calorini, *et al.* (2002) *Clin. Exp. Metastasis* 19:259-264; Brzoza, *et al.* (2004) *J. Immunol.* 173:2641-2651; Brzoza, *et al.* (2004) *J. Immunol.* 173:2641-2651; Cleveland, *et al.* (1996) *Infection Immunity* 64:1906-1912; Andersson, *et al.* (1998) *J. Immunol.* 161:5600-5606).

[0127] U.S. Patent Publication Nos. 2004/0228877 and 2004/0197343, each of which is incorporated by reference herein in its entirety, describe *Listeria* useful in some embodiments of the present invention. U.S. Patent Publication No. 2005/0249748, incorporated by

reference herein in its entirety, further describes *Listeria* and polynucleotides useful in some embodiments of the present invention.

(a). Secretory or signal sequences.

[0128] The present invention embraces a nucleic acid encoding a secretory sequence, or encoding a listerial protein, or a fragment thereof, suitable for use as a fusion protein partner. What is encompassed is a nucleic acid encoding:

- i. a secretory sequence,
- ii. a signal sequence,
- iii. a listerial polypeptide containing its native secretory sequence,
- iv. a listerial protein with its native secretory sequence replaced with that of another listerial protein,
- v. a listerial protein with its native secretory sequence replaced with the secretory sequence of a non-listerial bacterial protein,
- vi. a non-secreted listerial protein, or fragment thereof, not containing any secretory sequence; and
- vii. a non-listerial bacterial secretory sequence fused with, and in frame with, a non-secreted listerial protein, or fragment thereof.

[0129] These embodiments can encompass the following listerial proteins, and fragments or domains thereof:

- i. Listeriolysin (LLO). The secretory signal sequence of listeriolysin O (hly gene) has been identified (see, e.g., Lety, *et al.* (2003) Microbiol. 149:1249-1255).
- ii. ActA. The ribosomal binding site, promoter, and signal sequence have been identified for listerial ActA. The ribosomal binding site occurs 6 bp upstream of the start codon of the ActA gene (Vazquez-Boland, *et al.* (1992) Infect. Immunity 60:219-230).
- iii. Internalins. All of the internalin (Inl) proteins contain an N-terminal sequence of 30-35 amino acids with characteristics of bacterial signal peptides (see, e.g., Dramsi, *et al.* (1997) Infect. Immunity 65:1615-1625).
- iv. p60 (iap gene). A 27-amino acid region between the start codon and nucleotide 524 functions as a signal sequence, and directs transport of p60 across the *Listeria* cell membrane (Kohler, *et al.* (1990) Infect. Immunity 58:1943-1950). Kohler, *et al.*, *supra*, also disclose a purine-rich ribosome (16S RNA) binding site of the p60 mRNA of *L. monocytogenes*.

[0130] Table 1 discloses a number of non-limiting examples of signal peptides for use in fusing with a fusion protein partner sequence such as a heterologous antigen. The SignalP

algorithm can be used to determine signal sequences in Gram positive bacteria. This program is available on the world wide web at: cbs.dtu.dk/services/SignalP/. Signal peptides tend to contain three domains: a positively charged N-terminus (1-5 residues long); a central hydrophobic comain (7-15 residues long); and a neutral but polar C-terminal domain (see, e.g., Lety, *et al.* (2003) Microbiology 149:1249-1255; Paetzel, *et al.* (2000) Pharmacol. Ther. 87:27-49). As signal peptides and secretory sequences encoded by a *Listeria* genome, or by a genome or plasmid of another bacterium, are not necessarily codon optimized for optimal expression in *Listeria*, the present invention also provides nucleic acids originating from the *Listeria* genome, or from a genome or plasmid of another bacterium, that are altered by codon optimized for expressing by a *L. monocytogenes*. The present invention is not to be limited to polypeptide and peptide antigens that are secreted, but also embraces polypeptides and peptides that are not secreted or cannot be secreted from a *Listeria* or other bacterium.

Table 1. Bacterial signal pathway. Signal peptides are identified by the signal peptidase site.

Signal peptidase site (cleavage site represented by ')	Gene	Genus/species
secA1 pathway		
TEA'KD (SEQ ID NO:126)	<i>hly</i> (LLO)	<i>Listeria monocytogenes</i>
VYA'DT (SEQ ID NO: 127)	Usp45	<i>Lactococcus lactis</i> (see, e.g., Steidler, <i>et al.</i> (2003) Nat. Biotech. 21:785-789; Schotte, <i>et al.</i> (2000) Enzyme Microb. Technol. 27:761-765).
IQA'EV (SEQ ID NO: 128)	<i>pag</i> (protective antigen)	<i>Bacillus anthracis</i>
secA2 pathway		
ASA'ST (SEQ ID NO:129)	<i>iap</i> (invasion-associated protein) p60	<i>Listeria monocytogenes</i>
VGA'FG (SEQ ID NO: 130)	NamA lmo2691 (autolysin)	<i>Listeria monocytogenes</i>
AFA'ED (SEQ ID NO: 131)	* BA_0281 (NLP/P60 Family)	<i>Bacillus anthracis</i>
VQA'AE (SEQ ID NO: 132)	* <i>atl</i> (autolysin)	<i>Staphylococcus aureus</i>
Tat pathway		
DKA'LT (SEQ ID NO: 133)	lmo0367	<i>Listeria monocytogenes</i>
VGA'FG (SEQ ID NO: 134)	PhoD (alkaline phosphatase)	<i>Bacillus subtilis</i>
* Bacterial autolysins secreted by sec pathway (not determined whether secA1 or secA2). Secretory sequences are encompassed by the indicated nucleic acids encoded by the <i>Listeria</i> EGD genome (GenBank Acc. No. NC_003210) at, e.g., nucleotides 45434-456936 (inlA); nucleotides 457021-457125 (inlB); nucleotides 1860200-1860295 (inlC); nucleotides 286219-287718 (inlE); nucleotides 205819-205893 (<i>hly</i> gene; LLO) (see also GenBank Acc. No. P13128); nucleotides 209470-209556 (<i>ActA</i>) (see also GenBank Acc. No. S20887). The referenced nucleic acid sequences, and corresponding translated amino acid sequences, and the cited amino acid sequences, and the corresponding nucleic acid sequences associated with or cited in that reference, are incorporated by reference herein in their entirety.		

[0131] In some embodiments, the polypeptide comprising the heterologous antigen that is expressed by the *Listeria* comprises a signal sequence which is a non-listerial signal sequence. In some embodiments, the signal sequence is a secA1 signal peptide. In some other embodiments, the signal sequence is a secA2 or Tat signal peptide. In some embodiments, the signal peptide is an actA signal peptide. In some embodiments, the signal peptide used to effect secretion of the heterologous antigen is selected from p60 signal sequence, a *Listeria monocytogenes* LLO signal sequence, a *Bacillus anthracis* Protective Antigen (BaPa) signal sequence, a *Lactococcus lactis* usp45 signal sequence, and a *Bacillus subtilis* PhoD signal sequence. Signal peptides that can be used for the expression and secretion of heterologous antigens are described in, e.g., U.S. Patent Publication No. 2005/0249748, incorporated by reference herein in its entirety.

(b). Codon optimization.

[0132] The present invention, in certain embodiments, provides codon optimization of a nucleic acid heterologous to *Listeria*, or of a nucleic acid endogenous to *Listeria*. The optimal codons utilized by *L. monocytogenes* for each amino acid are shown (Table 2). A nucleic acid is codon-optimized if at least one codon in the nucleic acid is replaced with a codon that is more frequently used by *L. monocytogenes* for that amino acid than the codon in the original sequence.

[0133] Normally, at least one percent of any non-optimal codons are changed to provide optimal codons, more normally at least five percent are changed, most normally at least ten percent are changed, often at least 20% are changed, more often at least 30% are changed, most often at least 40%, usually at least 50 % are changed, more usually at least 60% are changed, most usually at least 70% are changed, optimally at least 80% are changed, more optimally at least 90% are changed, most optimally at least 95% are changed, and conventionally 100% of any non-optimal codons are codon-optimized for *Listeria* expression (Table 2).

Table 2. Optimal codons for expression in *Listeria*.

Amino Acid	A	R	N	D	C	Q	E	G	H	I
Optimal <i>Listeria</i> codon	GCA	CGU	AAU	GAU	UGU	CAA	GAA	GGU	CAU	AUU
Amino Acid	L	K	M	F	P	S	T	W	Y	V

Optimal <i>Listeria</i> codon	UUA	AAA	AUG	UUU	CCA	AGU	ACA	UGG	UAU	GUU
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[0134] In some embodiments, the nucleic acid encoding the heterologous antigen is codon-optimized for expression in *Listeria monocytogenes*. In some embodiments, the polynucleotide encoding the fusion protein comprising the modified ActA or an alternative signal peptide sequence and the heterologous antigen is codon-optimized.

[0135] Additional information regarding the codon-optimization of nucleotide sequences for expression in *Listeria monocytogenes* can be found in U.S. Patent Publication No. 2005/0249748, incorporated by reference herein in its entirety.

(c). **Virulence factors and attenuation.**

[0136] *L. monocytogenes* expresses various genes and gene products that contribute to invasion, growth, or colonization of the host (Table 3). Some of these are classed as "virulence factors." These virulence factors include ActA, listeriolysin (LLO), protein 60 (p60), internalin A (inlA), internalin B (inlB), phosphatidylcholine phospholipase C (PC-PLC), phosphatidylinositol-specific phospholipase C (PI-PLC; plcA gene). A number of other internalins have been characterized, e.g., InlC2, InlD, InlE, and InlF (Dramsi, *et al.* (1997) Infect. Immunity 65:1615-1625). Mpl, a metalloprotease that processes proPL-PLC to active PL-PLC, is also a virulence factor (Chakraborty, *et al.* (2000) Int. J. Med. Microbiol. 290:167-174; Williams, *et al.* (2000) J. Bact. 182:837-841). In some embodiments, a virulence gene is a gene that encodes a virulence factor. Without limiting the present invention to the attenuated genes disclosed herein, the present invention supplies a *Listeria* that is altered, mutated, or attenuated in one or more of the sequences of Table 3.

[0137] In some embodiments, the virulence gene is a prf-A dependent gene. In other embodiments, the virulence gene is a prf-A independent gene.

[0138] In some embodiments, the *Listeria* comprises an attenuating mutation in *actA* and/or *inlB*. In some embodiments, a polynucleotide encoding a heterologous antigen has been integrated into the *actA* and/or *inlB* gene.

[0139] DNA repair genes can also be the target of an attenuating mutation. Mutating or deleting a DNA repair gene can result in an attenuated bacterium (see, e.g., Darwin and Nathan (2005) Infection Immunity 73:4581-4587).

Table 3. Sequences of *L. monocytogenes* nucleic acids and proteins.

Protein/Gene	Nucleotides	GenBank Acc. No.
Actin assembly inducing protein precursor (ActA gene)	209470-211389 (coding sequence) 209456-211389 (gene)	NC_003210
ActA in various <i>L. monocytogenes</i> subtypes.	--	AF497169; AF497170; AF497171; AF497172; AF497173; AF497174; AF497175; AF497176; AF497177; AF497178; AF497179; AF497180; AF497181; AF497182; AF497183 (Lasa, <i>et al.</i> (1995) Mol. Microbiol. 18:425-436).
Listeriolysin O precursor (LLO) (hly gene)	205819-207408	NC_003210
Internalin A (InlA)	454534-456936	NC_003210
Internalin B (InlB)	457021-458913	NC_003210
SvpA	---	Bierne, <i>et al.</i> (2004) J. Bacteriol. 186:1972-1982; Borezee, <i>et al.</i> (2000) Microbiology 147:2913-2923.
p104 (a.k.a. LAP)		Pandiripally, <i>et al.</i> (1999) J. Med. Microbiol. 48:117-124; Jaradat, <i>et al.</i> (2003) Med. Microbiol. Immunol. 192:85-91.
Phosphatidylinositol-specific phospholipase C (PI-PLC) (plcA gene)	204624-205577	NC_003210
Phosphatidylcholine-specific phospholipase C (PC-PLC) (plcB gene)	1-3031	X59723
Zinc metalloprotease precursor (Mpl)	207739-209271	NC_003210
p60 (protein 60; invasion associated protein (iap)).	Complement of 618932-620380	NC_003210 (Lenz, <i>et al.</i> (2003) Proc. Natl. Acad. Sci. USA 100:12432-12437).
Sortase	966245-966913	NC_003210
Listeriolysin positive regulatory protein (PrfA gene)	203607-203642	NC_003210
Listeriolysin positive regulatory protein (PrfA gene)	1-801	AY318750

PrfB gene	2586114-2587097	NC_003210
FbpA gene	570 amino acids	Dramsi, <i>et al.</i> (2004) Mol. Microbiol. 53:639-649.
Auto gene	--	Cabanes, <i>et al.</i> (2004) Mol. Microbiol. 51:1601-1614.
Ami (amidase that mediates adhesion)	--	Dussurget, <i>et al.</i> (2004) Annu. Rev. Microbiol. 58:587-610.
dlt operon (dltA; dltB; dltC; dltD).	487-2034 (dltA)	GenBank Acc. No: AJ012255 (Abachin, <i>et al.</i> (2002) Mol. Microbiol. 43:1-14.)
prfA boxes	--	Dussurget, <i>et al.</i> (2002) Mol. Microbiol. 45:1095-1106.
Htp (sugar-P transporter)	1-1386	GenBank Acc. No. AJ315765 (see, e.g., Milohanic, <i>et al.</i> (2003) Mol. Microbiol. 47:1613-1625).
The referenced nucleic acid sequences, and corresponding translated amino acid sequences, and the cited amino acid sequences, and the corresponding nucleic acid sequences associated with or cited in that reference, are incorporated by reference herein in their entirety.		

[0140] Listeriolysin (LLO) biology is described (see, e.g., Glomski, *et al.* (2003) Infect. Immun. 71:6754-6765; Gedde, *et al.* (2000) Infect. Immun. 68:999-1003; Glomski, *et al.* (2002) J. Cell Biol. 156:1029-1038; Dubail, *et al.* (2001) Microbiol. 147:2679-2688; Dramsi and Cossart (2002) J. Cell Biol. 156:943-946). ActA biochemistry and physiology is disclosed (see, e.g., Machner, *et al.* (2001) J. Biol. Chem. 276:40096-40103; Lauer, *et al.* (2001) Mol. Microbiol. 42:1163-1177; Portnoy, *et al.* (2002) J. Cell Biol. 158:409-414). Internalin biochemistry and physiology is available (see, e.g., Bierne and Cossart (2000) J. Cell Sci. 115:3357-3367; Schluter, *et al.* (1998) Infect. Immun. 66:5930-5938; Dormann, *et al.* (1997) Infect. Immun. 65:101-109). Sortase proteins are described (see, e.g., Bierne, *et al.* (2002) Mol. Microbiol. 43:869-881). Two phospholipases, PI-PLC (encoded by plcA gene) and PC-PLC (encoded by plcB gene) are disclosed (see, e.g., Camilli, *et al.* (1993) Mol. Microbiol. 8:143-157; Schuster, *et al.* (1998) Infect. Immun. 66:5930-5938). Protein p60 is described (Pilgrim, *et al.* (2003) Infect. Immun. 71:3473-3484).

[0141] The invention also contemplates a *Listeria* attenuated in at least one regulatory factor, e.g., a promoter or a transcription factor. The following concerns promoters. ActA expression is regulated by two different promoters (Lauer, *et al.* (2002) J. Bacteriol.

184:4177-4186). Together, *inlA* and *inlB* are regulated by five promoters (Lingnau, *et al.* (1995) *Infect. Immun.* 63:3896-3903). The transcription factor *prfA* is required for transcription of a number of *L. monocytogenes* genes, e.g., *hly*, *plcA*, *ActA*, *mpl*, *prfA*, and *iap*. *PrfA*'s regulatory properties are mediated by, e.g., the *PrfA*-dependent promoter (*PinlC*) and the *PrfA*-box. The present invention, in certain embodiments, provides a nucleic acid encoding inactivated, mutated, or deleted in at least one of *ActA* promoter, *inlB* promoter, *PrfA*, *PinlC*, *PrfA*-box, and the like (see, e.g., Lalic-Mullthaler, *et al.* (2001) *Mol. Microbiol.* 42:111-120; Shetron-Rama, *et al.* (2003) *Mol. Microbiol.* 48:1537-1551; Luo, *et al.* (2004) *Mol. Microbiol.* 52:39-52). *PrfA* can be made constitutively active by a Gly145Ser mutation, Gly155Ser mutation, or Glu77Lys mutation (see, e.g., Mueller and Freitag (2005) *Infect. Immun.* 73:1917-1926; Wong and Freitag (2004) *J. Bacteriol.* 186:6265-6276; Ripio, *et al.* (1997) *J. Bacteriol.* 179:1533-1540).

[0142] Attenuation can be effected by, e.g., heat-treatment or chemical modification. Attenuation can also be effected by genetic modification of a nucleic acid that modulates; e.g., metabolism, extracellular growth, or intracellular growth, genetic modification of a nucleic acid encoding a virulence factor, such as *listerial prfA*, *ActA*, listeriolysin (LLO), an adhesion mediating factor (e.g., an internalin such as *inlA* or *inlB*), *mpl*, phosphatidylcholine phospholipase C (PC-PLC), phosphatidylinositol-specific phospholipase C (PI-PLC; *plcA* gene), any combination of the above, and the like. Attenuation can be assessed by comparing a biological function of an attenuated *Listeria* with the corresponding biological function shown by an appropriate parent *Listeria*.

[0143] The present invention, in other embodiments, provides a *Listeria* that is attenuated by treating with a nucleic acid targeting agent, such as a cross-linking agent, a psoralen, a nitrogen mustard, cis-platin, a bulky adduct, ultraviolet light, gamma irradiation, any combination thereof, and the like. Typically, the lesion produced by one molecule of cross-linking agent involves cross-linking of both strands of the double helix. The *Listeria* of the invention can also be attenuated by mutating at least one nucleic acid repair gene, e.g., *uvrA*, *uvrB*, *uvrAB*, *uvrC*, *uvrD*, *uvrAB*, *phrA*, and/or a gene mediating recombinational repair, e.g., *recA*. Moreover, the invention provides a *Listeria* attenuated by both a nucleic acid targeting agent and by mutating a nucleic acid repair gene. Additionally, the invention encompasses treating with a light sensitive nucleic acid targeting agent, such as a psoralen, and/or a light sensitive nucleic acid cross-linking agent, such as psoralen, followed by exposure to ultraviolet light.

[0144] In some embodiments, the *Listeria* of the invention is attenuated. Attenuated *Listeria* useful in the present invention are described in, e.g., in U.S. Pat. Publ. Nos. 2004/0228877 and 2004/0197343, each of which is incorporated by reference herein in its entirety. Various assays for assessing whether a particular strain of *Listeria* has the desired attenuation are provided, e.g., in U.S. Pat. Publ. Nos. 2004/0228877, 2004/0197343, and 2005/0249748, each of which is incorporated by reference herein in its entirety.

(d). *Listeria* strains.

[0145] The invention supplies a number of listerial species and strains for making or engineering an attenuated *Listeria* of the present invention (Table 4). Each of the references listed in Table 4 is incorporated by reference herein in its entirety. The *Listeria* of the present invention is not to be limited by the species and strains disclosed in this table.

Table 4. Strains of *Listeria* suitable for use in the present invention, e.g., as a vaccine or as a source of nucleic acids.

<i>L. monocytogenes</i> 10403S wild type.	Bishop and Hinrichs (1987) J. Immunol. 139:2005-2009; Lauer, <i>et al.</i> (2002) J. Bact. 184:4177-4186.
<i>L. monocytogenes</i> DP-L4056 (phage cured). The prophage-cured 10403S strain is designated DP-L4056.	Lauer, <i>et al.</i> (2002) J. Bact. 184:4177-4186.
<i>L. monocytogenes</i> DP-L4027, which is DP-L2161, phage cured, deleted in hly gene.	Lauer, <i>et al.</i> (2002) J. Bact. 184:4177-4186; Jones and Portnoy (1994) Infect. Immunity 65:5608-5613.
<i>L. monocytogenes</i> DP-L4029, which is DP-L3078, phage cured, deleted in ActA.	Lauer, <i>et al.</i> (2002) J. Bact. 184:4177-4186; Skoble, <i>et al.</i> (2000) J. Cell Biol. 150:527-538.
<i>L. monocytogenes</i> DP-L4042 (delta PEST)	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> DP-L4097 (LLO-S44A).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> DP-L4364 (delta lplA; lipoate protein ligase).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> DP-L4405 (delta inlA).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> DP-L4406 (delta inlB).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> CS-L0001 (delta ActA-delta inlB).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> CS-L0002 (delta ActA-delta lplA).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> CS-L0003 (L461T-delta lplA).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> DP-L4038 (delta ActA-LLO L461T).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.

<i>L. monocytogenes</i> DP-L4384 (S44A-LLO L461T).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> . Mutation in lipoate protein ligase (LplA1).	O'Riordan, <i>et al.</i> (2003) Science 302:462-464.
<i>L. monocytogenes</i> DP-L4017 (10403S hly (L461T) point mutation in hemolysin gene.	U.S. Provisional Pat. Appl. Ser. No. 60/490,089 filed July 24, 2003.
<i>L. monocytogenes</i> EGD.	GenBank Acc. No. AL591824.
<i>L. monocytogenes</i> EGD-e.	GenBank Acc. No. NC_003210. ATCC Acc. No. BAA-679.
<i>L. monocytogenes</i> strain EGD, complete genome, segment 3/12	GenBank Acc. No. AL591975
<i>L. monocytogenes</i> .	ATCC Nos. 13932; 15313; 19111-19120; 43248-43251; 51772-51782.
<i>L. monocytogenes</i> DP-L4029 deleted in <i>uvrAB</i> .	U.S. Provisional Pat. Appl. Ser. No. 60/541,515 filed February 2, 2004; U.S. Provisional Pat. Appl. Ser. No. 60/490,080 filed July 24, 2003.
<i>L. monocytogenes</i> DP-L4029 deleted in <i>uvrAB</i> treated with a psoralen.	U.S. Provisional Pat. Appl. Ser. No. 60/541,515 filed February 2, 2004.
<i>L. monocytogenes</i> ActA/ <i>inlB</i> - double mutant.	Deposited with ATCC on October 3, 2003. Acc. No. PTA-5562.
<i>L. monocytogenes</i> <i>lplA</i> mutant or <i>hly</i> mutant.	U.S. Pat. Applic. No. 20040013690 of Portnoy, <i>et al.</i>
<i>L. monocytogenes</i> DAL/DAT double mutant.	U.S. Pat. Applic. No. 20050048081 of Frankel and Portnoy.
<i>L. monocytogenes</i> str. 4b F2365.	GenBank Acc. No. NC_002973.
<i>Listeria ivanovii</i>	ATCC No. 49954
<i>Listeria innocua</i> Clip1 1262.	GenBank Acc. No. NC_003212; AL592022.
<i>Listeria innocua</i> , a naturally occurring hemolytic strain containing the PrfA-regulated virulence gene cluster.	Johnson, <i>et al.</i> (2004) Appl. Environ. Microbiol. 70:4256-4266.
<i>Listeria seeligeri</i> .	Howard, <i>et al.</i> (1992) Appl. Environ. Microbiol. 58:709-712.
<i>Listeria innocua</i> with <i>L. monocytogenes</i> pathogenicity island genes.	Johnson, <i>et al.</i> (2004) Appl. Environ. Microbiol. 70:4256-4266.
<i>Listeria innocua</i> with <i>L. monocytogenes</i> internalin A gene, e.g., as a plasmid or as a genomic nucleic acid.	See, e.g., Lingnau, <i>et al.</i> (1995) Infection Immunity 63:3896-3903; Gaillard, <i>et al.</i> (1991) Cell 65:1127-1141).
The present invention encompasses reagents and methods that comprise the above listerial strains, as well as these strains that are modified, e.g., by a plasmid and/or by genomic integration, to contain a nucleic acid encoding one of, or any combination of, the following genes: <i>hly</i> (LLO; listeriolysin); <i>iap</i> (p60); <i>inlA</i> ; <i>inlB</i> ; <i>inlC</i> ; <i>dal</i> (alanine racemase); <i>daaA</i> (<i>dat</i> ; D-amino acid aminotransferase); <i>plcA</i> ; <i>plcB</i> ; <i>ActA</i> ; or any nucleic acid that mediates growth, spread, breakdown of a single walled vesicle, breakdown of a double walled vesicle, binding to a host cell, uptake by a host cell. The present invention is not to be limited by the particular strains disclosed above.	

(e). Antigenes.

[0146] The present invention, in certain embodiments, provides a nucleic acid encoding at least one antigen, an antigen with one or more conservative changes, one or more epitopes from a specified antigen, or a peptide or polypeptide that is immunologically cross-reactive with an antigen (Table 5). The nucleic acids and antigens of the invention are not to be limited to those disclosed in the table.

Table 5. Antigens.

Antigen	Reference
Tumor antigens	
Mesothelin	GenBank Acc. No. NM_005823; U40434; NM_013404; BC003512 (see also, e.g., Hassan, <i>et al.</i> (2004) Clin. Cancer Res. 10:3937-3942; Muminova, <i>et al.</i> (2004) BMC Cancer 4:19; Iacobuzio-Donahue, <i>et al.</i> (2003) Cancer Res. 63:8614-8622).
Wilms' tumor-1 associated protein (Wt-1), including isoform A; isoform B; isoform C; isoform D.	WT-1 isoform A (GenBank Acc. Nos. NM_000378; NP_000369). WT-1 isoform B (GenBank Acc. Nos. NM_024424; NP_077742). WT-1 isoform C (GenBank Acc. Nos. NM_024425; NP_077743). WT-1 isoform D (GenBank Acc. Nos. NM_024426; NP_077744).
Stratum corneum chymotryptic enzyme (SCCE), and variants thereof.	GenBank Acc. No. NM_005046; NM_139277; AF332583. See also, e.g., Bondurant, <i>et al.</i> (2005) Clin. Cancer Res. 11:3446-3454; Santin, <i>et al.</i> (2004) Gynecol. Oncol. 94:283-288; Shigemasa, <i>et al.</i> (2001) Int. J. Gynecol. Cancer 11:454-461; Sepehr, <i>et al.</i> (2001) Oncogene 20:7368-7374.
MHC class I chain-related protein A (MICA); MHC class I chain-related protein A (MICB).	See, e.g., Groh, <i>et al.</i> (2005) Proc. Natl. Acad. Sci. USA 102:6461-6466; GenBank Acc. Nos. NM_000247; BC_016929; AY750850; NM_005931.
Gastrin and peptides derived from gastrin; gastrin/CCK-2 receptor (also known as CCK-B).	Harris, <i>et al.</i> (2004) Cancer Res. 64:5624-5631; Gilliam, <i>et al.</i> (2004) Eur. J. Surg. Oncol. 30:536-543; Laheru and Jaffee (2005) Nature Reviews Cancer 5:459-467.
Glypican-3 (an antigen of, e.g., hepatocellular carcinoma and melanoma).	GenBank Acc. No. NM_004484. Nakatsura, <i>et al.</i> (2003) Biochem. Biophys. Res. Commun. 306:16-25; Capurro, <i>et al.</i> (2003) Gastroenterol. 125:89-97; Nakatsura, <i>et al.</i> (2004) Clin. Cancer Res. 10:6612-6621).
Coactosin-like protein.	Nakatsura, <i>et al.</i> (2002) Eur. J. Immunol. 32:826-836; Laheru and Jaffee (2005) Nature Reviews Cancer 5:459-467.
Prostate stem cell antigen (PSCA).	GenBank Acc. No. AF043498; AR026974; AR302232 (see also, e.g., Argani, <i>et al.</i> (2001) Cancer Res. 61:4320-4324; Christiansen, <i>et al.</i> (2003) Prostate 55:9-19; Fuessel, <i>et al.</i> (2003) 23:221-228).

Prostate acid phosphatase (PAP); prostate-specific antigen (PSA); PSM; PSMA.	Small, <i>et al.</i> (2000) J. Clin. Oncol. 18:3894-3903; Altwein and Luboldt (1999) Urol. Int. 63:62-71; Chan, <i>et al.</i> (1999) Prostate 41:99-109; Ito, <i>et al.</i> (2005) Cancer 103:242-250; Schmittgen, <i>et al.</i> (2003) Int. J. Cancer 107:323-329; Millon, <i>et al.</i> (1999) Eur. Urol. 36:278-285.
Six-transmembrane epithelial antigen of prostate (STEAP).	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. NM_018234; NM_001008410; NM_182915; NM_024636; NM_012449; BC011802.
Prostate carcinoma tumor antigen-1 (PCTA-1).	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. L78132.
Prostate tumor-inducing gene-1 (PTI-1).	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442).
Prostate-specific gene with homology to G protein-coupled receptor.	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442).
Protease (an antrophen regulated serine protease).	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. BC096178; BC096176; BC096175.
Proteinase 3.	GenBank Acc. No. X55668.
Cancer-testis antigens, e.g., NY-ESO-1; SCP-1; SSX-1; SSX-2; SSX-4; GAGE, CT7; CT8; CT10; MAGE-1; MAGE-2; MAGE-3; MAGE-4; MAGE-6; LAGE-1.	GenBank Acc. No. NM_001327 (NY-ESO-1) (see also, e.g., Li, <i>et al.</i> (2005) Clin. Cancer Res. 11:1809-1814; Chen, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. U S A. 101(25):9363-9368; Kubuschok, <i>et al.</i> (2004) Int. J. Cancer. 109:568-575; Scanlan, <i>et al.</i> (2004) Cancer Immun. 4:1; Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047; Scanlan, <i>et al.</i> (2000) Cancer Lett. 150:155-164; Dalerba, <i>et al.</i> (2001) Int. J. Cancer 93:85-90; Ries, <i>et al.</i> (2005) Int. J. Oncol. 26:817-824.
MAGE-A1, MAGE-A2; MAGE-A3; MAGE-A4; MAGE-A6; MAGE-A9; MAGE-A10; MAGE-A12; GAGE-3/6; NT-SAR-35; BAGE; CA125.	Otte, <i>et al.</i> (2001) Cancer Res. 61:6682-6687; Lee, <i>et al.</i> (2003) Proc. Natl. Acad. Sci. USA 100:2651-2656; Sarcevic, <i>et al.</i> (2003) Oncology 64:443-449; Lin, <i>et al.</i> (2004) Clin. Cancer Res. 10:5708-5716.
GAGE-1; GAGE-2; GAGE-3; GAGE-4; GAGE-5; GAGE-6; GAGE-7; GAGE-8; GAGE-65; GAGE-11; GAGE-13; GAGE-7B.	De Backer, <i>et al.</i> (1999) Cancer Res. 59:3157-3165; Scarcella, <i>et al.</i> (1999) Clin. Cancer Res. 5:335-341.
HIP1R; LMNA; KIAA1416; Seb4D; KNSL6; TRIP4; MBD2; HCAC5; MAGEA3.	Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047.
DAM family of genes, e.g., DAM-1; DAM-6.	Fleishhauer, <i>et al.</i> (1998) Cancer Res. 58:2969-2972.
RCAS1.	Enjoji, <i>et al.</i> (2004) Dig. Dis. Sci. 49:1654-1656.
RU2.	Van Den Eynde, <i>et al.</i> (1999) J. Exp. Med. 190:1793-1800.
CAMEL.	Slager, <i>et al.</i> (2004) J. Immunol. 172:5095-5102; Slager, <i>et al.</i> (2004) Cancer Gene Ther. 11:227-236.

Colon cancer associated antigens, e.g., NY-CO-8; NY-CO-9; NY-CO-13; NY-CO-16; NY-CO-20; NY-CO-38; NY-CO-45; NY-CO-9/HDAC5; NY-CO-41/MBD2; NY-CO-42/TRIP4; NY-CO-95/KIAA1416; KNSL6; seb4D.	Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047.
N-Acetylglucosaminyl-transferase V (GnT-V).	Dosaka-Akita, <i>et al.</i> (2004) Clin. Cancer Res. 10:1773-1779.
Elongation factor 2 mutated (ELF2M).	Renkvist, <i>et al.</i> (2001) Cancer Immunol Immunother. 50:3-15.
HOM-MEL-40/SSX2	Neumann, <i>et al.</i> (2004) Int. J. Cancer 112:661-668; Scanlan, <i>et al.</i> (2000) Cancer Lett. 150:155-164.
BRDT.	Scanlan, <i>et al.</i> (2000) Cancer Lett. 150:155-164.
SAGE; HAGE.	Sasaki, <i>et al.</i> (2003) Eur. J. Surg. Oncol. 29:900-903.
RAGE.	See, e.g., Li, <i>et al.</i> (2004) Am. J. Pathol. 164:1389-1397; Shirasawa, <i>et al.</i> (2004) Genes to Cells 9:165-174.
MUM-1 (melanoma ubiquitous mutated); MUM-2; MUM-2 Arg-Gly mutation; MUM-3.	Gueguen, <i>et al.</i> (1998) J. Immunol. 160:6188-6194; Hirose, <i>et al.</i> (2005) Int. J. Hematol. 81:48-57; Baurain, <i>et al.</i> (2000) J. Immunol. 164:6057-6066; Chiari, <i>et al.</i> (1999) Cancer Res. 59:5785-5792.
LDLR/FUT fusion protein antigen of melanoma.	Wang, <i>et al.</i> (1999) J. Exp. Med. 189:1659-1667.
NY-REN series of renal cancer antigens.	Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047; Scanlan, <i>et al.</i> (1999) Cancer Res. 59:456-464.
NY-BR series of breast cancer antigens, e.g., NY-BR-62; NY-BR-75; NY-BR-85; NY-BR-62; NY-BR-85.	Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047; Scanlan, <i>et al.</i> (2001) Cancer Immunity 1:4.
BRCA-1; BRCA-2.	Stolier, <i>et al.</i> (2004) Breast J. 10:475-480; Nicoletto, <i>et al.</i> (2001) Cancer Treat Rev. 27:295-304.
DEK/CAN fusion protein.	von Lindern, <i>et al.</i> (1992) Mol. Cell. Biol. 12:1687-1697.
Ras, e.g., wild type ras, ras with mutations at codon 12, 13, 59, or 61, e.g., mutations G12C; G12D; G12R; G12S; G12V; G13D; A59T; Q61H. K-RAS; H-RAS; N-RAS.	GenBank Acc. Nos. P01112; P01116; M54969; M54968; P01111; P01112; K00654. See also, e.g., GenBank Acc. Nos. M26261; M34904; K01519; K01520; BC006499; NM_006270; NM_002890; NM_004985; NM_033360; NM_176795; NM_005343.

BRAF (an isoform of RAF).	Tannapfel, <i>et al.</i> (2005) <i>Am. J. Clin. Pathol.</i> 123:256-260; Tsao and Sober (2005) <i>Dermatol. Clin.</i> 23:323-333.
Melanoma antigens, including HST-2 melanoma cell antigens.	GenBank Acc. No. NM_206956; NM_206955; NM_206954; NM_206953; NM_006115; NM_005367; NM_004988; AY148486; U10340; U10339; M77481. See, e.g., Suzuki, <i>et al.</i> (1999) <i>J. Immunol.</i> 163:2783-2791.
Survivin	GenBank Acc. No. AB028869; U75285 (see also, e.g., Tsuruma, <i>et al.</i> (2004) <i>J. Translational Med.</i> 2:19 (11 pages); Pisarev, <i>et al.</i> (2003) <i>Clin. Cancer Res.</i> 9:6523-6533; Siegel, <i>et al.</i> (2003) <i>Br. J. Haematol.</i> 122:911-914; Andersen, <i>et al.</i> (2002) <i>Histol. Histopathol.</i> 17:669-675).
MDM-2	NM_002392; NM_006878 (see also, e.g., Mayo, <i>et al.</i> (1997) <i>Cancer Res.</i> 57:5013-5016; Demidenko and Blagosklonny (2004) <i>Cancer Res.</i> 64:3653-3660).
Methyl-CpG-binding proteins (MeCP2; MBD2).	Muller, <i>et al.</i> (2003) <i>Br. J. Cancer</i> 89:1934-1939; Fang, <i>et al.</i> (2004) <i>World J. Gastroenterol.</i> 10:3394-3398.
NA88-A.	Moreau-Aubry, <i>et al.</i> (2000) <i>J. Exp. Med.</i> 191:1617-1624.
Histone deacetylases (HDAC), e.g., HDAC5.	Waltregny, <i>et al.</i> (2004) <i>Eur. J. Histochem.</i> 48:273-290; Scanlan, <i>et al.</i> (2002) <i>Cancer Res.</i> 62:4041-4047.
Cyclophilin B (Cyp-B).	Tamura, <i>et al.</i> (2001) <i>Jpn. J. Cancer Res.</i> 92:762-767.
CA 15-3; CA 27.29.	Clinton, <i>et al.</i> (2003) <i>Biomed. Sci. Instrum.</i> 39:408-414.
Heat shock protein Hsp70.	Faure, <i>et al.</i> (2004) <i>Int. J. Cancer</i> 108:863-870.
GAGE/PAGE family, e.g., PAGE-1; PAGE-2; PAGE-3; PAGE-4; XAGE-1; XAGE-2; XAGE-3.	Brinkmann, <i>et al.</i> (1999) <i>Cancer Res.</i> 59:1445-1448.
MAGE-A, B, C, and D families. MAGE-B5; MAGE-B6; MAGE-C2; MAGE-C3; MAGE-3; MAGE-6.	Lucas, <i>et al.</i> (2000) <i>Int. J. Cancer</i> 87:55-60; Scanlan, <i>et al.</i> (2001) <i>Cancer Immun.</i> 1:4.
Kinesin 2; TATA element modulatory factor 1; tumor protein D53; NY	Scanlan, <i>et al.</i> (2001) <i>Cancer Immun.</i> 30:1-4.
Alpha-fetoprotein (AFP)	Grimm, <i>et al.</i> (2000) <i>Gastroenterol.</i> 119:1104-1112.
SART1; SART2; SART3; ART4.	Kumamuru, <i>et al.</i> (2004) <i>Int. J. Cancer</i> 108:686-695; Sasatomi, <i>et al.</i> (2002) <i>Cancer</i> 94:1636-1641; Matsumoto, <i>et al.</i> (1998) <i>Jpn. J. Cancer Res.</i> 89:1292-1295; Tanaka, <i>et al.</i> (2000) <i>Jpn. J. Cancer Res.</i> 91:1177-1184.
Preferentially expressed antigen of melanoma	Matsushita, <i>et al.</i> (2003) <i>Leuk. Lymphoma</i> 44:439-444; Oberthuer, <i>et al.</i> (2004) <i>Clin. Cancer Res.</i> 10:4307-4313.

(PRAME).	
Carcinoembryonic antigen (CEA), CAP1-6D enhancer agonist peptide.	GenBank Acc. No. M29540; E03352; X98311; M17303 (see also, e.g., Zaremba (1997) <i>Cancer Res.</i> 57:4570-4577; Sarobe, <i>et al.</i> (2004) <i>Curr. Cancer Drug Targets</i> 4:443-454; Tsang, <i>et al.</i> (1997) <i>Clin. Cancer Res.</i> 3:2439-2449; Fong, <i>et al.</i> (2001) <i>Proc. Natl. Acad. Sci. USA</i> 98:8809-8814).
HER-2/neu.	Disis, <i>et al.</i> (2004) <i>J. Clin. Immunol.</i> 24:571-578; Disis and Cheever (1997) <i>Adv. Cancer Res.</i> 71:343-371.
cdk4; cdk6; p16 (INK4); Rb protein.	Ghazizadeh, <i>et al.</i> (2005) <i>Respiration</i> 72:68-73; Ericson, <i>et al.</i> (2003) <i>Mol. Cancer Res.</i> 1:654-664.
TEL; AML1; TEL/AML1.	Stams, <i>et al.</i> (2005) <i>Clin. Cancer Res.</i> 11:2974-2980.
Telomerase (TERT).	Nair, <i>et al.</i> (2000) <i>Nat. Med.</i> 6:1011-1017.
707-AP.	Takahashi, <i>et al.</i> (1997) <i>Clin. Cancer Res.</i> 3:1363-1370.
Annexin, e.g., Annexin II.	Zimmerman, <i>et al.</i> (2004) <i>Virchows Arch.</i> 445:368-374.
BCR/ABL; BCR/ABL p210; BCR/ABL p190; CML-66; CML-28.	Cobaldda, <i>et al.</i> (2000) <i>Blood</i> 95:1007-1013; Hakansson, <i>et al.</i> (2004) <i>Leukemia</i> 18:538-547; Schwartz, <i>et al.</i> (2003) <i>Semin. Hematol.</i> 40:87-96; Lim, <i>et al.</i> (1999) <i>Int. J. Mol. Med.</i> 4:665-667.
BCL2; BCL6; CD10 protein.	Iqbal, <i>et al.</i> (2004) <i>Am. J. Pathol.</i> 165:159-166.
CDC27 (this is a melanoma antigen).	Wang, <i>et al.</i> (1999) <i>Science</i> 284:1351-1354.
Sperm protein 17 (SP17); 14-3-3-zeta; MEMD; KIAA0471; TC21.	Arora, <i>et al.</i> (2005) <i>Mol. Carcinog.</i> 42:97-108.
Tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2).	GenBank Acc. No. NM_001922. (see also, e.g., Bronte, <i>et al.</i> (2000) <i>Cancer Res.</i> 60:253-258).
gp100/pmel-17.	GenBank Acc. Nos. AH003567; U31798; U31799; U31807; U31799 (see also, e.g., Bronte, <i>et al.</i> (2000) <i>Cancer Res.</i> 60:253-258).
TARP.	See, e.g., Clifton, <i>et al.</i> (2004) <i>Proc. Natl. Acad. Sci. USA</i> 101:10166-10171; Virok, <i>et al.</i> (2005) <i>Infection Immunity</i> 73:1939-1946.
Tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2).	GenBank Acc. No. NM_001922. (see also, e.g., Bronte, <i>et al.</i> (2000) <i>Cancer Res.</i> 60:253-258).
Melanocortin 1 receptor (MC1R); MAGE-3; gp100; tyrosinase; dopachrome tautomerase (TRP-2); MART-1.	Salazar-Onfray, <i>et al.</i> (1997) <i>Cancer Res.</i> 57:4348-4355; Reynolds, <i>et al.</i> (1998) <i>J. Immunol.</i> 161:6970-6976; Chang, <i>et al.</i> (2002) <i>Clin. Cancer Res.</i> 8:1021-1032.
MUC-1; MUC-2.	See, e.g., Davies, <i>et al.</i> (1994) <i>Cancer Lett.</i> 82:179-184; Gambus, <i>et al.</i> (1995) <i>Int. J. Cancer</i> 60:146-148; McCool, <i>et al.</i> (1999) <i>Biochem. J.</i> 341:593-600.
Spas-1.	U.S. Published Pat. Appl. No. 20020150588 of Allison, <i>et al.</i>
CASP-8; FLICE; MACH.	Mandruzzato, <i>et al.</i> (1997) <i>J. Exp. Med.</i> 186:785-793.
CEACAM6; CAP-1.	Duxbury, <i>et al.</i> (2004) <i>Biochem. Biophys. Res. Commun.</i> 317:837-843; Morse, <i>et al.</i> (1999) <i>Clin. Cancer Res.</i> 5:1331-1338.
HMGB1 (a DNA binding protein and cytokine).	Brezniceanu, <i>et al.</i> (2003) <i>FASEB J.</i> 17:1295-1297.

ETV6/AML1.	Codrington, <i>et al.</i> (2000) Br. J. Haematol. 111:1071-1079.
Mutant and wild type forms of adenomatous polyposis coli (APC); beta-catenin; c-met; p53; E-cadherin; cyclooxygenase-2 (COX-2).	Clements, <i>et al.</i> (2003) Clin. Colorectal Cancer 3:113-120; Gulmann, <i>et al.</i> (2003) Appl. Immunohistochem. Mol. Morphol. 11:230-237; Jungck, <i>et al.</i> (2004) Int. J. Colorectal. Dis. 19:438-445; Wang, <i>et al.</i> (2004) J. Surg. Res. 120:242-248; Abutaily, <i>et al.</i> (2003) J. Pathol. 201:355-362; Liang, <i>et al.</i> (2004) Br. J. Surg. 91:355-361; Shirakawa, <i>et al.</i> (2004) Clin. Cancer Res. 10:4342-4348.
Renal cell carcinoma antigen bound by mAB G250.	Mulders, <i>et al.</i> (2003) Urol. Clin. North Am. 30:455-465; Steffens, <i>et al.</i> (1999) Anticancer Res. 19:1197-1200.
EphA2	See, e.g., U.S. Patent Publication No. 2005/0281783 A1; Genbank Accession No. NM_004431 (human); Genbank Accession No. NM_010139 (Mouse); Genbank Accession No. AB038986 (Chicken, partial sequence); GenBank Accession Nos. NP_004422, AAH37166, and AAA53375 (human); GenBank Accession Nos. NP_034269 (mouse), AAH06954 (mouse), XP_345597 (rat), and BAB63910 (chicken).
<i>Francisella tularensis</i> antigens	
<i>Francisella tularensis</i> A and B.	Complete genome of subspecies Schu S4 (GenBank Acc. No. AJ749949); of subspecies Schu 4 (GenBank Acc. No. NC_006570). Outer membrane protein (43 kDa) Bevanger, <i>et al.</i> (1988) J. Clin. Microbiol. 27:922-926; Porsch-Ozcurumez, <i>et al.</i> (2004) Clin. Diagnostic. Lab. Immunol. 11:1008-1015). Antigenic components of <i>F. tularensis</i> include, e.g., 80 antigens, including 10 kDa and 60 kDa chaperonins (Havlasova, <i>et al.</i> (2002) Proteomics 2:857-86), nucleoside diphosphate kinase, isocitrate dehydrogenase, RNA-binding protein Hfq, the chaperone ClpB (Havlasova, <i>et al.</i> (2005) Proteomics 5:2090-2103). See also, e.g., Oyston and Quarry (2005) Antonie Van Leeuwenhoek 87:277-281; Isherwood, <i>et al.</i> (2005) Adv. Drug Deliv. Rev. 57:1403-1414; Biagini, <i>et al.</i> (2005) Anal. Bioanal. Chem. 382:1027-1034.
Malarial antigens	
Circumsporozoite protein (CSP); SSP2; HEP17; Exp-1 orthologs found in <i>P. falciparum</i> ; and LSA-1.	See, e.g., Haddad, <i>et al.</i> (2004) Infection Immunity 72:1594-1602; Hoffman, <i>et al.</i> (1997) Vaccine 15:842-845; Oliveira-Ferreira and Daniel-Ribeiro (2001) Mem. Inst. Oswaldo Cruz, Rio de Janeiro 96:221-227. CSP (see, e.g., GenBank Acc. No. AB121024). SSP2 (see, e.g., GenBank Acc. No. AF249739). LSA-1 (see, e.g., GenBank Acc. No. Z30319).
Ring-infected erythrocyte surface protein (RESA); merozoite surface protein 2 (MSP2); Spf66; merozoite surface protein 1 (MSP1); 195A; BVp42.	See, e.g., Stirnadel, <i>et al.</i> (2000) Int. J. Epidemiol. 29:579-586; Krzych, <i>et al.</i> (1995) J. Immunol. 155:4072-4077. See also, Good, <i>et al.</i> (2004) Immunol. Rev. 201:254-267; Good, <i>et al.</i> (2004) Ann. Rev. Immunol. 23:69-99. MSP2 (see, e.g., GenBank Acc. No. X96399; X96397). MSP1 (see, e.g., GenBank Acc. No. X03371). RESA (see, e.g., GenBank Acc. No. X05181; X05182).
Apical membrane antigen 1 (AMA1).	See, e.g., Gupta, <i>et al.</i> (2005) Protein Expr. Purif. 41:186-198. AMA1 (see, e.g., GenBank Acc. No. A_13; AJ494905; AJ490565).
Viruses and viral antigens	
Hepatitis A	GenBank Acc. Nos., e.g., NC_001489; AY644670; X83302; K02990; M14707.
Hepatitis B	Complete genome (see, e.g., GenBank Acc. Nos. AB214516; NC_003977; AB205192; AB205191; AB205190; AJ748098; AB198079; AB198078; AB198076; AB074756).
Hepatitis C	Complete genome (see, e.g., GenBank Acc. Nos. NC_004102; AJ238800;

	AJ238799; AJ132997; AJ132996; AJ000009; D84263).
Hepatitis D	GenBank Acc. Nos, e.g. NC_001653; AB118847; AY261457.
Human papillomavirus, including all 200+ subtypes (classed in 16 groups), such as the high risk subtypes 16, 18, 30, 31, 33, 45.	See, e.g., Trimble, <i>et al.</i> (2003) Vaccine 21:4036-4042; Kim, <i>et al.</i> (2004) Gene Ther. 11:1011-1018; Simon, <i>et al.</i> (2003) Eur. J. Obstet. Gynecol. Reprod. Biol. 109:219-223; Jung, <i>et al.</i> (2004) J. Microbiol. 42:255-266; Damasus-Awatai and Freeman-Wang (2003) Curr. Opin. Obstet. Gynecol. 15:473-477; Jansen and Shaw (2004) Annu. Rev. Med. 55:319-331; Roden and Wu (2003) Expert Rev. Vaccines 2:495-516; de Villiers, <i>et al.</i> (2004) Virology 324:17-24; Hussain and Paterson (2005) Cancer Immunol. Immunother. 54:577-586; Molijn, <i>et al.</i> (2005) J. Clin. Virol. 32 (Suppl. 1) S43-S51. GenBank Acc. Nos. AY686584; AY686583; AY686582; NC_006169; NC_006168; NC_006164; NC_001355; NC_001349; NC_005351; NC_001596).
Human T-cell lymphotropic virus (HTLV) types I and II, including the HTLV type I subtypes Cosmopolitan, Central African, and Austro-Melanesian, and the HTLV type II subtypes IIa, IIb, IIc, and IId.	See, e.g., Capdepon, <i>et al.</i> (2005) AIDS Res. Hum. Retrovirus 21:28-42; Bhigjee, <i>et al.</i> (1999) AIDS Res. Hum. Restrovirus 15:1229-1233; Vandamme, <i>et al.</i> (1998) J. Virol. 72:4327-4340; Vallejo, <i>et al.</i> (1996) J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 13:384-391. HTLV type I (see, e.g., GenBank Acc. Nos. AY563954; AY563953. HTLV type II (see, e.g., GenBank Acc. Nos. L03561; Y13051; AF139382).
Coronaviridae, including Coronaviruses, such as SARS-coronavirus (SARS-CoV), and Toroviruses.	See, e.g., Brian and Baric (2005) Curr. Top. Microbiol. Immunol. 287:1-30; Gonzalez, <i>et al.</i> (2003) Arch. Virol. 148:2207-2235; Smits, <i>et al.</i> (2003) J. Virol. 77:9567-9577; Jamieson, <i>et al.</i> (1998) J. Infect. Dis. 178:1263-1269 (GenBank Acc. Nos. AY348314; NC_004718; AY394850).
Rubella virus.	GenBank Acc. Nos. NC_001545; AF435866.
Mumps virus, including the genotypes A, C, D, G, H, and I.	See, e.g., Orvell, <i>et al.</i> (2002) J. Gen. Virol. 83:2489-2496. See, e.g., GenBank Acc. Nos. AY681495; NC_002200; AY685921; AF201473.
Coxsackie virus A including the serotypes 1, 11, 13, 15, 17, 18, 19, 20, 21, 22, and 24 (also known as Human enterovirus C; HEV-C).	See, e.g., Brown, <i>et al.</i> (2003) J. Virol. 77:8973-8984. GenBank Acc. Nos. AY421768; AY790926; X67706.
Coxsackie virus B, including subtypes 1-6.	See, e.g., Ahn, <i>et al.</i> (2005) J. Med. Virol. 75:290-294; Patel, <i>et al.</i> (2004) J. Virol. Methods 120:167-172; Rezig, <i>et al.</i> (2004) J. Med. Virol. 72:268-274. GenBank Acc. No. X05690.
Human enteroviruses including, e.g., human enterovirus A (HEV-A, CAV2 to CAV8, CAV10, CAV12, CAV14, CAV16, and	See, e.g., Oberste, <i>et al.</i> (2004) J. Virol. 78:855-867. Human enterovirus A (GenBank Acc. Nos. NC_001612); human enterovirus B (NC_001472); human enterovirus C (NC_001428); human enterovirus D (NC_001430). Simian enterovirus A (GenBank Acc. No. NC_003988).

EV71) and also including HEV-B (CAV9, CBV1 to CBV6, E1 to E7, E9, E11 to E21, E24 to E27, E29 to E33, and EV69 and E73), as well as HEV.	
Polioviruses including PV1, PV2, and PV3.	See, e.g., He, <i>et al.</i> (2003) J. Virol. 77:4827-4835; Hahsido, <i>et al.</i> (1999) Microbiol. Immunol. 43:73-77. GenBank Acc. No. AJ132961 (type 1); AY278550 (type 2); X04468 (type 3).
Viral encephalitides viruses, including equine encephalitis, Venezuelan equine encephalitis (VEE) (including subtypes IA, IB, IC, ID, IIIC, IIID), Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), St. Louis encephalitis, Murray Valley (Australian) encephalitis, Japanese encephalitis, and tick-born encephalitis.	See, e.g., Hoke (2005) Mil. Med. 170:92-105; Estrada-Franco, <i>et al.</i> (2004) Emerg. Infect. Dis. 10:2113-2121; Das, <i>et al.</i> (2004) Antiviral Res. 64:85-92; Aguilar, <i>et al.</i> (2004) Emerg. Infect. Dis. 10:880-888; Weaver, <i>et al.</i> (2004) Arch. Virol. Suppl. 18:43-64; Weaver, <i>et al.</i> (2004) Annu. Rev. Entomol. 49:141-174. Eastern equine encephalitis (GenBank Acc. No. NC_003899; AY722102); Western equine encephalitis (NC_003908).
Human herpesviruses, including cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus-1 (HHV-1), HHV-2, HHV-3, HHV-4, HHV-5, HHV-6, HHV-7, HHV-8, herpes B virus, herpes simplex virus types 1 and 2 (HSV-1, HSV-2), and varicella zoster virus (VZV).	See, e.g., Studahl, <i>et al.</i> (2000) Scand. J. Infect. Dis. 32:237-248; Padilla, <i>et al.</i> (2003) J. Med. Virol. 70 (Suppl. 1) S103-S110; Jainkittivong and Langlais (1998) Oral Surg. Oral Med. 85:399-403. GenBank Nos. NC_001806 (herpesvirus 1); NC_001798 (herpesvirus 2); X04370 and NC_001348 (herpesvirus 3); NC_001345 (herpesvirus 4); NC_001347 (herpesvirus 5); X83413 and NC_000898 (herpesvirus 6); NC_001716 (herpesvirus 7). Human herpesviruses types 6 and 7 (HHV-6; HHV-7) are disclosed by, e.g., Padilla, <i>et al.</i> (2003) J. Med. Virol. 70 (Suppl. 1) S103-S110. Human herpesvirus 8 (HHV-8), including subtypes A-E, are disclosed in, e.g., Treurnicht, <i>et al.</i> (2002) J. Med. Virol. 66:235-240.
HIV-1 including group M (including subtypes A to J) and group O (including any distinguishable subtypes) (HIV-2, including subtypes	See, e.g., Smith, <i>et al.</i> (1998) J. Med. Virol. 56:264-268. See also, e.g., GenBank Acc. Nos. DQ054367; NC_001802; AY968312; DQ011180; DQ011179; DQ011178; DQ011177; AY588971; AY588970; AY781127; AY781126; AY970950; AY970949; AY970948; X61240; AJ006287; AJ508597; and AJ508596.

A-E.	
Epstein-Barr virus (EBV), including subtypes A and B.	See, e.g., Peh, <i>et al.</i> (2002) Pathology 34:446-450. Epstein-Barr virus strain B95-8 (GenBank Acc. No. V01555).
Reovirus, including serotypes and strains 1, 2, and 3, type 1 Lang, type 2 Jones, and type 3 Dearing.	See, e.g., Barthold, <i>et al.</i> (1993) Lab. Anim. Sci. 43:425-430; Roner, <i>et al.</i> (1995) Proc. Natl. Acad. Sci. USA 92:12362-12366; Kedl, <i>et al.</i> (1995) J. Virol. 69:552-559. GenBank Acc. No. K02739 (sigma-3 gene surface protein).
Cytomegalovirus (CMV) subtypes include CMV subtypes I-VII.	See, e.g., Chern, <i>et al.</i> (1998) J. Infect. Dis. 178:1149-1153; Vilas Boas, <i>et al.</i> (2003) J. Med. Virol. 71:404-407; Trincado, <i>et al.</i> (2000) J. Med. Virol. 61:481-487. GenBank Acc. No. X17403.
Rhinovirus, including all serotypes.	Human rhinovirus 2 (GenBank Acc. No. X02316); Human rhinovirus B (GenBank Acc. No. NC_001490); Human rhinovirus 89 (GenBank Acc. No. NC_001617); Human rhinovirus 39 (GenBank Acc. No. AY751783).
Adenovirus, including all serotypes.	AY803294; NC_004001; AC_000019; AC_000018; AC_000017; AC_000015; AC_000008; AC_000007; AC_000006; AC_000005; AY737798; AY737797; NC_003266; NC_002067; AY594256; AY594254; AY875648; AJ854486; AY163756; AY594255; AY594253; NC_001460; NC_001405; AY598970; AY458656; AY487947; NC_001454; AF534906; AY45969; AY128640; L19443; AY339865; AF532578.
Varicella-zoster virus, including strains and genotypes Oka, Dumas, European, Japanese, and Mosaic.	See, e.g., Loparev, <i>et al.</i> (2004) J. Virol. 78:8349-8358; Carr, <i>et al.</i> (2004) J. Med. Virol. 73:131-136; Takayama and Takayama (2004) J. Clin. Virol. 29:113-119.
Filoviruses, including Marburg virus and Ebola virus, and strains such as Ebola-Sudan (EBO-S), Ebola-Zaire (EBO-Z), and Ebola-Reston (EBO-R).	See, e.g., Geisbert and Jahrling (1995) Virus Res. 39:129-150; Hutchinson, <i>et al.</i> (2001) J. Med. Virol. 65:561-566. Marburg virus (see, e.g., GenBank Acc. No. NC_001608). Ebola virus (see, e.g., GenBank Acc. Nos. NC_006432; AY769362; NC_002549; AF272001; AF086833).
Arenaviruses, including lymphocytic choriomeningitis (LCM) virus, Lassa virus, Junin virus, and Machupo virus.	Junin virus, segment S (GenBank Acc. No. NC_005081); Junin virus, segment L (GenBank Acc. No. NC_005080).
Rabies virus.	See, e.g., GenBank Acc. Nos. NC_001542; AY956319; AY705373; AF499686; AB128149; AB085828; AB009663.
Arboviruses, including West Nile virus, Dengue viruses 1 to 4, Colorado tick fever virus, Sindbis virus,	Dengue virus type 1 (see, e.g., GenBank Acc. Nos. AB195673; AY762084). Dengue virus type 2 (see, e.g., GenBank Acc. Nos. NC_001474; AY702040; AY702039; AY702037). Dengue virus type 3 (see, e.g., GenBank Acc. Nos. AY923865; AT858043). Dengue virus type 4 (see, e.g., GenBank Acc. Nos. AY947539; AY947539;

Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae, Rhabdoviridae, Orthomyxoviridae, and the like.	AF326573). Sindbis virus (see, e.g., GenBank Acc. Nos. NC_001547; AF429428; J02363; AF103728). West Nile virus (see, e.g., GenBank Acc. Nos. NC_001563; AY603654).
Poxvirus including orthopoxvirus (variola virus, monkeypox virus, vaccinia virus, cowpox virus), yatapoxvirus (tanapox virus, Yaba monkey tumor virus), parapoxvirus, and molluscipoxvirus.	Variola virus (see, e.g., GenBank Acc. Nos. NC_001611; Y16780; X72086; X69198).
Yellow fever.	See, e.g., GenBank Acc. No. NC_002031; AY640589; X03700.
Hantaviruses, including serotypes Hantaan (HTN), Seoul (SEO), Dobrava (DOB), Sin Nombre (SN), Puumala (PUU), and Dobrava-like Saaremaa (SAAV).	See, e.g., Elgh, <i>et al.</i> (1997) J. Clin. Microbiol. 35:1122-1130; Sjolander, <i>et al.</i> (2002) Epidemiol. Infect. 128:99-103; Zeier, <i>et al.</i> (2005) Virus Genes 30:157-180. GenBank Acc. No. NC_005222 and NC_005219 (Hantavirus). See also, e.g., GenBank Acc. Nos. NC_005218; NC_005222; NC_005219.
Flaviviruses, including Dengue virus, Japanese encephalitis virus, West Nile virus, and yellow fever virus.	See, e.g., Mukhopadhyay, <i>et al.</i> (2005) Nature Rev. Microbiol. 3:13-22. GenBank Acc. Nos. NC_001474 and AY702040 (Dengue). GenBank Acc. Nos. NC_001563 and AY603654.
Measles virus.	See, e.g., GenBank Acc. Nos. AB040874 and AY486084.
Human parainfluenzaviruses (HPV), including HPV types 1-56.	Human parainfluenza virus 2 (see, e.g., GenBank Acc. Nos. AB176531; NC003443). Human parainfluenza virus 3 (see, e.g., GenBank Acc. No. NC_001796).
Influenza virus, including influenza virus types A, B, and C.	Influenza nucleocapsid (see, e.g., GenBank Acc. No. AY626145). Influenza hemagglutinin (see, e.g., GenBank Acc. Nos. AY627885; AY555153). Influenza neuraminidase (see, e.g., GenBank Acc. Nos. AY555151; AY577316). Influenza matrix protein 2 (see, e.g., GenBank Acc. Nos. AY626144). Influenza basic protein 1 (see, e.g., GenBank Acc. No. AY627897). Influenza polymerase acid protein (see, e.g., GenBank Acc. No. AY627896). Influenza nucleoprotein (see, e.g., GenBank Acc. No. AY627895).
Influenza A virus subtypes, e.g., swine viruses (SIV): H1N1 influenzaA and swine influenza virus.	Hemagglutinin of H1N1 (GenBank Acc. No. S67220). Influenza A virus matrix protein (GenBank Acc. No. AY700216). Influenza virus A H5N1 nucleoprotein (GenBank Acc. No. AY646426). H1N1 haemagglutinin (GenBank Acc. No. D00837). See also, GenBank Acc. Nos. BD006058; BD006055; BD006052. See also, e.g., Wentworth, <i>et al.</i> (1994) J. Virol. 68:2051-2058; Wells, <i>et al.</i> (1991) J.A.M.A. 265:478-481.

Respiratory syncytial virus (RSV), including subgroup A and subgroup B.	Respiratory syncytial virus (RSV) (see, e.g., GenBank Acc. Nos. AY353550; NC_001803; NC001781).
Rotaviruses, including human rotaviruses A to E, bovine rotavirus, rhesus monkey rotavirus, and human-RVV reassortments.	Human rotavirus C segment 8 (GenBank Acc. No. AJ549087); Human rotavirus G9 strain outer capsid protein (see, e.g., GenBank Acc. No. DQ056300); Human rotavirus B strain non-structural protein 4 (see, e.g., GenBank Acc. No. AY548957); human rotavirus A strain major inner capsid protein (see, e.g., GenBank Acc. No. AY601554).
Polyomavirus, including simian virus 40 (SV40), JC virus (JCV) and BK virus (BKV).	See, e.g., Engels, <i>et al.</i> (2004) <i>J. Infect. Dis.</i> 190:2065-2069; Vilchez and Butel (2004) <i>Clin. Microbiol. Rev.</i> 17:495-508; Shivapurkar, <i>et al.</i> (2004) <i>Cancer Res.</i> 64:3757-3760; Carbone, <i>et al.</i> (2003) <i>Oncogene</i> 2:5173-5180; Barbanti-Brodano, <i>et al.</i> (2004) <i>Virology</i> 318:1-9) (SV40 complete genome in, e.g., GenBank Acc. Nos. NC_001669; AF168994; AY271817; AY271816; AY120890; AF345344; AF332562).
Coltiviruses, including Colorado tick fever virus, Eyach virus.	Attoui, <i>et al.</i> (1998) <i>J. Gen. Virol.</i> 79:2481-2489. Segments of Eyach virus (see, e.g., GenBank Acc. Nos. AF282475; AF282472; AF282473; AF282478; AF282476; NC_003707; NC_003702; NC_003703; NC_003704; NC_003705; NC_003696; NC_003697; NC_003698; NC_003699; NC_003701; NC_003706; NC_003700; AF282471; AF282477).
Calciviruses, including the genogroups Norwalk, Snow Mountain group (SMA), and Saaporo.	Snow Mountain virus (see, e.g., GenBank Acc. No. AY134748).
Parvoviridae, including dependovirus, parvovirus (including parvovirus B19), and erythrovirus.	See, e.g., Brown (2004) <i>Dev. Biol. (Basel)</i> 118:71-77; Alvarez-Lafuente, <i>et al.</i> (2005) <i>Ann. Rheum. Dis.</i> 64:780-782; Ziyaeyan, <i>et al.</i> (2005) <i>Jpn. J. Infect. Dis.</i> 58:95-97; Kaufman, <i>et al.</i> (2005) <i>Virology</i> 332:189-198.
The present invention provides, but is not limited by, an attenuated <i>Listeria</i> comprising a nucleic acid that encodes at least one of the above-disclosed antigens, or at least one antigen encoded by one of the above-disclosed complete genomes. The present invention encompasses nucleic acids encoding mutants, muteins, splice variants, fragments, truncated variants, soluble variants, extracellular domains, intracellular domains, mature sequences, and the like, of the disclosed antigens. Provided are nucleic acids encoding epitopes, oligo- and polypeptides of these antigens. Also provided are codon optimized embodiments, that is, optimized for expression in <i>Listeria</i> . The cited references, GenBank Acc. Nos., and the nucleic acids, peptides, and polypeptides disclosed therein, are all incorporated herein by reference in their entirety.	

[0147] In some embodiments, the antigen is non-Listerial. In some embodiments, the antigen is from a cancer cell, tumor, or infectious agent. In some embodiments, the antigen is derived from an antigen from a cancer cell, tumor, or infectious agent. In some embodiments, an antigen that is "derived from" another antigen is a fragment or other derivative of the antigen. In some embodiments, the derived antigen comprises a fragment of at least 8 amino acids, at least 12 amino acids, at least 20 amino acids, at least 50 amino acids, at least 75 amino acids, at least 100 amino acids, or at least 200 amino acids. In some embodiments, the derivative of the antigen has at least about 80 % sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 98% sequence identity to the antigen from which it is derived, or a fragment thereof. In some embodiments, a derived antigen comprises an antigen deleted of its signal sequence and/or membrane anchor. In some embodiments, an antigen derived from another antigen comprises at least one MHC class I epitope and/or at least one MHC class II epitope from the original (full-length) antigen. In some embodiments, the antigen is a tumor antigen. Assays for testing the immunogenicity of antigens are described herein and are well known in the art.

[0148] In some embodiments, the heterologous antigen is a human tumor antigen or an antigen derived from a human tumor antigen. In some embodiments, the antigen derived from a tumor antigen consists of an amino acid sequence that exhibits at least about 65% sequence identity to the tumor antigen, at least 70% sequence identity to the tumor antigen, or at least about 75% sequence identity to the tumor antigen. In some embodiments, the derived antigen consists of an amino acid sequence that exhibits at least 85% sequence identity to the tumor antigen, at least 90% sequence identity to the tumor antigen, or at least about 95% sequence identity to the tumor antigen. In some embodiments, the antigen derived from the tumor antigen comprises at least 10, 20, 30, 40, 50, 75, 100, or 200 amino acids of the tumor antigen. In some embodiments, the antigen derived from the tumor antigen consists of at least 10, 20, 30, 40, 50, 75, 100, or 200 contiguous amino acids of the tumor antigen.

[0149] In some embodiments, the antigen is mesothelin, or derived from mesothelin. In some embodiments, the mesothelin is human. In some embodiments, the mesothelin is full-length (e.g., full length human mesothelin). In some embodiments, the antigen derived from mesothelin comprises mesothelin (e.g., human mesothelin) deleted in its signal sequence, deleted in its GPI anchor, or deleted in both the signal sequence and the GPI anchor. The polynucleotide encoding the mesothelin may be codon-optimized or non-codon optimized for expression in *Listeria*.

[0150] In some embodiments, the antigen derived from mesothelin consists of an amino acid sequence that exhibits at least about 65% sequence identity to human mesothelin, at least 70% sequence identity to human mesothelin, or at least about 75% sequence identity to human mesothelin. In some embodiments, the mesothelin polypeptide sequence consists of an amino acid sequence that exhibits at least 85% sequence identity to human mesothelin, at least 90% sequence identity to human mesothelin, or at least about 95% sequence identity to human mesothelin. In some embodiments, the antigen derived from mesothelin comprises at least 10, 20, 30, 40, 50, 75, 100, or 200 amino acids of a mesothelin polypeptide, such as human mesothelin or human mesothelin deleted of its signal peptide sequence and/or GPI anchor. In some embodiments, the antigen derived from mesothelin consists of at least 10, 20, 30, 40, 50, 75, 100, or 200 contiguous amino acids of a mesothelin polypeptide, such as human mesothelin or human mesothelin deleted of its signal peptide sequence and/or GPI anchor.

[0151] In certain embodiments, the antigen is prostate stem cell antigen (PSCA), or is an antigen derived from PSCA. In some embodiments, the PSCA is human PSCA. In some embodiments, the PSCA is full-length (e.g., full length human PSCA). In some embodiments, the antigen derived from PSCA comprises PSCA (e.g., human PSCA) deleted of (a) part or all its signal peptide region, (b) its GPI anchor region, or (c) its GPI anchor region and part or all of its signal peptide region. The polynucleotide encoding the PSCA may be codon-optimized or non-codon optimized for expression in *Listeria*.

[0152] Various assays for assessing whether a particular antigen derivative can stimulate the desired immunogenicity when expressed in *Listeria* are known to those of ordinary skill in the art. Specific examples are provided in the Examples below. Various assays are also provided, e.g., in U.S. Pat. Publ. Nos. 2004/0228877, 2004/0197343, and 2005/0249748, each of which is incorporated by reference herein in its entirety.

[0153] In some embodiments, the antigen derived from PSCA consists of an amino acid sequence that exhibits at least about 65% sequence similarity to human PSCA, at least 70% sequence similarity to human PSCA, or at least about 75% sequence similarity to human PSCA. In some embodiments, the PSCA polypeptide sequence consists of an amino acid sequence that exhibits at least 85% sequence similarity to human PSCA, at least 90% sequence similarity to human PSCA, or at least about 95% sequence similarity to human PSCA. In some embodiments, the antigen derived from PSCA comprises at least 10, 20, 30, 40, 50, 75, 100, or 200 amino acids of a PSCA polypeptide, such as human PSCA or human PSCA deleted of its signal peptide sequence and/or GPI anchor. In some embodiments, the

antigen derived from PSCA consists of at least 10, 20, 30, 40, 50, 75, 100, or 200 contiguous amino acids of a PSCA polypeptide, such as human PSCA or human PSCA deleted of its signal peptide sequence and/or GPI anchor.

[0154] In some embodiments, the antigen (e.g., heterologous antigen) comprises an EphA2 antigenic peptide (sometimes referred to as an "EphA2 antigenic polypeptide"), as defined and described in U.S. Patent Publication No. 2005/0281783 A1, which is hereby incorporated by reference herein in its entirety, including all sequences contained therein. In some embodiments, the antigen does not comprise an EphA2 antigenic peptide. In some embodiments, the EphA2 antigenic peptide that is used (or excluded from use) in the methods and compositions described herein can be any EphA2 antigenic peptide that is capable of eliciting an immune response against EphA2-expressing cells involved in a hyperproliferative disorder. Thus, in some embodiments, the EphA2 antigenic peptide can be an EphA2 polypeptide (e.g., the EphA2 polypeptide of SEQ ID NO:2 in U.S. Patent Publication No. 2005/0281783 A1, incorporated by reference herein in its entirety), or a fragment or derivative of an EphA2 polypeptide that (1) displays ability to bind or compete with EphA2 for binding to an anti-EphA2 antibody, (2) displays ability to generate antibody which binds to EphA2, (3) contains one or more T cell epitopes of EphA2, and/or (4) displays ability to generate a T cell response against an EphA2 peptide.

[0155] In some embodiments, the EphA2 antigenic peptide is a sequence encoded by one of the following nucleotide sequences, or a fragment or derivative thereof: Genbank Accession No. NM_004431 (Human); Genbank Accession No. NM_010139 (Mouse); or Genbank Accession No. AB038986 (Chicken, partial sequence). In some embodiments, the EphA2 antigenic peptide is full-length human EphA2 (e.g., SEQ ID NO:2 of U.S. Patent Publication No. 2005/0281783 A1, the polypeptide sequence shown in Figure 46A-B of the present application). In some embodiments, the EphA2 antigenic peptide comprises the extracellular domain of EphA2 (residue 22 to 554 of SEQ ID NO:2 of U.S. Patent Publication No. 2005/0281783 A1). In some other embodiments, the EphA2 antigenic peptide comprises the intracellular domain EphA2 (residue 558 to 976 of SEQ ID NO:2 of U.S. Patent Publication No. 2005/0281783 A1).

[0156] In some embodiments, the EphA2 antigenic peptide comprises the extracellular domain of EphA2 or the intracellular domain of EphA2. In some embodiments, the EphA2 antigenic peptide comprises more than one domain of the full length human EphA2. In some embodiments, the EphA2 antigenic peptides comprise the extracellular domain and the intracellular cytoplasmic domain, joined together. In some embodiments, the EphA2

antigenic peptide lacks the EphA2 transmembrane domain. In some embodiments, the EphA2 antigenic peptide comprises the EphA2 extracellular and intracellular domains and lacks the transmembrane domain of EphA2.

[0157] In some embodiments of the invention, the tyrosine kinase activity of EphA2 is ablated. Thus, EphA2 may contain deletions, additions or substitutions of amino acid residues that result in the elimination of tyrosine kinase activity. In some embodiments, a lysine to methionine substitution at position 646 is present. In some embodiments, the EphA2 antigenic peptide comprises full length EphA2 or a fragment thereof with a substitution of lysine to methionine at amino acid residue 646 of EphA2. In some embodiments, the EphA2 antigenic peptide comprises the extracellular and intracellular domains of EphA2, lacks the transmembrane domain of EphA2 and has a substitution of lysine to methionine at amino acid residue 646 of EphA2.

[0158] In some embodiments, the EphA2 antigenic peptide is a chimeric polypeptide comprising at least an antigenic portion of EphA2 and a second polypeptide.

[0159] In some embodiments, the fragments of EphA2 used in the methods and compositions of the invention may contain deletions, additions or substitutions of amino acid residues within the amino acid sequence encoded by an EphA2 gene. In some embodiments, mutations result in a silent change, thus producing a functionally equivalent EphA2 gene product. By "functionally equivalent," it is meant that the mutated EphA2 gene product has the same function as the wild-type EphA2 gene product, e.g., contains one or more epitopes of EphA2.

[0160] In some embodiments, the EphA2 antigenic peptide sequence comprises an amino acid sequence that exhibits at least about 65% sequence similarity to human EphA2, at least 70% sequence similarity to human EphA2, or at least about 75% sequence similarity to human EphA2. In some embodiments, the EphA2 polypeptide sequence comprises an amino acid sequence that exhibits at least 85% sequence similarity to human EphA2, at least 90% sequence similarity to human EphA2, or at least about 95% sequence similarity to human EphA2.

[0161] In some embodiments, the EphA2 antigenic peptide comprises at least 10, 20, 30, 40, 50, 75, 100, or 200 amino acids of an EphA2 polypeptide (e.g., SEQ ID NO:2 of U.S. Patent Publication No. 2005/0281783 A1). In some embodiments, the EphA2 antigenic peptide comprises at least 10, 20, 30, 40, 50, 75, 100, or 200 contiguous amino acids of an EphA2 polypeptide (e.g., SEQ ID NO:2 of U.S. Patent Publication No. 2005/0281783 A1). In some

embodiments, the polypeptide comprises all or a portion of the extracellular domain of an EphA2 polypeptide of SEQ ID NO:2 of U.S. Patent Publication No. 2005/0281783 A1.

[0162] In some embodiments, more than one EphA2 antigenic peptide, e.g., 2, 3, 4, 5, 6, or more EphA2 antigenic peptides, are encoded by nucleic acids in and/or expressed in the *Listeria* described herein. In some embodiments, a polycistronic expression cassette (e.g., a bicistronic expression cassette) is used in the engineered *Listeria* to express more than one fusion protein, each of which comprises an EphA2 antigenic peptide.

[0163] The invention supplies methods and reagents for stimulating immune response to infections, e.g., infections of the liver. These include infections from hepatotropic viruses and viruses that mediate hepatitis, e.g., hepatitis B virus, hepatitis C virus, and cytomegalovirus. The invention contemplates methods to treat other hepatotropic viruses, such as herpes simplex virus, Epstein-Barr virus, and dengue virus (see, e.g., Ahlenstiel and Rehmann (2005) *Hepatology* 41:675-677; Chen, *et al.* (2005) *J. Viral Hepat.* 12:38-45; Sun and Gao (2004) *Gastroenterol.* 127:1525-1539; Li, *et al.* (2004) *J. Leukoc. Biol.* 76:1171-1179; Ahmad and Alvarez (2004) *J. Leukoc. Biol.* 76:743-759; Cook (1997) *Eur. J. Gastroenterol. Hepatol.* 9:1239-1247; Williams and Riordan (2000) *J. Gastroenterol. Hepatol.* 15 (Suppl.)G17-G25; Varani and Landini (2002) *Clin. Lab.* 48:39-44; Rubin (1997) *Clin. Liver Dis.* 1:439-452; Loh, *et al.* (2005) *J. Virol.* 79:661-667; Shresta, *et al.* (2004) *Virology* 319:262-273; Fjaer, *et al.* (2005) *Pediatr. Transplant* 9:68-73; Li, *et al.* (2004) *World J. Gastroenterol.* 10:3409-3413; Collin, *et al.* (2004) *J. Hepatol.* 41:174-175; Ohga, *et al.* (2002) *Crit. Rev. Oncol. Hematol.* 44:203-215).

[0164] In another aspect, the present invention provides methods and reagents for the treatment and/or prevention of parasitic infections, e.g., parasitic infections of the liver. These include, without limitation, liver flukes (e.g., *Clonorchis*, *Fasciola hepatica*, *Opisthorchis*), *Leishmania*, *Ascaris lumbricoides*, *Schistosoma*, and helminths. Helminths include, e.g., nematodes (roundworms), cestodes (tapeworms), and trematodes (flatworms or flukes) (see, e.g., Tliba, *et al.* (2002) *Vet. Res.* 33:327-332; Keiser and Utzinger (2004) *Expert Opin. Pharmacother.* 5:1711-1726; Kaewkes (2003) *Acta Trop.* 88:177-186; Srivatanakul, *et al.* (2004) *Asian Pac. J. Cancer Prev.* 5:118-125; Stuafter, *et al.* (2004) *J. Travel Med.* 11:157-159; Nylen, *et al.* (2003) *Clin. Exp. Immunol.* 131:457-467; Bukte, *et al.* (2004) *Abdom. Imaging* 29:82-84; Singh and Sivakumar (2003) 49:55-60; Wyler (1992) *Parasitol. Today* 8:277-279; Wynn, *et al.* (2004) *Immunol. Rev.* 201:156-167; Asseman, *et al.* (1996) *Immunol. Lett.* 54:11-20; Becker, *et al.* (2003) *Mol. Biochem. Parasitol.* 130:65-74; Pockros and Capozza (2005) *Curr. Infect. Dis. Rep.* 7:61-70; Hsieh, *et al.* (2004) *J. Immunol.*

173:2699-2704; Korten, *et al.* (2002) J. Immunol. 168:5199-5206; Pockros and Capozza (2004) Curr. Gastroenterol. Rep. 6:287-296).

[0165] Yet another aspect of the present invention provides methods and reagents for the treatment and/or prevention of bacterial infections, e.g., by hepatotropic bacteria. Provided are methods and reagents for treating, e.g., *Mycobacterium tuberculosis*, *Treponema pallidum*, and *Salmonella spp* (see, e.g., Cook (1997) Eur. J. Gastroenterol. Hepatol. 9:1239-1247; Vankayalapati, *et al.* (2004) J. Immunol. 172:130-137; Sellati, *et al.* (2001) J. Immunol. 166:4131-4140; Jason, *et al.* (2000) J. Infectious Dis. 182:474-481; Kirby, *et al.* (2002) J. Immunol. 169:4450-4459; Johansson and Wick (2004) J. Immunol. 172:2496-2503; Hayashi, *et al.* (2004) Intern. Med. 43:521-523; Akcay, *et al.* (2004) Int. J. Clin. Pract. 58:625-627; de la Barrera, *et al.* (2004) Clin. Exp. Immunol. 135:105-113).

[0166] In a further embodiment, the heterologous of the present invention is derived from Human Immunodeficiency Virus (HIV), e.g., gp120; gp160; gp41; gag antigens such as p24gag or p55 gag, as well as protein derived from the pol, env, tat, vir, rev, nef, vpr, vpu, and LTR regions of HIV. The heterologous antigens contemplated include those from herpes simplex virus (HSV) types 1 and 2, from cytomegalovirus, from Epstein-Barr virus, or Varicella Zoster Virus. Also encompassed are antigens derived from a hepatitis virus, e.g., hepatitis A, B, C, delta, E, or G. Moreover, the antigens also encompass antigens from *Picornaviridae* (poliovirus; rhinovirus); *Caliciviridae*; *Togaviridae* (rubella; dengue); *Flaviviridae*; *Coronaviridae*; *Reoviridae*; *Birnaviridae*; *Rhabdoviridae*; *Orthomyxoviridae*; *Filoviridae*; *Paramyxoviridae* (mumps; measles); *Bunyviridae*; *Arenaviridae*; *Retroviridae* (HTLV-I; HIV-1); Papillomavirus, tick-borne encephalitis viruses, and the like.

[0167] In yet another aspect, the present invention provides reagents and methods for the prevention and treatment of bacterial and parasitic infections, e.g., *Salmonella*, *Neisseria*, *Borrelia*, *Chlamydia*, *Bordetella*, plasmodium, *Toxoplasma*, *Mycobacterium tuberculosis*, *Bacillus anthracis*, *Yersinia pestis*, Diphtheria, Pertussis, Tetanus, bacterial or fungal pneumonia, Otitis Media, Gonorrhea, Cholera, Typhoid, Meningitis, Mononucleosis, Plague, Shigellosis, Salmonellosis, Legionaire's Disease, Lyme disease, Leprosy, Malaria, Hookworm, *Onchocerciasis*, *Schistosomiasis*, Trypanosomes, Leshmania, Giardia, Amoebiasis, Filariasis, Borelia, and Trichinosis (see, e.g., Despommier, *et al.* (2000) Parasitic Diseases, 4th ed., Apple Trees Productions, New York, NY; U.S. Government (2002) 21st Century Collection Centers for Disease Control (CDC) Emerging Infectious Diseases (EID) - Comprehensive Collection from 1995 to 2002 with Accurate and Detailed Information on Dozens of Serious Virus and Bacteria Illnesses - Hantavirus, Influenza, AIDS, Malaria, TB,

Pox, Bioterrorism, Smallpox, Anthrax, Vaccines, Lyme Disease, Rabies, West Nile Virus, Hemorrhagic Fevers, Ebola, Encephalitis (Core Federal Information Series).

[0168] The present invention, at least in some embodiments, provides reagents and methods for treating a disorder or condition, or stimulating an immune response to a disorder or condition, that comprises both a cancer and infection. In some viral infections, for example, an antigen can be both a tumor antigen and a viral antigen (see, e.g., Montesano, *et al.* (1990) *Cell* 62:435-445; Ichaso and Dilworth (2001) *Oncogene* 20:7908-7916; Wilson, *et al.* (1999) *J. Immunol.* 162:3933-3941; Daemen, *et al.* (2004) *Antivir. Ther.* 9:733-742; Boudewijn, *et al.* (2004) *J. Natl. Cancer Inst.* 96:998-1006; Liu, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:14567-14571).

[0169] In some embodiments, the heterologous antigen shares at least one epitope with, or is immunologically cross-reactive with, an antigen from, or derived from, a cancer or infectious agent.

(f). DNA repair mutants and nucleic acid targeting agents.

[0170] The present invention, in other embodiments, provides *Listeria* mutants, where the mutant is defective in repair of DNA damage, including, e.g., the repair of UV-light induced DNA damage, radiation induced damage, interstrand cross-links, intrastrand cross-links, covalent adducts, bulky adduct-modified DNA, deamidated bases, depurinated bases, depyrimidinated bases, oxidative damage, psoralen adducts, cis-platin adducts, combinations of the above, and the like (Mu and Sancar (1997) *Prog. Nucl. Acid Res. Mol. Biol.* 56:63-81; Sancar (1994) *Science* 266:1954-1956; Lin and Sancar (1992) *Mol. Microbiol.* 6:2219-2224; Selby and Sancar (1990) 236:203-211; Grossman (1994) *Ann. N.Y. Acad. Sci.* 726:252-265). Provided is a *Listeria* mutated in, e.g., *uvrA*, *uvrB*, *uvrAB*, *uvrC*, any combination of the above, and the like.

[0171] Moreover, what is provided is a *Listeria* that comprises at least one interstrand cross-link in its genomic DNA, or at least two, at least three, at least four, at least five, at least ten, at least 20, at least 30, at least 40, at least 50, at least 100, or more, cross-links in its genomic DNA.

[0172] One embodiment of the present invention comprises *Listeria uvrAB* engineered to express a heterologous antigen, where the engineered bacterium is treated with a nucleic acid cross-linking agent, a psoralen compound, a nitrogen mustard compound, 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, or beta-alanine,N-(acridine-9-yl),2-[bis(2-

chloroethyl)amino]ethyl ester (see, e.g., U.S. Publ. Pat. Appl. No. US 2004/0197343 of Dubensky; Brockstedt, *et al* (2005) Nat. Med. 11:853-860).

(g) Hybridization under stringent conditions.

[0173] Hybridization of a polynucleotide such as a plasmid to a variant of that polynucleotide, bearing at least one mutation, can be accomplished under the following stringent conditions. The plasmid can be between 2-3 kb, 3-4 kb, 4-5 kb, 5-6 kb, 6-7 kb, and so on. The mutation can consist of 1-10 nucleotides (nt), 10-20 nt, 20-30 nt, 30-40 nt, 40-50 nt, 50-60 nt, 60-70 kb, 70-80 kb, 80-90 kb, 90-100 kb, and the like.

[0174] Stringent conditions for hybridization in formamide can use the following hybridization solution: 48 ml formamide; 24 ml 20 times SSC; 1.0 ml 2 M Tris Cl, pH 7.6; 1.0 ml 100 times Denhardt's solution; 5.0 ml water; 20 ml 50% dextran sulfate, 1.0 ml 10% sodium dodecylsulfate (total volume 100 ml). Hybridization can be for overnight at 42° C (see, e.g., (1993) Current Protocols in Molecular Biology, Suppl. 23, pages 6.3.3-6.3.4). More stringent hybridization conditions comprise use of the above buffer but at the temperature of 43°, 44°, 45°, 46°, 47°, 48°, 49°, 50°, 51°, 52°, 53°, 54°, and the like.

[0175] Stringent hybridization under aqueous conditions are 1% bovine serum albumin; 1 mM EDTA; 0.5 M NaHPO₄, pH 7.2, 7% sodium dodecyl sulfate, with overnight incubation at 65° C. More stringent aqueous hybridization conditions comprise the use of the above buffer, but at a temperature of 66°, 67°, 68°, 69°, 70°, 71°, 72°, 73°, 74°, 75°, and so on (see, e.g., (1993) Current Protocols in Molecular Biology, Suppl. 23, pages 6.3.3-6.3.4).

[0176] Increasing formamide concentration increases the stringency of hybridization. Mismatches between probe DNA and target DNA slows down the rate of hybridization by about 2-fold, for every 10% mismatching. Similarly, the melting temperature of mismatched DNA duplex decreases by about one degree centigrade for every 1.7% mismatching (Anderson (1999) Nucleic Acid Hybridization, Springer-Verlag, New York, N.Y., pp. 70-72; Tijssen (1993) Hybridization with Nucleic Acid Probes, Elsevier Publ. Co., Burlington, MA; Ross (ed.) (1998) Nucleic Acid Hybridization: Essential Techniques, John Wiley and Sons, Hoboken, NJ; U.S. Pat. No. 6,551,784 issued to Fodor, *et al.*).

[0177] The invention encompasses a variant first plasmid that hybridizes under stringent conditions to a second plasmid of the present invention, where both plasmids are functionally equivalent, and where hybridization is determinable by hybridizing the first plasmid directly to the second plasmid, or by hybridizing oligonucleotide probes spanning the entire length

(individually or as a collection of probes) of the first variant plasmid to the second plasmid, and so on.

[0178] The skilled artisan will be able to adjust, or elevate, the hybridization temperature to allow distinction between a probe nucleic acid and a target nucleic acid where the sequences of the probe and target differ by 5-10 nucleotides, 10-15 nucleotides, 15-20 nucleotides, 20-25 nucleotides, 25-30 nucleotides, 30-35 nucleotides, 35-40 nucleotides, 40-45 nucleotides, 45-50 nucleotides, 50-55 nucleotides, 55-60 nucleotides, 60-65 nucleotides, 65-70 nucleotides, 70-80 nucleotides, and the like.

[0179] The invention provides polynucleotides that hybridize under stringent conditions to each of the polynucleotides described herein or their complements.

III. SOME DETAILED EMBODIMENTS OF THE INVENTION.

(a). Integration by site-specific recombination and by homologous recombination.

[0180] In some embodiments, nucleic acids, polynucleotides, bacterial genomes including listerial genomes, and bacteria including *Listeria* and *Bacillus anthracis*, of the present invention are modified by site-specific recombination and/or by homologous recombination. Site specific recombinases are described (see, e.g., Landy (1993) Curr. Op. Biotechnol. 3:699-707; Smith and Thorpe (2002) Mol. Microbiol. 44:299-307; Groth and Calos (2004) J. Mol. Biol. 335:667-678; Nunes-Duby, *et al.* (1998) Nucleic Acids Res. 26:391-406; Sauer (1993) Methods Enzymol. 225:890-900). Transposition is distinguished from site-specific recombination (see, e.g., Hallett and Sherratt (1997) FEMS Microbiol. Rev. 21:157-178; Grindley (1997) Curr. Biol. 7:R608-R612).

A. Site-specific recombination.

[0181] The present invention provides systems for mediating site-specific integration into a nucleic acid, vector, or genome. By "system" is meant, a first nucleic acid encoding an integrase, as well as the expressed integrase polypeptide, a second nucleic acid encoding a phage attachment site (attPP'), and a third nucleic acid encoding a corresponding bacterial attachment site (attBB'). Generally, any given attPP' site corresponds to, or is compatible with, a particular attBB' site. The availability of the integration systems of the present invention allow for the integration of one or more nucleic acids into any given polynucleotide or genome.

[0182] The integration site of the present invention can be implanted at a pre-determined position in a listerial genome by way of site-specific integration at an existing site (e.g., at the

tRNA^{Arg} integration site or the comK integration site). In addition, or in the alternative, the integration system site can be implanted at a pre-determined location by way of homologous integration.

[0183] Homologous recombination can result in deletion of material from the integration site, or no deletion of material, depending on the design of the regions of homology (the “homologous arms”). Any deletion that occurs, during homologous recombination corresponds to the region of the target DNA that resides in between regions of the target DNA that can hybridize with the “homologous arms.” Homologous recombination can be used to implant an integration site (attBB') within a bacterial genome, for future use in site-specific recombination.

[0184] Figure 1 discloses a strategy for preparing the plasmid, pINT, for use in site-directed integration into a bacterial genome. pINT contains a chloramphenicol resistance gene and an erythromycin resistance gene (see, e.g., Roberts, *et al.* (1996) Appl. Environ. Microbiol. 62:269-270). When pINT mediates site-specific integration of a nucleic acid into the listerial genome, the antibiotic resistance genes can be subsequently eliminated by transient exposure to Cre recombinase. As shown in Figure 1, the antibiotic resistance genes reside in between a first loxP site and a second loxP site. Cre recombinase can catalyze removal of material residing in between the two loxP sites. Transient expression of Cre recombinase can be effected by electroporation by a plasmid encoding Cre recombinase, or by any number of other techniques.

[0185] The *Listeria* genome or chromosome of the present invention is modified using the plasmids pPL1, pPL2, and/or pINT1 (Lauer, *et al.* (2002) J. Bact. 184:4177-4186). The plasmid pPL1 (GenBank Acc. No. AJ417488) comprises a nucleic acid encoding U153 integrase, where this integrase catalyzes integration at the comK-attBB' location of the listerial genome (Lauer, *et al.* (2002) J. Bact. 184:4177-4186). The structure of comK is available (nucleotides 542-1114 of GenBank Acc. No. AF174588). pPL1 contains a number of restriction sites suitable for inserting a cassette. For example, in some embodiments, a cassette of the present invention encodes at least one heterologous antigen and a loxP-flanked region, where the loxP-flanked region comprises: a first nucleic acid encoding an integrase and a second nucleic acid encoding an antibiotic resistance factor. Some of the restriction sites are disclosed in Table 6. Restriction sites can also be introduced *de novo* by standard methods.

Table 6. Restriction sites in pPL1 and pPL2.

pPL1		pPL2	
Site	Cut position	Site	Cut position
<i>HindII</i>	56	<i>HindII</i>	56
<i>SmaI</i>	95	<i>SmaI</i>	95
<i>BamHI</i>	99	<i>BamHI</i>	99
<i>HindIII</i>	69	<i>Clal</i>	64
<i>NotI</i>	118	<i>NotI</i>	118
<i>Sall</i>	54	<i>Sall</i>	54
<i>KpnI</i>	37	<i>SpeI</i>	105
<i>PstI</i>	91	<i>KpnI</i>	37
<i>SacI</i>	139	<i>PstI</i>	91
<i>AatII</i>	5 and 175	<i>SacI</i>	139
<i>BalI</i>	490 (in chloramphenicol resistance gene)	<i>AatII</i>	5 and 175
<i>ScaI</i>	340 (in chloramphenicol resistance gene)	<i>AvaI</i>	48 and 93
<i>BaeI</i>	3942 and 3975 (in U153 integrase gene)	<i>BalI</i>	490 (in chloramphenicol resistance gene)
<i>BsePI</i>	3753 (in U153 integrase gene)	<i>ScaI</i>	340 (in chloramphenicol resistance gene)
<i>MluI</i>	4074 (in U153 integrase gene)	<i>AflIII</i>	3259 and 4328 (in PSA integrase gene)
--	--	<i>SnaBI</i>	4077 and 4177 (in PSA integrase gene)
--	--	<i>Eam1105I</i>	3263 (in PSA integrase gene)
--	--	<i>BseYI</i>	4357 (in PSA integrase gene)
--	--	<i>SwaI</i>	3353 (in PSA integrase gene)
--	--	<i>BglII</i>	4150 (in PSA integrase gene)

[0186] The skilled artisan will appreciate that the techniques used for preparing pPL1 and pPL2, and for using pPL1 and pPL2 to mediate site-specific integration, can be applied to the integrases, phage attachment sites (attPP'), and bacterial attachment sites (attBB'), of the present invention.

[0187] pPL2 (GenBank Acc. No. AJ417499) comprises a nucleic acid encoding PSA integrase, where this integrase catalyzes integration at the tRNA^{Arg} gene of the *L. monocytogenes* genome (Lauer, *et al.* (2002) J. Bact. 184:4177-4186). The 74 nucleotide tRNA^{Arg} gene is found at nucleotide 1,266,675 to 1,266,748 of *L. monocytogenes* strain EGD genome (see, e.g., GenBank Acc. No. NC_003210), and at nucleotides 1,243,907 to 1,243,980 of *L. monocytogenes* strain 4bF265 (see, e.g., GenBank Acc. No. NC_002973). pPL2 contains a number of restriction sites suitable for inserting a cassette. The present invention provides a cassette encoding, e.g., a heterologous antigen and loxP-flanked region, where the loxP-flanked region comprises: a first nucleic acid encoding an integrase and a second nucleic acid encoding an antibiotic-resistance factor. Some of the restriction sites are disclosed in Table 6. Standard methods can be used to introduce other restriction sites *de novo*.

[0188] A first embodiment of site-specific recombination involves integrase-catalyzed site-specific integration of a nucleic acid at an integration site located at a specific tRNA^{Arg} region of the *Listeria* genome.

[0189] A second embodiment uses integration of a nucleic acid at the ComK region of the *Listeria* genome.

[0190] Additional embodiments comprise prophage attachment sites where the target is found at, e.g., tRNA-Thr4 of *L. monocytogenes* F6854 φ F6854.3 (nucleotides 277,661-277,710 of *L. monocytogenes* EGD GenBank Acc. No. AL591983.1), tRNA-Lys4 of *L. innocua* 11262 φ 11262.1 (nucleotides 115,501-115,548 of GenBank Acc. No. AL596163.1); similar to *L. monocytogenes* 1262 of *L. innocua* 11262 phi11262.3; intergenic of *L. innocua* 11262 φ 11262.4 (nucleotides 162,123-162,143 of GenBank Acc. No. AL596169.1); and tRNA-Arg4 of *L. innocua* 11262 φ 11262.6 (nucleotides 15908-15922 of GenBank Acc. No. AL596173.1 of *L. innocua* or nucleotides 145,229-145,243 of GenBank Acc. No. AL591983.1 of *L. monocytogenes* EGD) (see, e.g., Nelson, *et al.* (2004) Nucleic Acids Res. 32:2386-2395)

[0191] A further embodiment of site-specific recombination comprises insertion of a loxP sites (or Frt site) by site-specific integration at the tRNA^{Arg} region or ComK region, where

insertion of the loxP sites is followed by Cre recombinase-mediated insertion of a nucleic acid into the *Listeria* genome.

[0192] pPL1 integrates at the comK-attBB' chromosomal location (6,101 bp; GenBank Acc. No. AJ417488). This integration is catalyzed by U153 integrase. The *L. monocytogenes* comK gene is disclosed (nucleotides 542-1114 of GenBank Acc. No. AF174588). The pPL1 integration site comprises nucleotides 2694-2696 of the plasmid sequence AJ417488. The following two PCR primers bracket the attachment site comK-attBB' of the *Listeria* genome: Primer PL60 is 5'-TGA AGT AAA CCC GCA CAC GATC-3' (SEQ ID NO:9); Primer PL61 is 5'-TGT AAC ATG GAG GTT CTG GCA ATC-3' (SEQ ID NO:10). The primer pair PL60 and PL61 amplifies comK-attBB' resulting in a 417 bp product in non-lysogenic strains, e.g., DP-L4056.

[0193] pPL2 integrates at the tRNA^{Arg}-attBB' chromosomal location (6,123 bp; GenBank Acc. No. AJ417449). This integration is catalyzed by PSA integrase. pPL2 is similar to pPL1, except that the PSA phage attachment site and U153 integrase of pPL1 were deleted and replaced with PSA integrase and the PSA phage attachment site. The pPL2 integration site comprises a 17 bp region that resides at nucleotides 2852-2868 of the plasmid pPL2 (AJ417449), with the corresponding bacterial region residing at nucleotides 1,266,733-1,266,749 of *L. monocytogenes* strain EGD genome (GenBank Acc. No. NC_003210).

[0194] For listeriphage A118, a phage closely related to U153 listeriphage, the attB position resides at nucleotides 187-189 of the 573 bp comK ORF (Loessner, *et al.* (2000) Mol. Microbiol. 35:324-340). This 573 bp ORG (nucleotide 542-1114 of GenBank Acc. No. AF174588) and the attB site (nucleotide 701-757 of GenBank Acc. No. AF174588) are both disclosed in GenBank Acc. No. AF174588. The attP site resides in the listeriphage A118 genome at nucleotides 23500-23444 (GenBank Acc. No. AJ242593).

[0195] The present invention provides reagents and methods for catalyzing the integration of a nucleic acid, e.g., a plasmid, at an integration site in a *Listeria* genome. The *L. monocytogenes* genome is disclosed (see, e.g., GenBank Acc. No. NC_003210; GenBank Acc. No. NC_003198, He and Luchansky (1997) Appl. Environ. Microbiol. 63:3480-3487, Nelson, *et al.* (2004) Nucl. Acids Res. 32:2386-2395; Buchrieser, *et al.* (2003) FEMS Immunol. Med. Microbiol. 35:207-213; Doumith, *et al.* (2004) Infect. Immun. 72:1072-1083; Glaser, *et al.* (2001) Science 294:849-852).

[0196] Suitable enzymes for catalyzing integration of a nucleic acid into a *Listeria* genome include, e.g., U153 integrase (see, e.g., complement of nucleotides 2741-4099 of GenBank Acc. No. AJ417488: Lauer, *et al.* (2002) J. Bact. 184:4177-4186) and PSA integrase (see,

e.g., complement of nucleotides 19,413-20,567 of PSA phage genome (37,618 bp genome) (GenBank Acc. No. NC_003291)).

[0197] A similar or identical nucleotide sequence for tRNA^{Arg} gene, and for the core integration site that is found within this gene, has been disclosed for a number of strains of *L. monocytogenes*. The *L. monocytogenes* strain EGD complete genome (2,944,528 bp total) (GenBank Acc. No. NC_003210) contains an integration site in the tRNA^{Arg} gene. The 74 nucleotide tRNA^{Arg} gene is found at nucleotide 1,266,675 to 1,266,748 of GenBank Acc. No. NC_003210. Similarly, the tRNA^{Arg} gene occurs in *L. monocytogenes* strain 4bF265 (GenBank Acc. No. NC_002973) at nucleotides 1,243,907 to 1,243,980. The sequence of tRNA^{Arg} gene for *L. monocytogenes* strain WSLC 1042 is disclosed in Lauer, *et al.* (2002) J. Bact. 184:4177-4186. Lauer, *et al.*, *supra*, disclose the bacterial core integration site and the corresponding phage core integration site.

[0198] Residence in a functional cluster establishes function of nucleic acids residing in that cluster. The function of a bacterial gene, or bacteriophage gene, can be identified according to its grouping in a functional cluster with other genes of known function, its transcriptional direction as relative to other genes of similar function, and occurrence on one operon with other genes of similar function (see, e.g., Bowers, *et al.* (2004) Genome Biology 5:R35.1-R35.13). For example, the gene encoding phage integrase has been identified in the genomes of a number of phages (or phages integrated into bacterial genomes), where the phage integrase gene resides in a lysogeny control cluster, where this cluster contains a very limited number of genes (three genes to nine genes) (see, e.g., Loessner, *et al.* (2000) Mol. Microbiol. 35:324-340; Zimmer, *et al.* (2003) Mol. Microbiol. 50:303-317; Zimmer, *et al.* (2002) J. Bacteriol. 184:4359-4368).

[0199] The phage attachment site (attPP') resides essentially immediately adjacent to the phage integrase gene. According to Zhao and Williams, the integrase gene (int) and attP are typically adjacent, facilitating their co-evolution (Zhao and Williams (2002) J. Bacteriol. 184:859-860). For example, in phiC31 phage, phage integrase is encoded by nucleotide (nt): 38,447 to 40,264, while the attP site resides nearby at nt 38,346 to 38,429. PhiC31 phage integrase does not require cofactors for catalyzing the integration reaction, and can function in foreign cellular environments, such as mammalian cells (see, e.g., Thorpe and Smith (1998) Proc. Natl. Acad. Sci. USA 95:5505-5510; Groth, *et al.* (2000) Proc. Natl. Acad. Sci. USA 97:5995-6000; GenBank Acc. No. AJ006589). Furthermore, for phage SM1, phage HP1, phage phi3626, for various actinomycete bacteriophages (intM gene), phage lambda, and for phage Aa phi23, the integrase gene and attP site are located immediately next

to each other. The integrase gene and attP site can occur together in small group of genes known as a "lysogeny control cluster." Methods for determining the genomic location, approximate size, maximally active size, and/or minimal size of an attPP' site (or attP site) are available (see, e.g., Zimmer, *et al.* (2002) *J. Bacteriol.* 184:4359-4368; Siboo, *et al.* (2003) *J. Bacteriol.* 185:6968-6975; Mayer, *et al.* (1999) *Infection Immunity* 67:1227-1237; Alexander, *et al.* (2003) *Microbiology* 149:2443-2453; Hoess and Landy (1978) *Proc. Natl. Acad. Sci. USA* 75:5437-5441; Resch (2005) *Sequence and analysis of the DNA genome of the temperate bacteriophage Aaphi23*, Inauguraldissertation, Univ. Basel; Campbell (1994) *Ann. Rev. Microbiol.* 48:193-222).

[0200] The present invention provides a vector for use in modifying a listerial genome, where the vector encodes phiC31 phage integrase, phiC31 attPP' site, and where the listerial genome was modified to include the phiC31 attBB' site. A bacterial genome, e.g., of *Listeria* or *B. anthracis*, can be modified to include an attBB' site by homologous recombination. The phiC31 attBB' site is disclosed by Thorpe and Smith (1998) *Proc. Natl. Acad. Sci. USA* 95:5505-5510. The amino acid sequence of phiC31 integrase is disclosed below (GenBank Acc. No. AJ414670):

```
MTQGVVTGVDITYAGAYDRQSRERENSSAASPATQRSANEDKAADLQREVERDGGFRFRFVGHFSEAPGT
SAFGTAERPEFERILNECRAGRLNMIIVYDVSFRSLKVMDAIPIVSELLALGVTIVSTQEGVFRQGN
VMDLIHLIMRLDASHKESSLKSAKILDTKNLQRELGGYVGGKAPYGFELVSETKEITRNGRMVNVVIN
KLAHSTTPLTGPFEPDVIRWWWREIKTHKHLPFKPGSQAAIHGPSITGLCKRMDADAVPTRGETIG
KKTASSAWDPATVMRILRDPRIAGFAAEVIYKKKPDGTPPTKIEGYRIQRDPITLRPVELDCGPITIEP
AEWYELQAWLDGRGRGKGLSRGQAILSAMDKLYCECGAVMTSKRGEESIKDSYRCRRRKVVDPSAPGQ
HEGTCNVSMALDKFVAERIFNKIRHAEGDEETLALLWEAARRFGKLTEAPEKSGERANLVAERADAL
NALEELYEDRAAGAYDGPVGRKHFRKQQAALTLRQGAEEERLAELEAAEAPKLPLDQWFPEDADADPT
GPKSWWGRASVDDKRVFVGLFVDKIVVTKSTTGRGQGTPIEKRASITWAKPPTDDDEDDAQDGTEDVA
A (GenBank Acc. No. AJ414670) (SEQ ID NO:11)
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[0201] The present invention provides the following relevant phiC31 target attBB' sites, and functional variants thereof:

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TGACGGTCTCGAAGCCGCGGTGCGGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTAC
TCCACCTCACCCATCTGGTCCA (SEQ ID NO:12) (see, e.g., Thorpe and Smith (1998) Proc.
Natl. Acad. Sci. USA 95:5505-5510).
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gtcgacgatgtaggtcacggtctcgaagccgcggtgcggtgccagggcggtgcccttgggct
ccccgggcggtactccacctcacccatctggtccatcatgatgaacgggtcgaggtggcgg
tagttgatcccggcgaacgcgcggcgccaggggaagccctcgccctcgaaaccgctgggccc
ggtggtcacggtgagcacgggacgtgcgacggcggtcgggcggtgcggtatcgcggggcagcg
tcagcggttctcgacggtcacggcgggcatgtcgac (SEQ ID NO:13) (GenBank Acc. No.
X60952)
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[0202] Furthermore, the invention provides the following relevant phiC31 attPP' sites, and functional variants thereof:

AAGGGGTTGTGACCGGGGTGGACACGTACGCGGGTGCTTACGACCGTCAGTCGC
GCGAGCGCGAGAATTC (SEQ ID NO:14) (see, e.g., GenBank Acc. Nos. X57036 and
AJ006589; Thorpe and Smith (1998) Proc. Natl. Acad. Sci. USA 95:5505-5510).

[0203] The present invention encompasses a vector that encodes a phage integrase and a functionally active attPP' site, but does not encode the phage integrase and attPP' site of pPL1. Also encompassed is a vector that encodes a phage integrase and a functionally active attPP' site, but does not encode the phage integrase and attPP' site of pPL2. Moreover, the present invention encompasses a vector that encodes a phage integrase and a functionally active attPP' site, but does not encode the phage integrase and attPP' site of pPL1 or of pPL2.

[0204] The present invention encompasses a vector useful for integrating a heterologous nucleic acid into a bacterial genome that encodes a phage integrase and a functionally active attPP' site, but does not encode the phage integrase and attPP' site of U153 phage. Also encompassed is a vector, useful for integrating a heterologous nucleic acid into a bacterial genome, that encodes a phage integrase and a functionally active attPP' site, but does not encode the phage integrase and attPP' site of PSA phage. Moreover, the present invention encompasses a vector, useful for integrating a heterologous nucleic acid into a bacterial genome, that encodes a phage integrase and a functionally active attPP' site, but does not encode the phage integrase and attPP' site from any of U153 phage and PSA phage. In another aspect, the present invention encompasses a vector, useful for integrating a heterologous nucleic acid into a bacterial genome, that encodes a phage integrase and a functionally active attPP' site, but does not encode the phage integrase and attPP' site of A118 phage. Further encompassed by the invention is a vector, useful for integrating a heterologous nucleic acid into a bacterial genome, that encodes a phage integrase and a functionally active attPP' site, but does not encode the phage integrase and attPP' site from any of A118 phage, U153 phage, or PSA phage.

B. Homologous recombination.

[0205] The target site for homologous recombination can be an open reading frame, a virulence gene, a gene of unknown function, a pseudogene, a region of DNA shown to have no function, a gene that mediates growth, a gene that mediates spread, a regulatory region, a

region of the genome that mediates listerial growth or survival, a gene where disruption leads to attenuation, an intergenic region, and the like.

[0206] To give a first example, once a nucleic acid encoding an antigen (operably linked with a promoter) is implanted into a virulence gene, the result is two fold, namely the inactivation of the virulence gene, plus the creation of an expressable antigen.

[0207] The invention provides a *Listeria* bacterium comprising an expression cassette, integrated via homologous recombination (or by allelic exchange, and the like), in a *listerial* virulence gene. Integration can be with or without deletion of a corresponding nucleic acid from the *listerial* genome.

[0208] The expression cassette can be operably linked with one or more promoters of the virulence gene (promoters already present in the parental or wild type *Listeria*). Alternatively, the expression cassette can be operably linked with both: (1) One or more promoters supplied by the expression cassette; and (2) One or more promoters supplied by the parent or wild type *Listeria*.

[0209] In some embodiments, the expression cassette can be operably linked with one or more promoters supplied by the expression cassette, and not at all operably linked with any promoter of the *Listeria*.

[0210] Without implying any limitation, the virulence factor gene can be one or more of *actA*, *inlB*, both *actA* and *inlB*, as well as one or more of the genes disclosed in Table 3. In another aspect, homologous recombination can be at the locus of one or more genes that mediate growth, spread, or both growth and spread.

[0211] In another aspect, the invention provides a *Listeria* bacterium having a polynucleotide, where the polynucleotide comprises a nucleic acid (encoding a heterologous antigen) integrated at the locus of a virulence factor. In some embodiments, integration is by homologous recombination. In some embodiments, the invention provides integration in a regulatory region of the virulence factor gene, in an open reading frame (ORF) of the virulence factor gene, or in both a regulatory region and the ORF of the virulence factor. Integration can be with deletion or without deletion of all or part of the virulence factor gene.

[0212] Expression of the nucleic acid encoding the heterologous antigen can be mediated by the virulence factor's promoter, where this promoter is operably linked and with the nucleic acid. For example, a nucleic acid integrated in the *actA* gene can be operably linked with the *actA* promoter. Also, a nucleic acid integrated at the locus of the *inlB* gene can be operably linked and in frame with the *inlB* promoter. In addition, or as an alternative, the regulation of

expression of the open reading frame can be mediated entirely by a promoter supplied by the nucleic acid.

[0213] The expression cassette and the above-identified nucleic acid can provide one or more listerial promoters, one or more bacterial promoters that are non-listerial, an actA promoter, an inlB promoter, and any combination thereof. The promoter mediates expression of the expression cassette. Also, the promoter mediates expression of the above-identified nucleic acid. Moreover, the promoter is operably linked with the ORF.

[0214] In some embodiments, integration into the virulence gene, or integration at the locus of the virulence gene, results in deletion of all or part of the virulence gene, and/or disruption of regulation of the virulence gene. In some embodiments, integration results in an attenuation of the virulence gene, or in inactivation of the virulence gene. Moreover, the invention provides a promoter that is prfA-dependent, a promoter that is prfA-independent, a promoter of synthetic origin, a promoter of partially synthetic origin, and so on.

[0215] Provided is a method for manufacturing the above-disclosed *Listeria*. Also provided are methods of using the above-disclosed *Listeria* for expressing the expression cassette or for expressing the above-identified nucleic acid. Moreover, in some embodiments, what is provided are methods for stimulating a mammalian immune system, comprising administering the above-disclosed *Listeria* to a mammal.

[0216] To give another example, once a bacterial attachment site (attBB') is implanted in a virulence gene, the result is two fold, namely the inactivation of that gene, plus the creation of a tool that enables efficient integration of a nucleic acid at that attBB' site.

[0217] In directing homologous integration of the pKSV7 plasmid, or another suitable plasmid, into the listerial genome, the present invention provides a region of homology that is normally at least 0.01 kb, more normally at least 0.02 kb, most normally at least 0.04 kb, often at least 0.08 kb, more often at least 0.1 kb, most often at least 0.2 kb, usually at least 0.4 kb, most usually at least 0.8 kb, generally at least 1.0 kb, more generally at least 1.5 kb, and most generally at least 2.0 kb.

[0218] Figure 2 demonstrates a strategy using pKSV7 in homologous recombination into a bacterial genome. In Step 1, the plasmid crosses over with a region of homology in the genome. In Step 2, the plasmid integrates into the genome, producing a merodiploid intermediate. WXYZ represents any sequence in the pKSV7, such as an antibiotic-resistance encoding gene. Step 3 shows a second crossover, while Step 4 shows elimination of the "body" of the pKSV7 plasmid and elimination of WXYZ. Subsequent treatment with

Cre recombinase, e.g., by transient expression of Cre recombination, catalyzes removal of material between the loxP sites.

[0219] Figure 3 shows a method for preparing an insert, where the insert is placed into pKSV7. The insert mediates homologous recombination into a listerial genome, resulting in integration of various elements into the listerial genome (nucleic acids encoding an antigen, loxP sites, and an antibiotic resistance gene). Subsequent treatment with Cre recombinase catalyzes removal of material between the loxP sites.

[0220] Figure 4 shows a method for preparing an insert, where the insert is placed into pKSV7. The insert mediates homologous recombination into a listerial genome, resulting in integration of various elements into the listerial genome (nucleic acid encoding an antigen). Nucleic acids encoding loxP sites and an antibiotic resistance gene are encoded by a modified pKSV7. Subsequent treatment with Cre recombinase, e.g., by transient expression of Cre recombination, catalyzes removal of material between the loxP sites.

[0221] Figure 5 discloses an embodiment that results in only integration with no deletion. Subsequent treatment with Cre recombinase, e.g., by transient expression of Cre recombination, catalyzes removal of material between the loxP sites.

[0222] The reagents and methods of the present invention, prepared by homologous recombination, are not limited to use of pKSV7, or to derivatives thereof. Other vectors suitable for homologous recombination are available (see, e.g., Merlin, *et al.* (2002) *J. Bacteriol.* 184:4573-4581; Yu, *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:5978-5983; Smith (1988) *Microbiol. Revs.* 52:1-28; Biswas, *et al.* (1993) *J. Bact.* 175:3628-3635; Yu, *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:5978-5983; Datsenko and Wanner (2000) *Proc. Natl. Acad. Sci. USA* 97:6640-6645; Zhang, *et al.* (1998) *Nature Genetics* 20:123-128).

[0223] For integrating a nucleic acid by way of homologous recombination, bacteria are electroporated with a pKSV7, where the pKSV7 encodes a heterologous protein or where the pKSV7 contains an expression cassette. Bacteria are selected by plating on BHI agar media (or media not based on animal proteins) containing a suitable antibiotic, e.g., chloramphenicol (0.01 mg/ml), and incubated at the permissive temperature of 30°C. Single cross-over integration into the bacterial chromosome is selected by passaging several individual colonies for multiple generations at the non-permissive temperature of 41°C in medium containing the antibiotic. Finally, plasmid excision and curing (double cross-over) is achieved by passaging several individual colonies for multiple generations at the permissive temperature of 30°C in BHI media not containing the antibiotic.

[0224] Homologous recombination can be used to insert a nucleic acid into a target DNA, with or without deletion of material from the target DNA. A vector that mediates homologous recombination includes a first homologous arm (first nucleic acid), a second homologous arm (second nucleic acid), and a third nucleic acid encoding a heterologous antigen that resides in between the two homologous arms. Regarding the correspondence of the homologous arms and the target genomic DNA, the target regions can abut each other or the target regions can be spaced apart from each other. Where the target regions abut each other, the event of homologous recombination merely results in insertion of the third nucleic acid. But where the target regions are spaced apart from each other, the event of homologous recombination results in insertion of the third nucleic acid and also deletion of the DNA residing in between the two target regions.

[0225] Homologous recombination at the *inlB* gene can be mediated by pKSV7, where the pKSV7 contains the following central structure. The following central structure consists essentially of a first homologous arm (upstream of *inlB* gene in a *L. monocytogenes* genome), a region containing *KpnI* and *BamHI* sites (underlined), and a second homologous arm (downstream of *inlB* gene in *L. monocytogenes*). The region containing *KpnI* and *BamHI* sites is suitable for receiving an insert, where the insert also contains *KpnI* and *BamHI* sites at the 5'-prime and 3'-prime end (or 3'-end and 5'-end):

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CCAAATTAGCGATCTTACACCATTGGCTAATTTAACAAGAATCACCCAACTAGG
GTTGAATGATCAAGCATGGACAAATGCACCAGTAACTACAAAGCAAATGTATC
CATTCCAAACACGGTGAAAAATGTGACTGGCGCTTTGATTGCACCTGCTACTAT
TAGCGATGGCGGTAGTTACGCAGAACCGGATATAACATGGAACCTTACCTAGTTA
TACAAATGAAGTAAGCTATACCTTTAGCCAACTGTCACTATTGGAAAAGGAAC
GACAACATTTAGTGGAACCGTGACGCAGCCACTTAAGGCAATTTTAAATGCTAA
GTTTCATGTGGACGGCAAAGAAACAACCAAGAAGTGGAAGCTGGGAATTTATT
GACTGAACCAGCTAAGCCCGTAAAAGAAGGTCACACATTTGTTGGTTGGTTTGA
TGCCCAAACAGGCGGAACCTAAGTGGAATTTCACTACGGATAAAATGCCGACAAA
TGACATCAATTTATATGCACAATTTAGTATTAACAGCTACACAGCAACCTTTGA
GAATGACGGTGTAACAACATCTCAAACAGTAGATTATCAAGGCTTGTTACAAGA
ACCTACACCACCAACAAAAGAAGGTTATACTTTCAAAGGCTGGTATGACGCAAA
AACTGGTGGTGACAAGTGGGATTTGCAACTAGCAAAATGCCTGCTAAAAACAT
CACCTTATATGCCCAATATAGCGCCAATAGCTATACAGCAACGTTTGATGTTGA
TGGAATCAACGACTCAAGCAGTAGACTATCAAGGACTTCTAAAAGAACCAAA
GGCACCACGAAAGCCGGATATACTTTCAAAGGCTGGTATGACGAAAAACAGA
TGGGAAAAAATGGGATTTTGCGACGGATAAAATGCCAGCAAAATGACATTACGCT
GTACGCTCAATTTACGAAAAATCCTGTGGCACCACCAACAACTGGAGGGAACAC
ACCGCCTACAACAAATAACGGCGGGAATACTACACCACCTCCGCAAATATAACC
TGGAAGCGACACATCTAACACATCAACTGGGAATTCAGCCAGCACACAAGTAC
AATGAACGCTTATGACCTTTATAATTCAAAGAAGCTTCACTCCCTACAACCTGG
CGATAGCGATAATGCGCTCTACCTTTTGTAGGGTTATTAGCAGTAGGAACCTGC
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AATGGCTCTTACTAAAAAGCACGTGCTAGTAAATAGAAGTAGTGTAAGAGCT
 AGATGTGGTTTTTCGGACTATATCTAGCTTTTTTATTTTTTAATAACTAGAAATCA
 AGGAGAGGATAGTGGTACCTTGGTGAGCTCCCTACGAAAAGCTACAACCTTAAA
 TTCATGAAAAAAGAACTGATTCTGCTGAAAACGGATCAGTTCTTTTTCTTTAGA
 CTTATTTTTTACAAAACCTTTTCGATAATTTCCATATTCTGGGGTCTGTCTTTGC
 TTTCAAGTACAGAAATATCACGAACAATGCTATCTAATTTAATTTTTTCCATTT
 CAAATTCTATTTTTTGTGGAGCAGATCGTATTTACTCGTAAGAAGCTGTTGGA
 TATTGGCTCCGACAACGCAGTCTGGGTTGGTTTTTGGATCAACGTGAATTAAT
 TCGTATTGCCTTCTATACTCTTATAAACATCAAGCAGTGAATTTCTTCTGGTG
 GTCTAGCAAGAATCGGATTTGCTTTGCCAGTCTGCGTAGTAATTAATCAGCTT
 TTTTTAAATTACTCATGATTTTCTAATGTTAGCAGGATTTGTTTTTACGCTAC
 CAGCAATAATTTCACTCGATAACAAATTCGTATTTTTAAAAATTTCTATATAAG
 CCAAAATGTGGATAGCATCGCTAAATTGGATAGAGTATTTCAATTTTTTCAATC
 CTTTCAAATTTTCTCCTTGACTTATCTTATCATAATGTTTATTATAAAGGTGTA
 AATTATAAATGTACAGCTTTAGTGTTAAAAAATTTAAAGGAGTGGTTTAAATGA
 CTTATTTAGTAAGTGGTGCAACAGGTGGACTTGGAGGCTACGCATTAAATTATT
 TGAAAGAGCTGGTTCCCATGTCCGATATTTATGCTTTAGTTCGTAGCGAAGAAA
 AAGGTACAGACTTGAAAGCAGCAGGATTTAATATCCGTATTGGTGATTATAGTG
 ATGTAGAATCAATGAAGCAAGCATTTCGCAGGCATCGACCGCTATTATTGTTT
 CAGGAGCACCTGGTAATCGCCAAGTAGAACACGAAAATGTGGTAAATGCGGCAA
 AAGAAGCAGGCGTTTCTTACATCGCTTACACAAGTTTCGCGGGCGCAGATAAAT
 CCACAAGCGCTTTAGCAGAAGATCATTCTTTACCGAAAAAGTAATCGAAAAAT
 CCGGAATCGCGCACACTTTCTTGCGTAACAACTGGTACTTCGAAAAATGAAATGC
 CGATGATCGGTGGCGCATTGAGTGCTGGAAAATTTGTATACGCTGCTGAAAATG
 GAAAAGTTGGCTGGGCATTAAACGCGAATACGCAGAAGTAGCCGCAAAAGCTG
 TTGCGGACGCTGACTTCCCAGAAATCCTTGAATTATCTGGCCCACTCATGCAAT
 TCGTAATCATGTATAGCTGTTTCTGTGTGAAATTGTTATCCGCTCACAAATC
 CACACAACATACGAGCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAG
 TGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAA
 ACCTGTCGTGCCAGCTGGACTAAAAGGCATGCAATTCA (SEQ ID NO:15)

[0226] The following is the region of the “central region” that contains *KpnI* and *BamHI* sites for inserting an expression cassette: GGTACCTTGGTGAGCTC (SEQ ID NO:121).

[0227] The upstream homologous arm is shown below (upstream of *inlB* gene). The present sequences are from *L. monocytogenes* 10403S. The following provides comparison with another listerial strain, *L. monocytogenes* 4bF2365. In this strain, the *inlB* gene resides at nt 196,241-198,133 (GenBank AE017323; segment 2 of 10 segments). The upstream homologous arm, disclosed here for *L. monocytogenes* 10403S, has a corresponding sequence in *L. monocytogenes* 4bF2365 at nt 194,932 to 196,240 (GenBank AE017323; segment 2 of 10 segments). The downstream homologous arm, disclosed here for *L. monocytogenes* 10403S, has a corresponding (but not totally identical) sequence in *L. monocytogenes* 4bF2365 at nt 198,134 to 199,629 (GenBank AE017323; segment 2 of 10 segments).

(upstream homologous arm)

CCAAATTAGCGATCTTACACCATTTGGCTAATTTAACAAGAATCACCCAACTAGG
 GTTGAATGATCAAGCATGGACAAATGCACCAGTAACTACAAAGCAAATGTATC

CATTCCAAACACGGTGAAAAATGTGACTGGCGCTTTGATTGCACCTGCTACTAT
 TAGCGATGGCGGTAGTTACGCAGAACCGGATATAACATGGAACCTACCTAGTTA
 TACAAATGAAGTAAGCTATACCTTTAGCCAACCTGTCACTATTGGAAAAGGAAC
 GACAACATTTAGTGGAACCGTGACGCAGCCACTTAAGGCAATTTTTAATGCTAA
 GTTTCATGTGGACGGCAAAGAAACAACCAAAGAAGTGGAAGCTGGGAATTTATT
 GACTGAACCAGCTAAGCCCGTAAAGAAGGTCACACATTTGTTGGTTGGTTTGA
 TGCCCAAACAGGCGGAACCTAAGTGGAATTTTCAGTACGGATAAAATGCCGACAAA
 TGACATCAATTTATATGCACAATTTAGTATTAACAGCTACACAGCAACCTTTGA
 GAATGACGGTGTAACAACATCTCAAACAGTAGATTATCAAGGCTTGTTACAAGA
 ACCTACACCACCAACAAAAGAAGGTTATACCTTTCAAAGGCTGGTATGACGCAAA
 AACTGGTGGTGACAAGTGGGATTTTCGCAACTAGCAAAATGCCTGCTAAAAACAT
 CACCTTATATGCCCAATATAGCGCCAATAGCTATACAGCAACGTTTGATGTTGA
 TGGAAAATCAACGACTCAAGCAGTAGACTATCAAGGACTTCTAAAAGAACCAAA
 GGCACCAACGAAAGCCGGATATACTTTCAAAGGCTGGTATGACGAAAAAACAGA
 TGGGAAAAAATGGGATTTTGCGACGGATAAAATGCCAGCAAAATGACATTACGCT
 GTACGCTCAATTTACGAAAAATCCTGTGGCACCACCAACAACCTGGAGGGAACAC
 ACCGCCTACAACAAATAACGGCGGGAATACTACACCACCTTCGCAAAATATACC
 TGGAAGCGACACATCTAACACATCAACTGGGAATTCAGCCAGCACACAAGTAC
 AATGAACGCTTATGACCTTTATAATTCAAAGAAGCTTCACTCCCTACAACCTGG
 CGATAGCGATAATGCGCTCTACCTTTTGTAGGGTTATTAGCAGTAGGAAGTGC
 AATGGCTCTTACTAAAAAAGCACGTGCTAGTAAATAGAAGTAGTGTAAAGAGCT
 AGATGTGGTTTTTCGGACTATATCTAGCTTTTTTTATTTTTTAATAACTAGAATCA
 AGGAGAGGATAGT (SEQ ID NO:16)

[0228] The downstream homologous arm is shown below (downstream of inlB gene):

(downstream homologous arm)

CCTACGAAAAGCTACAACCTTTAAATTCATGAAAAAGAACTGATTCGCTGAAAA
 CGGATCAGTTCTTTTTTCTTTAGACTTATTTTTACAAAACTTTTCGATAATTT
 CCATATTCTGGGGTCTGTCTTTGCTTTCAAGTACAGAAATATCACGAACAATGC
 TATCTAATTTAATTTTTTCCATTTCAAATTCATTTTTTTGTTGGAGCAGATCGT
 ATTTACTCGTAAGAAGCTTGTGGATATTGGCTCCGACAACGCAGTCTGGGTTGG
 TTTTTGGATCAACGTGAATTAATTCGTATTGCCTTCTATACTCTTATAAACAT
 CAAGCAGTGAAATTTCTTCTGGTGGTCTAGCAAGAATCGGATTTGCTTTGCCAG
 TCTGCGTAGTAATTAATCAGCTTTTTTTAAATTACTCATGATTTTTCTAATGT
 TAGCAGGATTTGTTTTTACGCTACCAGCAATAATTTCACTCGATAACAAATTCG
 TATTTTTAAAAATTTCTATATAAGCCAAAATGTGGATAGCATCGCTAAATTGGA
 TAGAGTATTTTCATTTTTTTCAATCCTTTCAAATTTTCTCCTTGACTTATCTTAT
 CATAATGTTTTATTATAAAGGTGTAAATTATAAATGTACAGCTTTAGTGTTAAAA
 AATTTAAAGGAGTGGTTTAAATGACTTATTTAGTAACTGGTGCAACAGGTGGAC
 TTGGAGGCTACGCATTAAATTATTTGAAAGAGCTGGTTCCTATGTCCGATATTT
 ATGCTTTAGTTCGTAGCGAAGAAAAAGGTACAGACTTGAAAGCAGCAGGATTTA
 ATATCCGTATTGGTGATTATAGTGATGTAGAATCAATGAAGCAAGCATTTCGCAG
 GCATCGACCGGTATTATTTGTTTCAGGAGCACCTGGTAATCGCCAAGTAGAAC
 ACGAAAATGTGGTAAATGCGGCAAAAGAAGCAGGCGTTTCTTACATCGCTTACA
 CAAGTTTCGCGGGGCGCAGATAAATCCACAAGCGCTTTAGCAGAAGATCATTTCT
 TTACCGAAAAAGTAATCGAAAAATCCGGAATCGCGCACACTTTCTTGCCTAACA
 ACTGGTACTTCGAAAAATGAAATGCCGATGATCGGTGGCGCATTGAGTGCTGGAA
 AATTTGTATACGCTGCTGAAAATGGAAAAGTTGGCTGGGCATTAAAACGCGAAT
 ACGCAGAAGTAGCCGCAAAAGCTGTTGCGGACGCTGACTTCCAGAAATCCTTG
 AATTATCTGGCCCACTCATGCAATTCGTAATCATGTACATAGCTGTTTCCTGTGT
 GAAATTGTTATCCGCTCACAATTCACACACATACGAGCCGGAAGCATAAAGT

GTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCT
 CACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGGACTAAAAGGCAT
 GCAATTCA (SEQ ID NO:17)

[0229] Regarding insertion at the ActA gene in a listerial genome, the following discloses a suitable upstream and downstream homologous arms for mediating homologous-recombination integration at the ActA locus of *L. monocytogenes* 10403S:

(upstream homologous arm)

AGAATTTAGTTCCGCGAGTGGATGCTCATTTTTACGCAAGTGAAGTGTACGAATACTATAAAAAATGTCC
 ACCAAGTAGAGAGTCTAGATGGTAAAGGTGGAGAAAATTGATTCGTTTGTCCATTATGGCTTGAATTGC
 AATAATGCCTTTTGGGATGGCCAAGAAATCTTTATGGAGATGGGGACAAAAAGAAATTTCAAACCATT
 TTCATGCGCCAAAACATTTGTTGGTTCATGAACTAACGCATGCAGTTATCCAGTATTCGGCGGGATTGG
 AATACGAAGGGCAATCAGGTGCGCTAAACGAGTCGTTTCGCCGATGTTTTTGGTTATTTTATTGCGCCA
 AATCATTGGTTGATTGGTGAGGATGTCTGTGTGCGTGGGTTCGCGAGATGGGCGAATAAGAAGCATTA
 AGATCCTGACAAATATAATCAAGCGGCTCATATGAAGGATTACGAATCGCTTCCAATCACAGAGGAAG
 GCGACTGGGGCGGAGTTCATTATAATAGTGGTATCCCGAATAAAGCAGCCTATAATACTATCNCATAAA
 CTTGGAAAAGAAAAAACAGAACAGCTTTATTTTCGCGCCTTAAAGTACTATTTAACGAAAAAATCCCA
 GTTTACCGATGCGAAAAAAGCGCTTCAACAAGCAGCGAAAGATTTATATGGTGAAGATGCTTCTAAAA
 AAGTTGCTGAAGCTTGGGAAGCAGTTGGGGTTAACTGATTAAACAATGTTAGAGAAAAATTAATTCTC
 CAAGTGATATTCTTAAATAATTCATGAATATTTTTCTTATATTAGCTAATTAAGAAGATAATTAAC
 TGCTAATCCAATTTTAAACGGAATAAATTAGTGAAAATGAAGGCCGAATTTTCCTTGTCTAAAAAGG
 TTGTATTAGCGTATCACGAGGAGGGAGTATAA (SEQ ID NO:18)

[0230] The following discloses a suitable downstream homologous arm, for mediating insertion at the listerial ActA gene:

(homologous downstream arm)

AAACACAGAACGAAAGAAAAAGTGAGGTGAATGATATGAAATTCAAAAATGT
 GGTTCCTAGGTATGTGCTTGACCGCAAGTGTTCTAGTCTTTCCGGTAACGATA
 AAAGCAAATGCCTGTTGTGATGAATACTTACAAACACCCGAGCTCCGCATG
 ATATTGACAGCAAATTACACATAAACTTAGTTGGTCCGCGGATAACCCGAC
 AAATACTGACGTAAATACGCACTATTGGCTTTTTAAACAAGCGGAAAAAATA
 CTAGCTAAAGATGTAAATCATATGCGAGCTAATTTAATGAATGAACTTAAAA
 AATTCGATAAAACAAATAGCTCAAGGAATATATGATGCGGATCATAAAAATCC
 ATATTATGATACTAGTACATTTTTATCTCATTTTTATAATCCTGATAGAGAT
 AATACTTATTTGCCGGGTTTTGCTAATGCGAAAAATAACAGGAGCAAAGTATT
 TCAATCAATCGGTGACTGATTACCGAGAAGGGAATTTGACACAGCGTTTTTA
 TAAATTAGGCCTAGCAATCCATTATTATACGGATATTAGTCAACCTATGCAC
 GCCAATAATTTTACCGCAATATCATACCTCCAGGCTACCACTGTGCATATG
 AAAATTACGTAGATACCATTAACACAATTATCAAGCAACGGAAGACATGGT
 AGCAAAAAGATTTTGTCTCAGATGACGTGAAAGACTGGCTCTATGAAAATGCG
 AAAAGGGCGAAAGCGGACTACCCGAAAAATAGTCAATGCGAAAACTAAAAAAT
 CATATTTAGTAGGAAATTCGAATGGAAAAAGGATACAGTGGAACCTACTGG
 AGCTAGACTAAGAGATTACAGCAAACCTTGGCAGGTTTTTTAGAAATTTTGG
 TCTAAAAAACAAATGAATAACAATATTTAGGAATACATTCTTATCCACTCG
 TTAGCGGGTGGATATATTTTATGGGGAGGAAGTAAGCCAAATGTATATAAAA
 GGGAGGTTAATCTTTTCTTTGTAATGTAGTAATCGCGTTATGTTCCGAAG
 GGC (SEQ ID NO:19)

(b). LoxP-flanked antibiotic resistance genes.

[0231] The present invention, in some embodiments, provides reagents and methods for mediating the rapid or efficient excision of a first nucleic acid from a bacterial genome. The method depends on recombinase-mediated excision, where the recombinase recognizes heterologous recombinase binding sites that flank the first nucleic acid. The heterologous recombinase binding sites can be, for example, a pair of loxP sites or a pair of *flp* sites. To provide a non-limiting example, the first nucleic acid can encode a selection marker such as an antibiotic resistance gene.

[0232] The reagents of this embodiment include plasmids comprising two heterologous recombinase binding sites that flank the first nucleic acid; a bacterial genome comprising two heterologous recombinase binding sites that flank the first nucleic acid; and a bacterium containing a genome comprising two heterologous recombinase binding sites that flank the first nucleic acid.

[0233] The method of this embodiment is set forth in the following steps:

- i. Transfect a bacterium with a plasmid, where the plasmid can mediate integration of a first nucleic acid (flanked by a pair of heterologous recombinase binding sites) into the bacterial genome;
- ii. Allow integration of the first nucleic acid (flanked by two heterologous recombinase binding sites) into the bacterial genome. Without implying any limitation as to the mechanism, integration can be by way of site-specific recombination or homologous recombination;
- iii. Select for the bacterium containing the integrated first nucleic acid. Where the first nucleic acid encodes an antibiotic resistance gene, selection can involve culturing the bacterium in a medium containing the antibiotic. The selection step can result in a genotypically pure bacterium;
- iv. Treat the genotypically pure bacterium with conditions that facilitate recombinase-catalyzed excision of the first nucleic acid from the bacterial genome. Where the pair of heterologous recombinase binding sites are loxP sites, the recombinase can be Cre recombinase. Cre recombinase can be introduced into the bacterium by transfecting with a plasmid encoding this enzyme. In one embodiment, expression of Cre recombinase is transient. Cre recombinase and FLP recombinase use the same enzymatic reaction mechanism, and mediate precise site-specific excision between a pair of their specific target sequences;
- v. After allowing for Cre recombinase-catalyzed excision of the first nucleic acid, the bacterium can be cultured until the plasmid is lost by dilution or nuclease action;

vi. The resulting bacterium can be identified by the presence of the first nucleic acid in the genome. Also, the resulting bacterium can be identified by the loss of one of the two heterologous recombinase binding sites from the genome, that is, only one of the two sites will be left.

[0234] The above disclosure is not intended to limit the method to the recited steps, is not intended to limit the method to the disclosed order of steps, and is not intended to mean that all of these steps must occur. The invention is not necessarily limited to two heterologous recombinase binding sites. Polynucleotides containing two loxP sites and two Frt sites can be used, for example, where the two loxP sites flank a first nucleic acid, and the two Frt sites flank a second nucleic acid, and where transient expression of Cre recombinase allows excision of the first nucleic acid, and where transient expression of FLP recombinase (perhaps at a different time) results in excision of the second nucleic acid.

[0235] The canonical DNA target site for site-specific recombinases consists of two recombinase binding sites, where the two recombinase binding sites flank a core region (spacer region). The present invention provides two canonical DNA target sites (a pair of canonical DNA target sites), where the sites flank a first nucleic acid. LoxP is one type of canonical DNA target site. LoxP has two 13bp recombinase binding sites (13bp inverted repeats) that flank an 8bp core region or spacer. Thus, each loxP site is a sequence of 34 continuous nucleotides (34bp).

[0236] Cre recombinase and FLP recombinase are members of the integrase family of site-specific recombinases. Cre and FLP recombinase utilize a tyrosine residue to catalyze DNA cleavage. Cre recombinase recognizes lox sites, while FLP recombinase recognizes Frt sites.

[0237] Guidance for designing alternate and variant Lox sites and Frt sites is available. Where an alternate spacer region is desired, the skilled artisan will recognize that Cre recombinase-mediated excision is likely to require identical spacer regions in the first lox site and the second lox site (see, e.g., Araki, et al. (2000) *Nucleic Acids Res.* 30:e103; Nagy (2000) *Genetics* 26:99-109; Guo, et al. (1997) *Nature* 389:40-46; Sauer (1993) *Methods Enzymol.* 225:890-900; Langer, et al. (2002) *Nucleic Acids Res.* 30:3067-3077; Lath, et al. (2002) *Nucleic Acids Res.* 30:e115; Baer and Bode (2001) *Curr. Opinion Biotechnol.* 12:473-480; Nakano, et al. (2001) *Microbiol. Immunol.* 45:657-665).

[0238] The present invention contemplates a polynucleotide comprising a first lox site and a second lox site, where the pair of lox sites flanks a first nucleic acid, and where the first nucleic acid can encode, e.g., a selection marker, antibiotic resistance gene, regulatory region,

or antigen. Also contemplated is a polynucleotide comprising a first lox site and a second lox site, where the pair of lox sites flanks a first nucleic acid, and where the first nucleic acid can encode, e.g., a selection marker, antibiotic resistance gene, regulatory region, or antigen.

[0239] The skilled artisan will readily appreciate that variant Lox sites where the recombinase binding site is under 13bp are available, in light of reports that Cre recombinase can function with a recombinase binding site as short as 8-10 bp.

[0240] An alternate lox site, loxY is available, to provide a non-limiting example. The present invention contemplates a polynucleotide comprising a first loxY site and a second loxY site, where the pair of loxY sites flanks a first nucleic acid, and where the first nucleic acid can encode, e.g., a selection marker, an antibiotic resistance gene, a regulatory region, or an antigen, and so on. Note also, that the core region of loxP has alternating purine and pyrimidine bases. However, this alternating pattern is necessary for recognition by Cre recombinase, and the present invention encompasses LoxP site variants with mutated core regions (see, e.g., Sauer (1996) *Nucleic Acids Res.* 24:4608-4613; Hoess, et al. (1986) *Nucleic Acids Res.* 14:2287-2300).

[0241] The Frt site contains three 13bp symmetry elements and one 8bp core region (48bp altogether). FLP recombinase recognizes Frt as a substrate, as well as variant Frt sites, including Frt sites as short as 34bp, and Frt site with variant core regions (see, e.g., Schweizer (2003) *J. Mol. Microbiol. Biotechnol.* 5:67-77; Bode, et al. (2000) *Biol. Chem.* 381:801-813).

[0242] The present invention provides a polynucleotide containing a first loxP site and an operably linked second loxP site, wherein the first and second loxP sites flank a first nucleic acid, to provide a non-limiting example. It will be appreciated that the invention encompasses other heterologous recombinase binding sites, such as variants of loxP, as well as frt sites and frt site variants.

[0243] The term "operably linked," as it applies to a first loxP site and a second loxP site, where the two loxP sites flank a first nucleic acid, encompasses the following. Here, "operably linked" means that Cre recombinase is able to recognize the first loxP site and the second loxP site as substrates, and is able to catalyze the excision of the first nucleic acid from the bacterial genome. The term "operably linked" is not to be limited to loxP sites, as it encompasses any "heterologous recombinase binding sites" such as other lox sites, or frt sites. Also, the term "operably linked" is not to be limited to recombinase-catalyzed excision, the term also embraces recombinase-catalyzed integration. Moreover, the term "operably linked" is not to be limited to nucleic acids residing in a genome -- also

encompassed are nucleic acids residing in plasmids, intermediates used in genetic engineering, and the like.

[0244] Nucleic acids encoding recombinases are disclosed in Table 7A, and nucleic acid target sites recognized by these recombinases appear in Table 7B.

Table 7A. Recombinases.

Recombinase	Location and GenBank Accession No.
Cre recombinase	Nucleotides 5347-6195 (exon 1) and 6262-6465 (exon 1) of GenBank Acc. No. AJ627603.
FLP recombinase	Complement of nucleotides 4426-5697 of GenBank Acc. No. AF048702.
FLP recombinase	Complement of nucleotides 6054-7325 of GenBank Acc. No. AY597273.
FLP recombinase	Nucleotides 5570-6318, 1-523 of GenBank Acc. No. J01347. The upstream region of the coding sequence begins at nucleotide 5570, while the downstream region of the coding sequence ends at nucleotide 523.

Table 7B. Binding sites for recombinases.

Target site	Location and GenBank Accession No.
Target sites of FLP recombinase	
Frt	Nucleotides 260-307 of GenBank Acc. No. AY562545.
Frt	Nucleotides 464-511 of GenBank Acc.No. AY597272.
Frt	Nucleotides 3599-3646 of GenBank Acc. No. AY423864.
Target sites of Cre recombinase	
LoxP	Nucleotides 415-448 of GenBank Acc. No. AF143506.
LoxP	Nucleotides 118-151 of GenBank Acc. No. U51223.
LoxP	Nucleotides 1050-1083 of GenBank Acc. No. AY093430.
LoxP	Nucleotides 759-792 of GenBank Acc. No. AJ401047.

The referenced nucleic acid sequences, and corresponding translated amino acid sequences, and the cited amino acid sequences, and the corresponding nucleic acid sequences associated with or cited in that reference, are incorporated by reference herein in their entirety.

[0245] Nucleic acid sequences encoding various antibiotic resistance factors are disclosed (Table 8). Typical sequences are those encoding resistance to an antibiotic that is toxic to *Listeria* e.g., chloramphenicol acetyltransferase (CAT) (Table 8).

[0246] A first nucleic acid encoding the antibiotic resistance factor is operably linked to a ribosome binding site, a promoter, and contains a translation start site, and/or a translation stop site, and is flanked by two heterologous recombinase binding sites.

[0247] The invention provides a polynucleotide containing a pair of operably linked loxP sites flanking a first nucleic acid, and a second nucleic acid (not flanked by the loxP sites),

where the polynucleotide consists of a first strand and a second strand, and where the first nucleic acid has a first open reading frame (ORF) and the second nucleic acid has a second open reading frame (ORF). In one aspect, the first ORF is on the first strand, and the second ORF is also on the first strand. In another aspect, the first ORF is on the first strand and the second ORF is on the second strand. Yet another aspect provides a first ORF on the second strand and the second ORF on the first strand. Moreover, both ORFs can reside on the second strand. The present invention, in one aspect, provides a plasmid comprising the above-disclosed polynucleotide. Also provided is a *Listeria* containing the above-disclosed polynucleotide, where the polynucleotide can be on a plasmid and/or integrated in the genome. Each of the above-disclosed embodiments can comprise heterologous recombinase binding sites other than loxP. For example, lox variants, Frt sites, Frt variants, and recombinase binding sites unrelated to lox or Frt are available.

Table 8. Antibiotic resistance genes.

Antibiotic resistance gene.	GenBank Accession No.
Chloramphenicol (chloramphenicol acetyltransferase; CAT).	Complement of nucleotides 312-971 of GenBank Acc. No. AJ417488 (pPL1 of Lauer, <i>et al.</i>).
Chloramphenicol (CAT).	Complement of nucleotides 4898-5548 of GenBank Acc. No. AJ417488 (pPL1 of Lauer, <i>et al.</i>).
Chloramphenicol (CAT).	Complement of nucleotides 312-971 of GenBank Acc. No. AJ417449 (pPL2 of Lauer, <i>et al.</i>).
Chloramphenicol (CAT).	Complement of nucleotides 4920-5570 of GenBank Acc. No. AJ417449 (pPL2 of Lauer, <i>et al.</i>).
Chloramphenicol (CAT).	Nucleotides 3021-3680 of GenBank Acc. No. AJ007660.
Penicillin (penicillin-binding protein 2).	Nucleotides 25-1770 of GenBank Acc. No. X59629.
Erythromycin (erythromycin resistance determinant).	Nucleotides 864-1601 of GenBank Acc. No. AY680862.
Ampicillin (penicillin beta-lactamase).	Complement of nucleotides 3381-4311 of GenBank Acc. No. AJ401049.
Tetracycline (tetracycline resistance protein).	Complement of nucleotides of 4180-5454 of GenBank Acc. No. AY608912.
Gentamycin (aminoglycoside acetyltransferase).	Complement of nucleotides 1326-1859 of GenBank Acc. No. EVE414668.

(c). **ActA fusion protein partners, and derivatives thereof.**

A. Modified ActA fusion protein partners, polynucleotides encoding the fusion proteins comprising modified ActA, and *Listeria* comprising the polynucleotides

i. GENERAL.

[0248] The present invention, in certain aspects, provides a polynucleotide comprising a first nucleic acid encoding a modified ActA, operably linked and in frame with a second nucleic acid encoding a heterologous antigen. The invention also provides a *Listeria* containing the polynucleotide, where expression of the polynucleotide generates a fusion protein comprising the modified ActA and the heterologous antigen. The modified ActA can include the natural secretory sequence of ActA, a secretory sequence derived from another listerial protein, a secretory sequence derived from a non-listerial bacterial protein, or the modified ActA can be devoid of any secretory sequence.

[0249] The ActA-derived fusion protein partner finds use in increasing expression, increasing stability, increasing secretion, enhancing immune presentation, stimulating immune response, improving survival to a tumor, improving survival to a cancer, increasing survival to an infectious agent, and the like.

[0250] In one aspect, the invention provides a polynucleotide comprising a promoter operably linked to a nucleic acid sequence encoding a fusion protein, wherein the fusion protein comprises (a) modified ActA and (b) a heterologous antigen. In some embodiments, the promoter is ActA promoter. In some embodiments, the modified ActA comprises at least the first 59 amino acids of ActA. In some embodiments, the modified ActA comprises more than the first 59 amino acids of ActA. In some embodiments, the modified ActA is a fragment of ActA comprising the signal sequence of ActA (or is derived from a fragment of ActA comprising the signal sequence of ActA). In some embodiments, the modified ActA comprises at least the first 59 amino acids of ActA, but less than about the first 265 amino acids of ActA. In some embodiments, the modified ActA comprises more than the first 59 amino acids of ActA, but less than about the first 265 amino acids of ActA. In other words, in some embodiments, the modified ActA sequence corresponds to an N-terminal fragment of ActA (including the ActA signal sequence) that is truncated somewhere between amino acid 59 and about amino acid 265 of the Act A sequence. In some embodiments, the modified ActA comprises the first 59 to 200 amino acids of ActA, the first 59 to 150 amino acids of ActA, the first 59 to 125 amino acids of ActA, or the first 59 to 110 amino acids of ActA. In some embodiments, the modified ActA consists of the first 59 to 200 amino acids of ActA, the first 59 to 150 amino acids of ActA, the first 59 to 125 amino acids of ActA, or the first 59 to 110 amino acids of ActA. In some embodiments, the modified ActA comprises about

the first 65 to 200 amino acids of ActA, about the first 65 to 150 amino acids of ActA, about the first 65 to 125 amino acids of ActA, or about the first 65 to 110 amino acids of ActA. In some embodiments, the modified ActA consists of about the first 65 to 200 amino acids of ActA, about the first 65 to 150 amino acids of ActA, about the first 65 to 125 amino acids of ActA, or about the first 65 to 110 amino acids of ActA. In some embodiments, the modified ActA comprises the first 70 to 200 amino acids of ActA, the first 80 to 150 amino acids of ActA, the first 85 to 125 amino acids of ActA, the first 90 to 110 amino acids of ActA, the first 95 to 105 amino acids of ActA, or about the first 100 amino acids of ActA. In some embodiments, the modified ActA consists of the first 70 to 200 amino acids of ActA, the first 80 to 150 amino acids of ActA, the first 85 to 125 amino acids of ActA, the first 90 to 110 amino acids of ActA, the first 95 to 105 amino acids of ActA, or about the first 100 amino acids of ActA. In some embodiments, the modified ActA comprises amino acids 1-100 of ActA. In some embodiments, the modified ActA consists of amino acids 1-100 of ActA. In some embodiments, the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is immunologically cross-reactive with, or shares at least one epitope with, the cancer, tumor, or infectious agent. In some embodiments, the heterologous antigen is a tumor antigen or is derived from a tumor antigen. In some embodiments, the heterologous antigen is, or is derived from, human mesothelin. In some embodiments, the nucleic acid sequence encoding the fusion protein is codon-optimized for expression in *Listeria*. The invention provides plasmids and cells comprising the polynucleotide. The invention further provides a *Listeria* bacterium e.g., *Listeria monocytogenes*) comprising the polynucleotide, as well as vaccines comprising the *Listeria*. In some embodiments, the genomic DNA of the *Listeria* comprises the polynucleotide. In some embodiments, the polynucleotide is positioned in the genomic DNA at the site of the *actA* gene or the site of the *inlB* gene. In some embodiments, the *Listeria* comprises a plasmid comprising the polynucleotide. The invention further provides immunogenic and pharmaceutical compositions comprising the *Listeria*. The invention also provides methods for stimulating immune responses to the heterologous antigen in a mammal (e.g., a human), comprising administering an effective amount of the *Listeria* (or an effective amount of a composition comprising the *Listeria*) to the mammal. For instance, the invention also provides methods for stimulating immune responses to an antigen from, or derived from, a cancer or infectious agent, comprising administering an effective amount of the *Listeria* (or a composition comprising the *Listeria*) to a mammal having the cancer or infectious agent,

wherein the heterologous antigen shares at least one epitope with or is immunologically cross-reactive with the antigen from, or derived from, the cancer or infectious agent.

[0251] The preferred embodiment of antigen expression cassettes utilizing ActA-N-100 heterologous antigen fusion partner configurations is to functionally link the said antigen fusion construct with the native actA promoter and 5' untranslated region (UTR) RNA. PrfA-dependent transcription from the actA promoter results in synthesis of a 150 nucleotide 5' UTR RNA prior to the ActA protein GUG translation initiation site. *L. monocytogenes* mutants deleted of the actA promoter 5' UTR express low levels of ActA, resulting in a phenotype characterized by absence of intracellular actin recruitment, inability to spread from cell-to-cell, and attenuated, as compared to the wild-type parent bacterium (Wong et. al. defective Cellular Microbiology 6:155-166).

[0252] In some embodiments, the promoter used to express the fusion protein comprising the modified ActA and a heterologous antigen is an hly promoter. In another embodiments, the promoter is an actA promoter. These listerial promoters may, of instance, be derived from any strain of *Listeria monocytogenes*. For instance, the hly or actA promoter may be derived from the *L. monocytogenes* strain EGD. The EGD strain genome sequence is publicly available at GenBank Acc. No. AL591974 (complete genome segment 2/12) (hly promoter: nts 5581-5818; actA gene: nts 9456-11389), incorporated by reference herein in its entirety. Alternatively, the sequences used may be from another strain such as the *L. monocytogenes* strain 10403S.

[0253] In another aspect, the invention provides a polynucleotide comprising a first nucleic acid encoding a modified ActA, operably linked and in frame with, a second nucleic acid encoding a heterologous antigen. In some embodiments, the modified ActA comprises at least the first 59 amino acids of ActA, but less than about the first 265 amino acids of ActA. In some embodiments, the modified ActA comprises the first 59 to 200 amino acids of ActA, the first 59 to 150 amino acids of ActA, the first 59 to 125 amino acids of ActA, or the first 59 to 110 amino acids of ActA. In some embodiments, the modified ActA comprises the first 70 to 200 amino acids of ActA, the first 80 to 150 amino acids of ActA, the first 85 to 125 amino acids of ActA, the first 90 to 110 amino acids of ActA, the first 95 to 105 amino acids of ActA, or about the first 100 amino acids of ActA. In some embodiments, the first nucleic acid encodes amino acids 1-100 of ActA. In some embodiments, the polynucleotide is genomic. In some alternative embodiments, the polynucleotide is plasmid-based. In some embodiments, the polynucleotide is operably linked with a promoter. For instance, the polynucleotide may be operably linked with one or more of the following: (a) actA

promoter; or (b) a bacterial promoter that is not actA promoter. In some embodiments, the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is immunologically cross-reactive with, or shares at least one epitope with, the cancer, tumor, or infectious agent. In some embodiments, the heterologous antigen is, or is derived from human mesothelin. The invention further provides a *Listeria* bacterium e.g., *Listeria monocytogenes*) comprising the polynucleotide, as well as vaccines comprising the *Listeria*. In some embodiments, the *Listeria* is hMeso26 or hMeso38 (see Table 11 of Example VII, below). The invention also provides methods for stimulating immune responses to an antigen from, or derived from, a cancer or infectious agent, comprising administering the *Listeria* to a mammal having the cancer or infectious agent, wherein the heterologous antigen shares at least one epitope with or is immunologically cross-reactive with the antigen from, or derived from, the cancer or infectious agent.

[0254] In some embodiments, the *L. monocytogenes* native sequence encoding the first 100 amino acids of ActA is functionally linked in frame with a desired heterologous antigen sequence. In the some embodiments, the heterologous antigen sequence is synthesized according to the optimal codon usage of *L. monocytogenes*, a low GC percentage organism. In some embodiments, compositions utilizing the actA promoter together with the 5' untranslated sequences are desired.

[0255] In another aspect, the invention provides a polynucleotide comprising a first nucleic acid encoding a modified actA, where the modified actA comprises (a) amino acids 1-59 of actA, (b) an inactivating mutation in, deletion of, or truncation prior to, at least one domain for actA-mediated regulation of the host cell cytoskeleton, wherein the first nucleic acid is operably linked and in frame with a second nucleic acid encoding a heterologous antigen. In some embodiments, the domain is the cofilin homology region (KKRR (SEQ ID NO:23)). In some embodiments, the domain is the phospholipid core binding domain (KVFKKIKDAGKWVRDKI (SEQ ID NO:20)). In some embodiments, at least one domain comprises all four proline-rich domains (FPPPP (SEQ ID NO:21), FPPPP (SEQ ID NO:21), FPPPP (SEQ ID NO:21), FPIPP (SEQ ID NO:22)) of ActA. In some embodiments, the modified actA is actA-N100. In some embodiments, the polynucleotide is genomic. In some embodiments, the polynucleotide is not genomic. In some embodiments, the polynucleotide is operably linked with one or more of the following: (a) actA promoter; or (b) a bacterial (e.g., listerial) promoter that is not actA promoter. The invention further provides a *Listeria* bacterium (e.g., *Listeria monocytogenes*) comprising the polynucleotide, as well as vaccines

comprising the *Listeria*. In some embodiments, the *Listeria* is hMeso26 or hMeso38 (see Table 11 of Example VII, below). The invention also provides methods for stimulating immune responses to an antigen from, or derived from, a cancer or infectious agent, comprising administering the *Listeria* to a mammal having the cancer or infectious agent, wherein the heterologous antigen shares at least one epitope with or is immunologically cross-reactive with the antigen from, or derived from, the cancer or infectious agent. In some embodiments, the stimulating is relative to immune response without administering the *Listeria*. In some embodiments, the cancer comprises a tumor or pre-cancerous cell. In some embodiments, the infectious agent comprises a virus, pathogenic bacterium, or parasitic organism. In some embodiments, the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent. In some embodiments, the heterologous antigen is immunologically cross-reactive with, or shares at least one epitope with, the cancer, tumor, or infectious agent. In some embodiments, the heterologous antigen is, or is derived from, human mesothelin.

[0256] In some embodiments, what is provided is a polynucleotide comprising a first nucleic acid encoding a modified ActA comprising at least amino acids 1-59 of ActA, further comprising at least one modification in a wild type ActA sequence, wherein the at least one modification is an inactivating mutation in, deletion of, or truncation at or prior to, a domain specifically used for ActA-mediated regulation of the host cell cytoskeleton, wherein the first nucleic acid is operably linked and in frame with a second nucleic acid encoding a heterologous antigen.

[0257] Also encompassed is the above polynucleotide, where the at least one modification is an inactivating mutation in, deletion of, or termination at, comprising the cofilin homology region KKRR (SEQ ID NO:23). Moreover, what is encompassed is the above polynucleotide where the at least one modification is an inactivating mutation in, deletion of, or termination at, comprising the phospholipid core binding domain (KVFKKIKDAGKWVRDKI (SEQ ID NO:20)).

[0258] In yet another aspect, what is contemplated is the above polynucleotide, wherein the at least one modification comprises an inactivating mutation in, or deletion of, in each of the first proline-rich domain (FPPPP (SEQ ID NO:21)), the second proline-rich domain (FPPPP (SEQ ID NO:21)), the third proline-rich domain (FPPPP (SEQ ID NO:21)), and the fourth proline-rich domain (FPPIP (SEQ ID NO:22)), or a termination at the first proline-rich domain. In another aspect, what is provided is the above polynucleotide where the modified ActA is ActA-N100.

[0259] Yet another embodiment provides a *Listeria* bacterium comprising one or more of the above polynucleotide. The polynucleotide can be genomic, it can be plasmid-based, or it can reside on both a plasmid and the listerial genome. Also provided is the above *Listeria* where the polynucleotide is not genomic, as well as the above *Listeria* where the polynucleotide is not plasmidic. The *Listeria* can be *Listeria monocytogenes*, *L. innocua*, or some other listerial species.

[0260] Moreover, what is supplied by yet another embodiment, is a method of stimulating immune response to an antigen from, or derived from, a tumor, cancer cell, or infectious agent, comprising administering to a mammal the above-disclosed *Listeria* and where the heterologous antigen is shares at least one epitope with the antigen derived from the tumor, cancer cell, or infectious agent. What is also supplied is the above method, where the stimulating is relative to antigen-specific immune response in absence of the administering the *Listeria* (specific to the antigen encoded by the second nucleic acid).

[0261] Optionally, the heterologous antigen can be identical to the antigen from (or derived from) the tumor, cancer cell, or infectious agent.

[0262] ActA-N100 encompasses a nucleic acid encoding amino acids 1-100 of ActA, as well as the polypeptide expressed from this nucleic acid. (This numbering includes all of the secretory sequence of ActA.) What is provided is a polynucleotide comprising a first nucleic acid encoding ActA-N100 operably linked and in frame with a second nucleic acid encoding a heterologous antigen.

[0263] Yet another embodiment provides a *Listeria* bacterium comprising one or more of the above polynucleotide. The polynucleotide can be genomic, it can be plasmid-based, or it can reside on both a plasmid and the listerial genome. Also provided is the above *Listeria* where the polynucleotide is not genomic, as well as the above *Listeria* where the polynucleotide is not plasmidic. The *Listeria* can be *Listeria monocytogenes*, *L. innocua*, or some other listerial species.

[0264] Methods for using ActA-N100 are also available. Provided is a method for stimulating immune response to an antigen from, or derived from, a tumor, cancer cell, or infectious agent, comprising administering to a mammal the above-disclosed *Listeria*, and wherein the heterologous antigen is shares at least one epitope with the antigen derived from the tumor, cancer cell, or infectious agent. What is also provided is the above method, where the stimulating is relative to antigen-specific immune response in absence of the administering the *Listeria* (specific to the antigen encoded by the second nucleic acid).

Alternatively, the heterologous antigen can be identical to the antigen from, or derived from, the tumor, cancer cell, or infectious agent.

[0265] In some embodiments, the modified ActA consists of a fragment of ActA or other derivative of ActA in which the ActA signal sequence has been deleted. In some embodiments, the polynucleotides comprising nucleic acids encoding a fusion protein comprising such a modified ActA and the heterologous antigen further comprise a signal sequence that is not the ActA signal sequence. The ActA signal sequence is MGLNRFMRAMMVVFITANCITINPDIIFA (SEQ ID NO:125). In some embodiments, the modified ActA consists of amino acids 31-100 of ActA (i.e., ActA-N100 deleted of the signal sequence).

[0266] The present invention provides a polynucleotide comprising a first nucleic acid encoding a modified ActA, operatively linked and in frame with a second nucleic acid encoding a heterologous antigen. ActA contains a number of domains, each of which plays a part in binding to a component of the mammalian cytoskeleton, where the present invention contemplates removing one or more of these domains.

[0267] ActA contains a number of domains, including an N-terminal domain (amino acids 1-234), proline-rich domain (amino acids 235-393), and a C-terminal domain (amino acids 394-610). The first two domains have distinct effects on the cytoskeleton (Cicchetti, *et al.* (1999) J. Biol. Chem. 274:33616-33626). The proline-rich domain contains four proline-rich motifs. The proline-rich motifs are docking sites for the Ena/VASP family of proteins. Deletion of proline-rich domains of ActA strongly reduces actin filament assembly (Cicchetti, *et al.* (1999) J. Biol. Chem. 274:33616-33626). Machner, *et al.*, provides guidance for designing mutated proline-rich motifs that can no longer dock, where this guidance can be put to use for embodiments of the present invention (Machner, *et al.* (2001) J. Biol. Chem. 276:40096-40103). For example, the phenylalanine of the proline-rich motifs is critical. The present invention, in an alternate embodiment, provides a polynucleotide comprising a first nucleic acid encoding ActA, where the codons for the phenylalanine in each proline-rich motif is changed to an alanine codon, operably linked and in frame with a second nucleic acid encoding at least one heterologous antigen. In another aspect, the first nucleic acid encoding ActA comprises a proline to alanine mutation in only the first proline-rich motif, in only the second proline-rich motif, in only the third proline-rich motif, in only the fourth proline-rich motif, or any combination thereof. In another aspect, a nucleic acid encoding an altered ActA can encompass a mutation in a codon for one or more proline-rich

motifs in combination with a mutation or deletion in, e.g., cofilin homology region and/or the core binding sequence for phospholipids interaction.

[0268] What is also embraced, is a mutation of proline to another amino acid, e.g., serine. The above guidance in designing mutations is not to be limited to changing the proline-rich motifs, but applies as well to the cofilin homology region, the core binding sequence for phospholipids interaction, and any other motifs or domains that contribute to interactions of ActA with the mammalian cytoskeleton.

[0269] ActA contains a domain that is a "core binding sequence for phospholipids interaction" at amino acids 185-201 of ActA, where the function in phospholipids binding was demonstrated by binding studies (Cicchetti, *et al.* (1999) *J. Biol. Chem.* 274:33616-33626). According to Cicchetti, *et al.*, *supra*, phospholipids binding regulates the activities of actin-binding proteins.

[0270] ActA contains a cofilin homology region KKRR (SEQ ID NO:23). Mutations of the KKRR (SEQ ID NO:23) region abolishes the ActA's ability to stimulate actin polymerization (see, e.g., Baoujemaa-Paterski, *et al.* (2001) *Biochemistry* 40:11390-11404; Skoble, *et al.* (2000) *J. Cell. Biol.* 150:527-537; Pistor, *et al.* (2000) *J. Cell Sci.* 113:3277-3287).

[0271] The following concerns expression, by *L. monocytogenes*, of truncated actA derivatives truncated down from amino acid 263 to amino acid 59. Unlike other truncated derivatives, actA N59 was not expressed whereas all of the longer ones were expressed (Skoble, J. (unpublished)). The next longest derivative tested was actA-N101. Fusion protein constructs expressed from actA promoter, consisting of a first fusion protein partner that is actA secretory sequence, and a second fusion protein partner, resulted in much less protein secretion than where the first fusion protein partner was actA-N100. Regarding deletion constructs, good expression was also found where the first fusion protein partner was soluble actA with amino acids 31-59 deleted. Moreover, good expression was found where the first fusion protein partner was soluble actA with amino acids 31-165 deleted (Skoble, J. (unpublished)).

[0272] The present invention, in certain embodiments, provides a polynucleotide comprising a first nucleic acid encoding a modified ActA, comprising at least one modification, wherein the at least one modification is an inactivating mutation in, deletion of, or termination of the ActA polypeptide sequence at or prior to, a domain required for ActA-mediated regulation of the host cell cytoskeleton, and a second nucleic acid encoding a heterologous antigen. The modified ActA can be one resulting in impaired motility and/or decreased plaque size, and includes a nucleic acid encoding one of the mutants 34, 39, 48, and 56 (Lauer, *et al.* (2001)

Mol. Microbiol. 42:1163-1177). The present invention also contemplates a nucleic acid encoding one of the ActA mutants 49, 50, 51, 52, and 54. Also provides is a nucleic acid encoding one of the ActA mutants 40, 41, 42, 43, 44, 45, 45, and 47. Provided are mutants in the actin monomer binding region AB region, that is, mutants 41, 42, 43, and 44 (Lauer, *et al.* (2001) Mol. Microbiol. 42:1163-1177).

[0273] In another aspect, the modified ActA of the present invention can consist a deletion mutant, can comprise a deletion mutant, or can be derived from a deletion mutant ActA that is unable to polymerize actin in cells and/or unable to support plaque formation, or supported only sub-maximal plaque formation. These ActA deletion mutants include the nucleic acids encoding $\Delta 31-165$; $\Delta 136-200$; $\Delta 60-165$; $\Delta 136-165$; $\Delta 146-150$, $\Delta 31-58$; $\Delta 60-101$; and $\Delta 202-263$ and the like (Skoble, *et al.* (2000) J. Cell Biol. 150:527-537). Encompassed are nucleic acids encoding ActA deletion mutants that have narrower deletions and broader deletions. The following set of examples, which discloses deletions at the cofilin homology region, can optionally to each the ActA deletions set forth herein. The present invention provides nucleic acids encoding these deletions at the cofilin homology region: $\Delta 146-150$; $\Delta 145-150$; $\Delta 144-150$; $\Delta 143-150$; $\Delta 142-150$; $\Delta 141-150$; $\Delta 140-150$; $\Delta 139-150$; $\Delta 138-150$; $\Delta 137-150$; $\Delta 136-150$, and the like. Also encompassed are nucleic acids encoding ActA with the deletions: $\Delta 146-150$; $\Delta 146-151$; $\Delta 146-152$; $\Delta 146-153$; $\Delta 146-154$; $\Delta 146-155$; $\Delta 146-156$; $\Delta 146-157$; $\Delta 146-158$; $\Delta 146-159$; $\Delta 146-160$; and so on. Moreover, also embraced are nucleic acids encoding the deletion mutants: $\Delta 146-150$; $\Delta 145-151$; $\Delta 144-152$; $\Delta 143-153$; $\Delta 142-154$; $\Delta 141-155$; $\Delta 140-156$; $\Delta 139-157$; $\Delta 138-158$; $\Delta 137-159$; $\Delta 136-160$, and the like. Where there is a deletion at both the N-terminal end of the region in question, and at the C-terminal end, the sizes of these two deletions need not be equal to each other.

[0274] Deletion embodiments are also provided, including but not limited to the following. What is provided is a nucleic acid encoding full length actA, an actA missing the transmembrane anchor, or another variant of actA, where the actA is deleted in a segment comprising amino acids (or in the alternative, consisting of the amino acids):

31-59, 31-60, 31-61, 31-62, 31-63, 31-64, 31-65, 31-66, 31-67, 31-68, 31-69, 31-70, 31-71, 31-72, 31-73, 31-74, 31-75, 31-76, 31-77, 31-78, 31-79, 31-80, 31-81, 31-82, 31-83, 31-84, 31-85, 31-86, 31-87, 31-88, 31-89, 31-90, 31-91, 31-92, 31-93, 31-94, 31-95, 31-96, 31-97, 31-98, 31-99, 31-100, 31-101, 31-102, 31-103, 31-104, 31-105, 31-106, 31-107, 31-108, 31-109, 31-110, 31-111, 31-112, 31-113, 31-114, 31-115, 31-116, 31-117, 31-118, 31-119, 31-120, 31-121, 31-122, 31-123, 31-124, 31-125, 31-126, 31-127, 31-128, 31-129, 31-130, 31-131, 31-132, 31-133, 31-134, 31-135, 31-136, 31-137, 31-138, 31-139, 31-140, 31-141,

31-142, 31-143, 31-144, 31-145, 31-146, 31-147, 31-148, 31-149, 31-150, 31-151, 31-152, 31-153, 31-154, 31-155, 31-156, 31-157, 31-158, 31-159, 31-160, 31-161, 31-162, 31-163, 31-164, 31-165, and the like.

[0275] In yet another aspect, what is supplied is a polypeptide containing a first nucleic acid encoding an actA derivative, and a second nucleic acid encoding a heterologous nucleic acid, where the actA derivative is soluble actA comprising a deletion or conservative amino acid mutation, and where the deletion or conservative amino acid mutation comprises (or in another embodiment, where the deletion consists of) amino acid: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, and so on.

[0276] What is also provided, in other embodiments, is a polynucleotide comprising a first nucleic acid encoding an altered ActA, operably linked and in frame with a second nucleic acid, encoding a heterologous antigen, where the first nucleic acid is derived from, for example, Δ ActA3 (amino acids 129-153 deleted); Δ ActA9 (amino acids 142-153 deleted); Δ ActA6 (amino acids 68-153 deleted); Δ ActA7 (amino acids 90-153 deleted); or Δ ActA8 (amino acids 110-153 deleted), and so on (see, e.g., Pistor, *et al.* (2000) J. Cell Science 113:3277-3287).

[0277] A number of derivatives of ActA, encompassing the start methionine (N-terminus) and prematurely terminated, resulting in a novel C-terminus. Some of these derivatives are reported in Skoble, *et al.* (2000) J. Cell Biol. 150:527-537). Nucleic acids encoding these derivatives were introduced into *L. monocytogenes*, to test expression. The ActA derivative terminating at amino acid 59 (ActA-N59) was not expressed by *L. monocytogenes*. In contrast, ActA-N101, and longer derivatives of ActA, were expressed. Fusion proteins (expressed from the ActA promoter) consisting of only the ActA signal sequence and a fusion protein partner, showed much less secretion than fusion proteins consisting of ActA-N100 and a fusion protein partner.

[0278] The truncation, deletion, or inactivating mutation, can reduce or eliminate the function of one or more of ActA's four FP₄ domains ((E/D)FPPPX(D/E) (SEQ ID NO: 135)). ActA's FP₄ domains mediate binding to the following proteins: mammalian enabled (Mena); Ena/VASP-like protein (Evl); and vasodilator-stimulated phosphoprotein (VASP) (Machner, *et al.* (2001) J. Biol. Chem. 276:40096-40103). Hence, the nucleic acid of the present invention encodes a truncated ActA, deleted or mutated in one or more of its FP₄ domains, thereby reducing or preventing binding to Mena, Evl, and/or VASP. Provided is a nucleic acid encoding a truncated, partially deleted or mutated ActA and a heterologous antigen, where the truncation, partial deletion, or mutation, occurs at amino acids 236-240; amino acids 270-274; amino acids 306-310; and/or amino acids 351-355 of ActA (numbering of Machner, *et al.* (2001) J. Biol. Chem. 276:40096-40103).

[0279] The present invention provides a polynucleotide comprising a first nucleic acid encoding an ActA variant, and a second nucleic acid encoding at least one heterologous antigen, where the ActA variant is ActA deleted in or mutated in one "long repeat," two long repeats, or all three long repeats of ActA. The long repeats of ActA are 24-amino acid sequences located in between the FP₄ domains (see, e.g., Smith, *et al.* (1996) J. Cell Biol. 135:647-660). The long repeats help transform actin polymerization to a force-generating mechanism.

[0280] As an alternate example, what is provided is a nucleic acid encoding the following ActA-based fusion protein partner, using consisting language: What is provided is a nucleic acid encoding a fusion protein partner consisting of amino acids 1-50 of human actA (for example, GenBank Acc. No. AY512476 or its equivalent, where numbering begins with the start amino acid), amino acids 1-60; 1-61; 1-62; 1-63; 1-64; 1-65; 1-66; 1-67; 1-68; 1-69; 1-70; 1-72; 1-73; 1-74; 1-75; 1-76; 1-77; 1-78; 1-79; 1-80; 1-81; 1-82; 1-83; 1-84; 1-85; 1-86; 1-87; 1-88; 1-89; 1-90; 1-91; 1-92; 1-93; 1-94; 1-95; 1-96; 1-97; 1-98; 1-99; 1-100; 1-101; 1-102; 1-103; 1-104; 1-105; 1-106; 1-107; 1-108; 1-109; 1-110; 1-111; 1-112; 1-113; 1-114; 1-115; 1-116; 1-117; 1-118; 1-119; 1-120; 1-121; 1-122; 1-123; 1-124; 1-125; 1-126; 1-127; 1-128; 1-129; 1-130; 1-131; 1-132; 1-133; 1-134; 1-135; 1-136; 1-137; 1-138; 1-139; 1-140; 1-141; 1-142; 1-143; 1-144; 1-145; 1-146; 1-147; 1-148; 1-149; 1-150; 1-151; 1-152; 1-153; 1-154; 1-155; 1-156; 1-157; 1-158; 1-159; 1-160, and so on.

[0281] As yet another alternate example, what is provided is a nucleic acid encoding the following ActA-based fusion protein partner, using comprising language: What is provided is a nucleic acid encoding a fusion protein partner comprising amino acids 1-50 of human actA (for example, GenBank Acc. No. AY512476 or its equivalent, where numbering begins

with the start amino acid), amino acids 1-60; 1-61; 1-62; 1-63; 1-64; 1-65; 1-66; 1-67; 1-68; 1-69; 1-70; 1-72; 1-73; 1-74; 1-75; 1-76; 1-77; 1-78; 1-79; 1-80; 1-81; 1-82; 1-83; 1-84; 1-85; 1-86; 1-87; 1-88; 1-89; 1-90; 1-91; 1-92; 1-93; 1-94; 1-95; 1-96; 1-97; 1-98; 1-99; 1-100; 1-101; 1-102; 1-103; 1-104; 1-105; 1-106; 1-107; 1-108; 1-109; 1-110; 1-111; 1-112; 1-113; 1-114; 1-115; 1-116; 1-117; 1-118; 1-119; 1-120; 1-121; 1-122; 1-123; 1-124; 1-125; 1-126; 1-127; 1-128; 1-129; 1-130; 1-131; 1-132; 1-133; 1-134; 1-135; 1-136; 1-137; 1-138; 1-139; 1-140; 1-141; 1-142; 1-143; 1-144; 1-145; 1-146; 1-147; 1-148; 1-149; 1-150; 1-151; 1-152; 1-153; 1-154; 1-155; 1-156; 1-157; 1-158; 1-159; 1-160, and so on.

[0282] The contemplated nucleic acids encoding an actA-based fusion protein partner include nucleic acids encoding the actA-based fusion protein partner, where one or more nucleotides is altered to provide one or more conservative amino acid changes. What is contemplated is one conservative amino acid change, two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, or more, conservative amino acid changes. Moreover, what is contemplated is a nucleic acid encoding the actA-based fusion protein partner, comprising at least one mutation encoding at least one short deletion, or at least one short insertion, or any combination thereof.

[0283] Regarding the identity of the nucleic acid encoding ActA, and derivatives thereof, the codon for the start methionine can be a valine start codon. In other words, *Listeria* uses a valine start codon to encode methionine.

[0284] The contemplated invention encompasses ActA, and ActA deleted in one or more cytoskeleton-binding domains, ActA-N100 fusion protein partners, from all listerial species, including *L. monocytogenes* and *L. ivanovii* (Gerstel, *et al.* (1996) Infection Immunity 64:1929-1936; GenBank Acc. No. X81135; GenBank Acc. No. AY510073).

[0285] In some embodiments, the modified ActA consists of a sequence having at least about 80% sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, or at least about 95% sequence identity to a fragment of ActA comprising more than the first 59 amino acids of ActA and less than the first 380 amino acids of ActA. For instance, in some embodiments, the modified ActA consists of a sequence having at least about 80% sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, or at least about 95% sequence identity to ActA-N100. In some embodiments, the nucleic acid molecule encoding the modified ActA hybridizes under stringent conditions to a nucleic acid molecule encoding the ActA-N100 sequence, or its complement.

[0286] The present invention, in some embodiments, encompasses a polynucleotide comprising a first nucleic acid encoding actA-N100 operably linked and in frame with a second nucleic acid encoding a heterologous antigen, such as human mesothelin, or a derivative thereof. Human mesothelin was expressed from a number of constructs, where these constructs were created by site-directed integration or homologous integration into the *Listeria* genome. Some of these constructs are shown in Figure 6. Figure 6 discloses naturally occurring human mesothelin, which contains a signal sequence and a GPI-sequence. The signal sequence and GPI-sequence was deleted in the following examples, where the naturally occurring signal sequence was replaced with the *Bacillus anthracis* Protective Antigen secretory sequence (BaPA), with LLO-62, with LLO-60_{codon optimized} (LLO-60_{opt}), or with ActA-N100 (Figure 6). The sequence of ActA-N100 includes the naturally occurring secretory sequence of ActA.

ii. ABNORMAL CELL PHYSIOLOGY PRODUCED BY WILD TYPE ActA.

[0287] The modified ActA, of at least some embodiments of the invention, is changed to reduce or eliminate its interaction with the mammalian cytoskeleton. While the physiological function of ActA is to bind to the mammalian cytoskeleton and to allow actin-mediated movement of the *Listeria* bacterium through the cytoplasm, this binding is reduced or eliminated in the ActA component of the fusion protein.

[0288] Expression of soluble ActA in mammalian cytoplasm, by way of eukaryotic expression vectors, results in abnormalities of the cytoskeleton, e.g., “redistribution of F-actin,” and sequestration of the recombinant ActA at the location of “membrane protrusions.” In other words, the normal location of F-actin was changed, where its new location was in membrane protrusions. Moreover, “ActA stain co-distributed with that of F-actin in membrane protrusions.” Other abnormalities in mammalian cells included “loss of stress fibres.” It was observed that “the amino-terminal part of ActA is involved in the nucleation of actin filaments while the segment including the proline-rich repeat region promotes or controls polymerization” (Friederich, *et al.* (1995) EMBO J. 14:2731-2744). Moreover, according to Olazabal and Machesky, overexpressing a protein demonstrated to be similar to ActA, the WASP protein, causes “defects in actin organization that lead to malfunctions of cells” (Olazabal and Machesky (2001) J. Cell Biol. 154:679-682). The title of a publication (“*Listeria* protein ActA mimics WASP family proteins”) indicates this similarity (Boujemaa-Paterski, *et al.* (2001) Biochemistry 40:11390-11404).

[0289] Introducing certain domains of ActA into a mammalian cell disrupts the host cell cytoplasm. In detail, microinjecting ActA's repeat oligoproline sequence induces "loss of stress fibers," "dramatic retraction of peripheral membranes," and "accumulation of filamentous actin near the retracting peripheral membrane" (Southwick and Purich (1994) *Proc. Natl. Acad. Sci. USA* 91:5168-5172). ActA, a protein expressed by *Listeria*, sequesters or "highjacks" or utilizes various cytoskeleton related proteins, including the Arp2/3 complex and actin (Olazabal, *et al.* (2002) *Curr. Biol.* 12:1413-1418; Zalevsky, *et al.* (2001) *J. Biol. Chem.* 276:3468-3475; Brieher, *et al.* (2004) *J. Cell Biol.* 165:233-242).

[0290] The ActA-based fusion protein partner, of the present invention, has a reduced polypeptide length when compared to ActA lacking the transmembrane domain. The ActA-based fusion protein partner provides reduced disruption of actin-dependent activity such as immune presentation, host cell proliferation, cell polarity, cell migration, endocytosis, sealing of detached vesicles, movement of endocytotic vesicles, secretion, cell polarity, and response to wounds (wound healing) (see, e.g., Setterblad, *et al.* (2004) *J. Immunol.* 173:1876-1886; Tskvitaria-Fuller, *et al.* (2003) *J. Immunol.* 171:2287-2295). Without implying any limitation on the invention, reduced disruption in this context is relative to that found with full-length ActA, with ActA deleted only in the transmembrane domain, or with ActA truncated at the transmembrane domain. ActA lacking the membrane anchor sequence produces a "discernable redistribution of actin" in mammalian cells (see, e.g., Pistor, *et al.* (1994) *EMBO J.* 13:758-763).

[0291] Actin-dependent activities of the cell include immune cell functions, wound healing, capping, receptor internalization, phagocytosis, Fc-receptor clustering and Fc-receptor mediated phagocytosis, utilize actin (see, e.g., Kwiatkowska, *et al.* (2002) *J. Cell Biol.* 116:537-550; Ma, *et al.* (2001) *J. Immunol.* 166:1507-1516; Fukatsu, *et al.* (2004) *J. Biol. Chem.* 279:48976-48982; Botelho, *et al.* (2002) *J. Immunol.* 169:4423-4429; Krishnan, *et al.* (2003) *J. Immunol.* 170:4189-4195; Gomez-Garcia and Kornberg (2004) *Proc. Natl. Acad. Sci. USA* 101:15876-15880; Kusner, *et al.* (2002) *J. Biol. Chem.* 277:50683-50692; Roonov-Jessen and Peterson (1996) *J. Cell Biol.* 134:67-80; Choma, *et al.* (2004) *J. Cell Science* 117:3947-3959; Miki, *et al.* (2000) *Am. J. Physiol. Lung Cell. Mol. Physiol.* 278:L13-L18; Fujimoto, *et al.* (2000) *Traffic* 1:161-171; Zualmann, *et al.* (2000) *J. Cell Biol.* 150:F111-F116; Olazabal, *et al.* (2002) *Curr. Biol.* 12:1413-1418; Magdalena, *et al.* (2003) *Molecular Biology of the Cell* 14:670-684).

[0292] ActA is degraded (in the mammalian cytoplasm) by way of the "N-end rule pathway." (see, e.g., Moors, *et al.* (1999) Cellular Microbiol. 1:249-257; Varshavsky (1996) Proc. Natl. Acad. Sci. USA 93:12142-12149).

B. Rare codons of ActA; immunogenicity of ActA.

[0293] The ActA coding region contains a number of codons that are non-optimal for *L. monocytogenes*. Of these, a number occur in the listerial genome at a frequency of 25% or less than that of the most commonly used codon. The following provides a codon analysis for *L. monocytogenes* 10403S ActA. In the codons encoding amino acids 101-400, rare codons for glutamate (GAG) occur 12 times; rare codons for lysine (AAG) occurs three times; rare codons for isoleucine (ATA) occurs three times; rare codons for arginine (CGG) occurs once; rare codons for glutamine (CAG) occurs once; and rare codons for leucine (CTG; CTC) occurs three times. The following commentary relates to non-optimal codons, not just to rare codons. Moreover, in the codons encoding amino acids 101-400 (300 codons), non-optimal codons (this is in addition to the rare codons) occur 152 times (out of 300 codons total).

[0294] ActA is a major target for immune response by humans exposed to *L. monocytogenes* (see, e.g., Grenningloh, *et al.* (1997) Infect. Immun. 65:3976-3980). In some embodiments, the present invention provides an ActA-based fusion protein partner, where the ActA-based fusion protein partner has reduced immunogenicity, e.g., contains fewer epitopes than full-length ActA or is modified to provide epitopes of reduced immunogenicity.

[0295] The reagents and methods of the present invention provide a nucleic acid encoding an ActA, a truncated ActA, and/or a mutated ActA (e.g., a point mutation or a deletion), having a reduced number of antigenic epitopes, or that lacks one or more regions of increased antigenicity. Regions of increased antigenicity, as determined by a Welling plot, include amino acids 85-90; 140-150; 160-190; 220-230; 250-260; 270-280; 305-315; 350-370; 435-445; 450-460; 490-520; 545-555; and 595-610, of GenBank Acc. No. X59723. ActA has been identified as an immunogenic protein (see, e.g., Grenningloh, *et al.* (1997) Infection Immunity 65:3976-3980; Darji, *et al.* (1998) J. Immunol. 161:2414-2420; Niebuhr, *et al.* (1993) Infect. Immun. 61:2793-2802; Lingnau, *et al.* (1995) Infect. Immun. 63:3896-3903). The immunogenic properties of ActA increase with expression of soluble forms of actin, e.g., actin lacking all or part of its C-terminal region (amino acids 394-610 using numbering of Mourrain, *et al.* (1997) Proc. Natl. Acad. Sci. USA 94:10034-10039) (see also, e.g., Darji, *et al.* (1998) J. Immunol. 161:2414-2420; Cicchetti, *et al.* (1999) J. Biol. Chem. 274:33616-

33626). Hence, where a truncated, partially deleted, or mutated ActA of the present invention lacks (or functionally lacks) a domain used for membrane-binding, thereby resulting in increased immunogenicity, the present invention provides for further truncations or mutations in order to reduce immunogenicity of the truncated ActA.

C. Assays to measure binding of ActA derivatives, to cytoskeletal proteins, and ActA-dependent movement of *Listeria*.

[0296] Assays for determining recruiting of actin, or other proteins, to ActA, or to variants of ActA, are available. Recruiting can reasonably be assessed by bacterial movement assays, that is, assays that measure actin-dependent rate of *Listeria* movement in eukaryotic cell extracts or inside a eukaryotic cell (see, e.g., Marchand, *et al.* (1995) *J. Cell Biol.* 130:331-343). Bacterial movement assays can distinguish between *Listeria* expressing wild type ActA, and *Listeria* expressing mutant versions of ActA, for example, mutant ActA that lacks FP₄ domains (Smith, *et al.* (1996) *J. Cell Biol.* 135:647-660).

[0297] Recruitment can also be assessed by measuring local actin concentration at the surface of ActA-coated beads or at the surface of ActA-expressing bacteria. Bead-based assays are described (see, e.g., Machner, *et al.* (2001) *J. Biol. Chem.* 276:40096-40103; Fradelizi, *et al.* (2001) *Nature Cell Biol.* 3:699-707; Theriot, *et al.* (1994) *Cell* 76:505-517; Smith, *et al.* (1995) *Mol. Microbiol.* 17:945-951; Cameron, *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:4908-4913). Ultracentrifugation can assess the number of cytoskeletal proteins bound to ActA (see, e.g., Machner, *et al.*, *supra*).

[0298] Assays available to the skilled artisan include, e.g., the spontaneous actin polymerization assay; the elongation from the barbed end assay; and the elongation from the pointed end (see, e.g., Zalevsky, *et al.* (2001) *J. Biol. Chem.* 276:3468-3475). Methods are also available for assessing polarity of ActA-induced actin polymerization (see, e.g., Mogilner and Oster (2003) *Biophys. J.* 84:1591-1605; Noireauz, *et al.* (2000) *Biophys. J.* 78:1643-1654).

(d). SecA2-secreted proteins for use as fusion protein partner.

[0299] The present invention provides a family of SecA2 listerial secretory proteins useful as fusion protein partners with a heterologous antigen. The secretory protein-derived fusion protein partner finds use in increasing expression, increasing stability, increasing secretion, enhancing immune presentation, stimulating immune response, improving survival to a tumor, improving survival to a cancer, increasing survival to an infectious agent, and the like.

[0300] The contemplated listerial secretory proteins include p60 autolysin; N-acetyl-muramidase (NamA); penicillin-binding protein 2B (PBP-2B) (GenBank Acc. No. NC_003210); pheromone transporter (OppA) (complement to nt 184,539-186,215 of GenBank Acc. No. AL591982); maltose/maltodextrin ABC transporter (complement to nt 104,857-105,708 of GenBank Acc. No. AL591982); antigenic lipoprotein (Csa) (nt 3646-4719 of GenBank Acc. No. AL591982); and conserved lipoprotein, e.g., of *L. monocytogenes* EGD (see, e.g., Lenz, *et al.* (2003) Proc. Natl. Acad. Sci. USA 100:12432-12437; Lenz and Portnoy (2002) Mol. Microbiol. 45:1043-1056).

[0301] p60 is encoded by an open reading frame of 1,452 bp, has an N-terminal signal sequence, an SH3 domain in the N-terminal region, a central region containing threonine-asparagine repeats, and a C-terminal region encompassing the autolysin catalytic site (see, e.g., Pilgrim, *et al.* (2003) Infect. Immun. 71:3473-3484). p60 is also known as invasion-associated protein (iap) (GenBank Acc. No. X52268; NC_003210).

[0302] The present invention provides a polynucleotide comprising a first nucleic acid encoding p60, or a p60 derivative, and a second nucleic acid encoding a heterologous antigen. The p60 or p60 derivatives encompass a full length p60 protein (e.g., from *L. monocytogenes*, *L. innocua*, *L. ivanovii*, *L. seeligeri*, *L. welshimeri*, *L. murrayi*, and/or *L. grayi*), truncated p60 proteins consisting essentially of the N-terminal 70 amino acids; a truncated p60 protein deleted in the region that catalyses hydrolysis; signal sequences from a p60 protein; or a p60 protein with its signal sequence replaced with a different signal sequence (e.g., the signal sequence of ActA, LLO, PFO, or BaPA), and a second nucleic acid encoding a heterologous antigen. The p60 signal sequence (27 amino acids) is:

MNMKKATIAATAGIAVTAFAPTIASA (SEQ ID NO:24) (Bubert, *et al.* (1992) J. Bacteriol. 174:8166-8171; Bubert, *et al.* (1992) Appl. Environ. Microbiol. 58:2625-2632; J. Bacteriol. 173:4668-4674). The N-acetyl-muramidase signal sequence (52 amino acids) is: MDRKFIKPGIILLIVAFLLVVSINVGAETGGSRTAQVNLTTTSQQAFIDEILPA (SEQ ID NO:25) (nt 2679599 to 2681125 of GenBank Acc. No. NC_003210; GenBank Acc. No. AY542872; nt 2765101 to 2766627 of GenBank Acc. No. NC_003212; Lenz, *et al.* (2003) Proc. Natl. Acad. Sci. USA 100:12432-12437).

[0303] The present invention provides a p60 variant, for example, where the codons for amino acids 69 (L) and 70 (Q) are changed to provide a unique *Pst* I restriction site, where the *Pst* I site finds use in insertion a nucleic acid encoding a heterologous antigen.

[0304] Contemplated is nucleic acid encoding a fusion protein comprising a SecA2-pathway secreted protein and a heterologous antigen. Also contemplated is a nucleic acid encoding a

fusion protein comprising a derivative or truncated version of a SecA2-pathway secreted protein and a heterologous antigen. Moreover, what is contemplated is a *Listeria* bacterium comprising a nucleic acid encoding a fusion protein comprising a SecA2-pathway secreted protein and a heterologous antigen, or comprising a nucleic acid encoding a fusion protein comprising a derivative or truncated version of a SecA2-pathway secreted protein and a heterologous antigen.

(e) Mesothelin.

[0305] Human mesothelin cDNA is 2138 bp, contains an open reading frame of 1884 bp, and encodes a 69 kD protein. The mesothelin precursor protein contains 628 amino acids, and a furin cleavage site (RPRFRR at amino acids 288-293). Cleavage of the 69 kD protein generates a 40 kD membrane-bound protein (termed "mesothelin") plus a 31 kD soluble protein called megakaryocyte-potentiating factor (MPF). Mesothelin has a lipophilic sequence at its C-terminus, which is removed and replaced by phosphatidyl inositol, which causes mesothelin to be membrane-bound. Mesothelin contains a glycosylphosphatidyl inositol anchor signal sequence near the C-terminus. Mesothelin's domains, expression of mesothelin by cancer and tumor cells, and antigenic properties of mesothelin, are described (see, e.g., Hassan, *et al.* (2004) Clin. Cancer Res. 10:3937-3942; Ryu, *et al.* (2002) Cancer Res. 62:819-826; Thomas, *et al.* (2003) J. Exp. Med. 200:297-306; Argani, *et al.* (2001) Clin. Cancer Res. 7:3862-3868; Chowdhury, *et al.* (1998) Proc. Natl. Acad. Sci. USA 95:669-674; Chang and Pastan (1996) Proc. Natl. Acad. Sci. USA 93:136-140; Muminova, *et al.* (2004) BMC Cancer 4:19; GenBank Acc. Nos. NM_005823 and NM_013404; U.S. Pat. No. 5,723,318 issued to Yamaguchi, *et al.*).

[0306] Human mesothelin, deleted in mesothelin's signal sequence, is shown below:

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RTLAGETGQEAAPLDGVLTNPPNISSLSPRQLLGFPACAEVSGLSTERV
RELAVALAQKNVKLSTEQLRCLAHRLSEPPEDLDALPLDLLLFLNPDAFS
GPQACTRFFSRITKANVDLLPRGAPERQRLPAALACWGVGRSLLSEADV
RALGGLACDLPGRFVAESAELVLLPRLVSCPGPLDQDQQAARAALQGGGP
PYGPPSTWSVSTMDALRGLLPVLGQPIRSIPQGIVAAWRQRSSRDPSWR
QPERTILRPRFRREVEKTACPSGKKAREIDESLIFYKKWELEACVDAALL
ATQMDRVNAIPFTYEQLDVLKHKLDELYPQGYVESVIQHLGYLFLKMSPE
DIRKWNVTSLLETLKALLEVNKGHEMSPQVATLIDRFVKGRGQLDKDTLDT
LTAFYPGYLCSLSPHEELSSVPPSSIWAVRPQDLDTCDPRQLDVLYPKARL
AFQNMNGSEYFVKIQSFLGGAPTEDLKALSQQNVSMDLATFMKLRTDAVL
PLTVAEVQKLLGPHVEGLKAEERHRPVRDWILRQRQDDDLDTLGLGLQGGI
PNGYLVLDLSVQEAALSGTPCLLGPGPVLTVLALLLASTLA (SEQ ID NO:26)

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[0307] Human mesothelin, deleted in mesothelin's signal sequence and also deleted in mesothelin's GPI-anchor, is disclosed below:

RTLAGE TGQEAAPLDGVL TNPPNISSLSPRQLLGFP CAEVSG LSTERV
 RELAV ALAQKNVKLSTEQLRCLAHRLSEPPEDLDALPLDLLFLNPDAFS
 GPQACTRFFSRITKANVDLLPRGAPERQRLLP AALACWGVRGSLLEADV
 RALGGLACDLPGRFVAESA EVLLPRLVSCPGPLDQDQQAARAALQGGGP
 PYGPPSTWSVSTMDALRGLLPVLGQPIIRSIPQGIVAAWRQRSSRDPSWR
 QPERTILRPRFRREVEKTACPSGKKAREIDESLIFYKKWELEACVDAALL
 ATQMDRVNAIPFTYEQLDVLKHKLDELYPQGYPESVIQHLGYLFLKMSPE
 DIRKWNVTSLLETLKALLEVNKGHEMSPQVATLIDRFVKGRGQLDKDTLDT
 LTA FYPGYLC SLPEELSSVPPSSIWAVRPQDLDTCDPRQLDVLYPKARL
 AFQNMNGSEYFVKIQSFLGGAPTEDLKALSQQNVSM DLATFMKLRTDAVL
 PLTVAEVQKLLGPHVEGLKAEERHRPVRDWILRQRQDDLDLGLGLQG (SEQ ID
 NO:27)

[0308] The following documents are hereby incorporated by reference (see, e.g., U.S. Pat. No. 5,723,318 issued to Yamaguchi, *et al.*; U.S. Pat. No. 6,153,430 issued to Pastan, *et al.*; U.S. Pat. No. 6,809,184 issued to Pastan, *et al.*; U.S. Patent Applic. Publ. Pub. No.: US 2005/0214304 of Pastan, *et al.*; International Publ. No. WO 01/95942 of Pastan, *et al.*).

(f) Site of Integration

[0309] The present invention provides a polynucleotide comprising a first nucleic acid that mediates growth or spread in a wild type or parent *Listeria*, wherein the first nucleic acid is modified by integration of a second nucleic acid encoding at least one antigen. In one aspect, the integration results in attenuation of the *Listeria*. In another aspect, the integration does not result in attenuation of the *Listeria*. In yet another aspect, the parent *Listeria* is attenuated, and the integration results in further attenuation. Furthermore, as another non-limiting example, the parent *Listeria* is attenuated, where the integration does not result in further measurable attenuation.

[0310] Embodiments further comprising modification by integrating in the first nucleic acid, a third nucleic acid encoding at least one antigen, a fourth nucleic acid encoding at least one antigen, a fifth nucleic acid encoding at least one antigen, or the like, are also provided.

[0311] Without implying any limitation, the antigen can be a heterologous antigen (heterologous to the *Listeria*), a tumor antigen or an antigen derived from a tumor antigen, an infectious agent antigen or an antigen derived from an infectious agent antigen, and the like.

[0312] The first nucleic acid can be the actA gene or inlB gene. Integration can be at a promoter or regulatory region of actA or inlB, and/or in the open reading frame of actA or

inlB, where the integration attenuates the *Listeria*, as determinable under appropriate conditions. Integration can be accompanied by deletion of a part or all of the promoter or regulatory region of actA or inlB, or with deletion of part or all of the open reading frame of actA or inlB, or with deletion of both the promoter or regulatory region plus part or all of the open reading frame of actA or inlB, where the integration attenuates the *Listeria*, as determinable under appropriate conditions.

[0313] For each of the above-disclosed embodiments, the present invention provides a *Listeria* bacterium containing the polynucleotide. The polynucleotide can be genomic.

[0314] In some embodiments, the first nucleic acid that is modified by integration of a second nucleic acid encoding at least one antigen mediates growth or spread in a wild type or parent *Listeria*. In some embodiments, the first nucleic acid that is modified mediates cell to cell spread. In some embodiments, the first nucleic acid is actA.

[0315] In some embodiments, the first nucleic acid that is modified by integration of a second nucleic acid encoding at least one antigen, comprises a gene identified as one of the following: hly gene (encodes listeriolysin O; LLO); internalin A; internalin B; actA; SvpA; p104 (a.k.a. LAP); lplA; phosphatidylinositol-specific phospholipase C (PI-PLC) (plcA gene); phosphatidylcholine-specific phospholipase C (PC-PLC) (plcB gene); zinc metalloprotease precursor (Mpl gene); p60 (protein 60; invasion associated protein (iap); sortase; listeriolysin positive regulatory protein (PrfA gene); PrfB gene; FbpA gene; Auto gene; Ami (amidase that mediates adhesion); dlt operon (dltA; dltB; dltC; dltD); any prfA boxe; or Htp (sugar-P transporter).

[0316] Moreover, what is embraced is a *Listeria* comprising the above polynucleotide. The polynucleotide can be genomic. In one aspect, the *Listeria* can be *Listeria monocytogenes*. Provided is each of the above-disclosed embodiments, wherein the integration results in attenuation of the *Listeria*, as determinable under appropriate conditions. Also provided is each of the above-disclosed embodiments, wherein the integration does not result in attenuation of the *Listeria*, as determinable under appropriate conditions. In yet another aspect, the parent *Listeria* is attenuated, and the integration results in further attenuation. Furthermore, as another example, the parent *Listeria* is attenuated, where the integration does not result in further measurable attenuation.

[0317] In another aspect, first nucleic acid can be genomic. In another aspect, the integration can be mediated by homologous recombination, where the integration does not result in any deletion of the first nucleic acid, where the integration results in deletion of all or part of the first nucleic acid, where the first nucleic acid contains a promoter or other

regulatory region and where the second nucleic acid is operably linked and/or in frame with the promoter or other regulatory region, and where the first nucleic acid contains a promoter or other regulatory region and where the second nucleic acid is not at all operably linked and/or in frame with the promoter or other regulatory region.

[0318] The term “gene modified by integration” encompasses, but is not limited to, “a locus of integration that is the gene.”

[0319] What is also embraced by the present invention is a polynucleotide comprising a first nucleic acid that mediates growth or spread in a wild type or parent *Listeria*, where the first nucleic acid comprises all or part of a pathogenicity island or virulence gene cluster, wherein the all or part of the pathogenicity island or virulence gene cluster is modified by integration of a second nucleic acid encoding at least one antigen, wherein the integration results in attenuation of the *Listeria*, as determinable under appropriate conditions. Pathogenicity islands and virulence gene clusters are disclosed (see, e.g., Chakraborty, et al. (2000) Int. J. Med. Microbiol. 290:167-174; Vazquez-Boland, et al. (2001) Clin. Microbiol. Revs. 14:584-640). The gene that mediates growth and spread is not limited to a gene that specifically mediates virulence, but encompasses growth-mediating genes such those that mediate energy production (e.g., glycolysis, Krebs cycle, cytochromes), anabolism and/or catabolism of amino acids, sugars, lipids, minerals, purines, and pyrimidines, and genes that mediate nutrient transport, transcription, translation, and/or replication, and the like.

[0320] In another aspect, what is provided is a polynucleotide comprising a first nucleic acid that mediates growth or spread in a wild type or parent *Listeria*, wherein the nucleic acid is modified by integration of a plurality of nucleic acids encoding an antigen or antigens.

[0321] The integration can be within the second nucleic acid without any corresponding deletion of the second nucleic acid. Alternatively, the integration can be within the second nucleic acid with a corresponding deletion of the second nucleic acid, or a portion thereof. Where the first nucleic acid in the wild type or parent *Listeria* comprises a promoter and/or other regulatory site, the integration can be in the promoter and/or regulatory site.

[0322] Where the first nucleic acid comprises a promoter and/or other regulatory site, the present invention provides an integrated second nucleic acid, where the second nucleic acid comprises a coding region that is operably linked and in-frame with the promoter and/or regulatory site. As an alternative, the present invention provides an integrated second nucleic acid, where the second nucleic acid comprises a coding region that is not operably linked and in-frame with the promoter and/or regulatory site. Provided is each of the above embodiments, where the integrated nucleic acid (second nucleic acid) comprises a promoter

and/or regulatory site, where the promoter and/or regulatory site can take the place of, or alternatively can operate in addition to, a promoter and/or other regulatory site present in the first nucleic acid.

[0323] In one aspect, the first nucleic acid comprises (or in the alternative, consists of) a promoter or other regulatory element, and the second nucleic acid is operably linked with the promoter and/or other regulatory element. In another aspect, the second nucleic acid encoding an antigen further comprises a promoter and/or other regulatory element.

[0324] The first nucleic acid need not encode any polypeptide, as the first nucleic acid can be a regulatory region or box. The following concerns integration as mediated by, for example, homologous integration. The invention provides the above polynucleotide, wherein the second nucleic acid is integrated without deletion of any of the first nucleic acid.

[0325] In one embodiment, the first nucleic acid mediates growth but not spread. In another embodiment, the first nucleic acid mediates spread but not growth. In yet another embodiment, the first nucleic acid mediates both growth and spread. In one aspect, the integration reduces or eliminates the growth, reduces or eliminates the spread, or reduces or eliminates both growth and spread.

[0326] Moreover, in one embodiment the first nucleic acid has the property that its inactivation results in at least 10% reduction of growth, sometimes in at least 20% reduction of growth, typically in at least 30% reduction of growth, more typically in at least 40% reduction of growth, most typically in at least 50% reduction in growth, often in at least 60% reduction in growth, more often in at least 70% reduction in growth, most often in at least 80% reduction in growth, conventionally at least 85% reduction in growth, more conventionally at least 90% reduction in growth, and most conventionally in at least 95% reduction in growth, and sometimes in at least 99% reduction in growth. In one aspect, the growth can be measured in a defined medium, in a broth medium, in agar, within a host cell, in the cytoplasm of a host cell, and the like.

[0327] Moreover, in one embodiment the first nucleic acid has the property that its inactivation results in at least 10% reduction of cell-to-cell spread, sometimes in at least 20% reduction of spread, typically in at least 30% reduction of spread, more typically in at least 40% reduction of spread, most typically in at least 50% reduction in spread, often in at least 60% reduction in spread, more often in at least 70% reduction in spread, most often in at least 80% reduction in spread, conventionally at least 85% reduction in spread, more conventionally at least 90% reduction in spread, and most conventionally in at least 95% reduction in spread, and sometimes in at least 99% reduction in spread. In one aspect, the growth can be

measured in a defined medium, in a broth medium, in agar, within a host cell, in the cytoplasm of a host cell, and the like.

[0328] Provided is a *Listeria* bacterium comprising each of the above-disclosed polynucleotides. In one aspect, the *Listeria* is *Listeria monocytogenes*. Without implying any limitation, the present invention contemplates each of the above polynucleotides that is genomic, plasmid based, or that is present in both genomic and plasmid based forms.

[0329] In each of the above-disclosed embodiments, integration can be mediated by site-specific integration. Site-specific integration involves a plasmidic attPP' site, which recognizes a genomic attBB' site. In certain embodiments, the attBB' site can be naturally present in a gene that mediates growth or spread. In other embodiments, the attBB' site can be integrated, e.g., by homologous integration, in the gene that mediates growth or spread, followed by site-specific integration of the above-disclosed second nucleic acid.

[0330] The present invention provides a *Listeria* containing a polynucleotide comprising a first nucleic acid that, in the wild type *Listeria* or parent *Listeria*, mediates growth or spread, or both growth and spread, wherein the nucleic acid is modified by integration of a second nucleic acid encoding an antigen. Yet one further example of each of the embodiments disclosed herein provides an integration that reduces or eliminates growth, reduces or eliminates spread, or reduces or eliminates both growth and spread.

[0331] What is also embraced is a polynucleotide comprising a first nucleic acid that mediates growth or spread of a wild type or parental *Listeria*, and where the first nucleic acid comprises a signal sequence or secretory sequence, wherein the first nucleic acid is modified by integration of a second nucleic acid encoding at least one antigen, and wherein the integration results in an attenuation of the *Listeria*, and where the integration operably links the signal or secretory sequence (encoded by the first nucleic acid) with an open reading frame encoding by the second nucleic acid. In one aspect, the above integration results in deletion of all of the polypeptide encoded by the first nucleic acid, except for the signal or secretory sequence encoded by the first nucleic acid (where the signal or secretory sequence remains intact).

[0332] Genomes comprising each of the polynucleotide embodiments described herein are further contemplated. Moreover, what is provided is a listerial genome comprising each of the above embodiments. Furthermore, the invention supplies a *Listeria* bacterium comprising each of the polynucleotide embodiments described herein.

[0333] In one embodiment, the invention provides *Listeria* (e.g., *Listeria monocytogenes*) in which the genome comprises a polynucleotide comprising a nucleic acid encoding a

heterologous antigen. In some embodiments, the nucleic acid encoding the heterologous antigen has been integrated into the genome by site-specific recombination or homologous recombination. In some embodiments, the presence of the nucleic acid in the genome attenuates the *Listeria*. In some embodiments, the nucleic acid encoding the heterologous antigen has been integrated into the locus of a virulence gene. In some embodiments, the nucleic acid encoding the heterologous antigen has been integrated into the *actA* locus. In some embodiments, the nucleic acid encoding the heterologous antigen has been integrated into the *inlB* locus. In some embodiments, the genome of the *Listeria* comprises a first nucleic acid encoding a heterologous antigen that has been integrated into a first locus (e.g., the *actA* locus) and a second nucleic acid encoding a second heterologous antigen that has been integrated into a second locus (e.g., the *inlB* locus). The first and second heterologous antigens may be identical to each other or different. In some embodiments, the first and second heterologous antigens differ from each other, but are derived from the same tumor antigen or infectious agent antigen. In some embodiments, the first and second heterologous antigens are each a different fragment of an antigen derived from a cancer cell, tumor, or infectious agent. In some embodiments, the integrated nucleic acid encodes a fusion protein comprising a modified ActA and the heterologous antigen. In some embodiments, at least two, at least three, at least four, at least five, at least six, or at least seven nucleic acid sequences encoding heterologous antigens have been integrated into the *Listeria* genome.

[0334] In some embodiments, a polynucleotide (or nucleic acid) described herein has been integrated into a virulence gene in the genome of the *Listeria*, wherein the integration of the polynucleotide (a) disrupts expression of the virulence gene; and/or (b) disrupts a coding sequence of the virulence gene. In some embodiments, the *Listeria* is attenuated by the disruption of the expression of the virulence gene and/or the disruption of the coding sequence of the virulence gene attenuates the *Listeria*. In some embodiments, the virulence gene is necessary for mediating growth or spread. In other embodiments, the virulence gene is not necessary for mediating growth or spread. In some embodiments, the virulence gene is a *prfA*-dependent gene. In some embodiments, the virulence gene is not a *prfA*-dependent gene. In some embodiments, the virulence gene is *actA* or *inlB*. In some embodiments, the expression of the virulence gene in which the polynucleotide/nucleic acid is integrated is disrupted at least 10%, at least 25%, at least 50%, at least 75%, at least 90%, or about 100% (relative to the expression of the virulence gene in the absence of the integrated polynucleotide/nucleic acid, as determined by measuring expression levels. Disruption of the coding sequence of the virulence gene encompasses alterations of the coding sequence of any

kind including frame-shift mutations, truncations, insertions, deletions, or replacements/substitutions. In some embodiments, all or part of the virulence gene is deleted during integration of the polynucleotide into the virulence gene. In other embodiments, none of the virulence gene is deleted during integration of the polynucleotide. In some embodiments, part or all of the coding sequence of the virulence gene is replaced by the integrated polynucleotide.

[0335] In some embodiments, multiple polynucleotides described herein have been integrated into the *Listeria* genome at one or more different sites. The multiple polynucleotides may be the same or different. In some embodiments, a first polynucleotide described herein has been integrated into the *actA* locus and/or a second polynucleotide described herein has been integrated into the *inlB* locus. In some embodiments, a first polynucleotide described herein has been integrated into the *actA* locus and a second polynucleotide described herein has been integrated into the *inlB* locus. The heterologous antigen encoded by the first polynucleotide may be the same or different as that encoded by the second polynucleotide. In some embodiments, the two heterologous antigens encoded by the integrated antigens differ, but are derived from the same antigen.

IV. THERAPEUTIC COMPOSITIONS AND USES.

(a). Therapeutic compositions.

[0336] The attenuated *Listeria*, vaccines, small molecules, biological reagents, and adjuvants that are provided herein can be administered to a host, either alone or in combination with a pharmaceutically acceptable excipient, in an amount sufficient to induce an appropriate immune response to an immune disorder, cancer, tumor, or infection. The immune response can comprise, without limitation, specific immune response, non-specific immune response, both specific and non-specific response, innate response, primary immune response, adaptive immunity, secondary immune response, memory immune response, immune cell activation, immune cell proliferation, immune cell differentiation, and cytokine expression.

[0337] "Pharmaceutically acceptable excipient" or "diagnostically acceptable excipient" includes but is not limited to, sterile distilled water, saline, phosphate buffered solutions, amino acid-based buffers, or bicarbonate buffered solutions. An excipient selected and the amount of excipient used will depend upon the mode of administration. Administration may be oral, intravenous, subcutaneous, dermal, intradermal, intramuscular, mucosal, parenteral, intraorgan, intralesional, intranasal, inhalation, intraocular, intramuscular, intravascular,

intranodal, by scarification, rectal, intraperitoneal, or any one or combination of a variety of well-known routes of administration. The administration can comprise an injection, infusion, or a combination thereof. Administration of the *Listeria* of the present invention by a non-oral route can avoid tolerance (see, e.g., Lecuit, *et al.* (2001) *Science* 292:1722-1725; Kirk, *et al.* (2005) *Transgenic Res.* 14:449-462; Faria and Weiner (2005) *Immunol. Rev.* 206:232-259; Kraus, *et al.* (2005) *J. Clin. Invest.* 115:2234-2243; Mucida, *et al.* (2005) *J. Clin. Invest.* 115:1923-1933). Methods are available for administration of *Listeria*, e.g., intravenously, subcutaneously, intramuscularly, intraperitoneally, orally, mucosal, by way of the urinary tract, by way of a genital tract, by way of the gastrointestinal tract, or by inhalation (Dustoor, *et al.* (1977) *Infection Immunity* 15:916-924; Gregory and Wing (2002) *J. Leukoc. Biol.* 72:239-248; Hof, *et al.* (1997) *Clin. Microbiol. Revs.* 10:345-357; Schluter, *et al.* (1999) *Immunobiol.* 201:188-195; Hof (2004) *Expert Opin. Pharmacother.* 5:1727-1735; Heymer, *et al.* (1988) *Infection* 16(Suppl. 2):S106-S111; Yin, *et al.* (2003) *Environ. Health Perspectives* 111:524-530).

[0338] The invention provides immunogenic compositions comprising any of the *Listeria* described herein. The invention further provides pharmaceutical compositions comprising any of the *Listeria* described herein and a pharmaceutically acceptable excipient. In some embodiments, the immunogenic compositions or pharmaceutical compositions are vaccines. In some embodiments, the composition comprising the *Listeria* is a vaccine that further comprises an adjuvant.

[0339] The following applies, optionally, to each of the embodiments disclosed herein. Provided is an administered reagent that is pure or purified, for example where the administered reagent can be administered to a mammal in a pure or purified form, i.e., alone, as a pharmaceutically acceptable composition, or in an excipient. Moreover, the following also can apply, optionally, to each of the embodiments disclosed herein. Provided is an administered reagent that is pure or purified, where the administered reagent can be administered in a pure or purified form, i.e., alone, as a pharmaceutically acceptable composition, or in an excipient, and where the reagent is not generated after administration (not generated in the mammal). In one embodiment, what might optionally apply to each of the reagents disclosed herein, is a polypeptide reagent that is administered as a pure or purified polypeptide (e.g., alone, as a pharmaceutically acceptable composition, or in an excipient), where the administered polypeptide reagent is not administered in the form of a nucleic acid encoding that polypeptide, and as a consequence, there is no administered nucleic acid that can generate the polypeptide inside the mammal.

[0340] The *Listeria* of the present invention can be stored, e.g., frozen, lyophilized, as a suspension, as a cell paste, or complexed with a solid matrix or gel matrix.

[0341] An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the route and dose of administration and the severity of side effects. An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the route and dose of administration and the severity of side effects. Guidance for methods of treatment and diagnosis is available (see, e.g., Maynard, *et al.* (1996) A Handbook of SOPs for Good Clinical Practice, Interpharm Press, Boca Raton, FL; Dent (2001) Good Laboratory and Good Clinical Practice, Urch Publ., London, UK).

[0342] The *Listeria* of the present invention can be administered in a dose, or dosages, where each dose comprises at least 1000 *Listeria* cells/kg body weight; normally at least 10,000 cells; more normally at least 100,000 cells; most normally at least 1 million cells; often at least 10 million cells; more often at least 100 million cells; typically at least 1 billion cells; usually at least 10 billion cells; conventionally at least 100 billion cells; and sometimes at least 1 trillion *Listeria* cells/kg body weight. The present invention provides the above doses where the units of *Listeria* administration is colony forming units (CFU), the equivalent of CFU prior to psoralen-treatment, or where the units are number of *Listeria* cells.

[0343] The *Listeria* of the present invention can be administered in a dose, or dosages, where each dose comprises between 10^7 and 10^8 *Listeria* per 70 kg body weight (or per 1.7 square meters surface area; or per 1.5 kg liver weight); 2×10^7 and 2×10^8 *Listeria* per 70 kg body weight (or per 1.7 square meters surface area; or per 1.5 kg liver weight); 5×10^7 and 5×10^8 *Listeria* per 70 kg body weight (or per 1.7 square meters surface area; or per 1.5 kg liver weight); 10^8 and 10^9 *Listeria* per 70 kg body weight (or per 1.7 square meters surface area; or per 1.5 kg liver weight); between 2.0×10^8 and 2.0×10^9 *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 5.0×10^8 to 5.0×10^9 *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^9 and 10^{10} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 2×10^9 and 2×10^{10} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 5×10^9 and 5×10^{10} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^{11} and 10^{12} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 2×10^{11} and 2×10^{12} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 5×10^{11} and 5×10^{12} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per

1.5 kg liver weight); between 10^{12} and 10^{13} *Listeria* per 70 kg (or per 1.7 square meters surface area); between 2×10^{12} and 2×10^{13} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 5×10^{12} and 5×10^{13} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^{13} and 10^{14} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 2×10^{13} and 2×10^{14} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); 5×10^{13} and 5×10^{14} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^{14} and 10^{15} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 2×10^{14} and 2×10^{15} *Listeria* per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); and so on, wet weight.

[0344] The mouse liver, at the time of administering the *Listeria* of the present invention, weighs about 1.5 grams. Human liver weighs about 1.5 kilograms.

[0345] Also provided is one or more of the above doses, where the dose is administered by way of one injection every day, one injection every two days, one injection every three days, one injection every four days, one injection every five days, one injection every six days, or one injection every seven days, where the injection schedule is maintained for, e.g., one day only, two days, three days, four days, five days, six days, seven days, two weeks, three weeks, four weeks, five weeks, or longer. The invention also embraces combinations of the above doses and schedules, e.g., a relatively large initial dose of *Listeria*, followed by relatively small subsequent doses of *Listeria*, or a relatively small initial dose followed by a large dose.

[0346] A dosing schedule of, for example, once/week, twice/week, three times/week, four times/week, five times/week, six times/week, seven times/week, once every two weeks, once every three weeks, once every four weeks, once every five weeks, and the like, is available for the invention. The dosing schedules encompass dosing for a total period of time of, for example, one week, two weeks, three weeks, four weeks, five weeks, six weeks, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, and twelve months.

[0347] Provided are cycles of the above dosing schedules. The cycle can be repeated about, e.g., every seven days; every 14 days; every 21 days; every 28 days; every 35 days; 42 days; every 49 days; every 56 days; every 63 days; every 70 days; and the like. An interval of non-dosing can occur between a cycle, where the interval can be about, e.g., seven days; 14 days; 21 days; 28 days; 35 days; 42 days; 49 days; 56 days; 63 days; 70 days; and the like. In this context, the term "about" means plus or minus one day, plus or minus two days, plus or

minus three days, plus or minus four days, plus or minus five days, plus or minus six days, or plus or minus seven days.

[0348] The present invention encompasses a method of administering *Listeria* that is oral. Also provided is a method of administering *Listeria* that is intravenous. Moreover, what is provided is a method of administering *Listeria* that is intramuscular. The invention supplies a *Listeria* bacterium, or culture or suspension of *Listeria* bacteria, prepared by growing in a medium that is meat based, or that contains polypeptides derived from a meat or animal product. Also supplied by the present invention is a *Listeria* bacterium, or culture or suspension of *Listeria* bacteria, prepared by growing in a medium that does not contain meat or animal products, prepared by growing on a medium that contains vegetable polypeptides, prepared by growing on a medium that is not based on yeast products, or prepared by growing on a medium that contains yeast polypeptides.

[0349] The present invention encompasses a method of administering *Listeria* that is not oral. Also provided is a method of administering *Listeria* that is not intravenous. Moreover, what is provided is a method of administering *Listeria* that is not intramuscular. The invention supplies a *Listeria* bacterium, or culture or suspension of *Listeria* bacteria, prepared by growing in a medium that is not meat based, or that does not contain polypeptides derived from a meat or animal product. Also supplied by the present invention is a *Listeria* bacterium, or culture or suspension of *Listeria* bacteria, prepared by growing in a medium based on vegetable products, that contains vegetable polypeptides, that is based on yeast products, or that contains yeast polypeptides.

[0350] Methods for co-administration with an additional therapeutic agent, e.g., a small molecule, antibiotic, innate immunity modulating agent, tolerance modulating agent, cytokine, chemotherapeutic agent, or radiation, are well known in the art (Hardman, *et al.* (eds.) (2001) Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill, New York, NY; Poole and Peterson (eds.) (2001) Pharmacotherapeutics for Advanced Practice: A Practical Approach, Lippincott, Williams & Wilkins, Phila., PA; Chabner and Longo (eds.) (2001) Cancer Chemotherapy and Biotherapy, Lippincott, Williams & Wilkins, Phila., PA).

[0351] The present invention provides reagents for administering in conjunction with an attenuated *Listeria*. These reagents include biological reagents such as: (1) Cytokines, antibodies, dendritic cells, attenuated tumor cells; (2) Small molecule reagents such as 5-fluorouracil, methotrexate, paclitaxel, docetaxel, cis-platin, gemcitabine; (3) Reagents that modulate regulatory T cells, such as cyclophosphamide, anti-CTLA4 antibody, anti-CD25

antibody (see, e.g., Hawryfar, *et al.* (2005) *J. Immunol.* 174:344-3351); and (4) Vaccines (including polypeptide vaccines, nucleic acid vaccines, attenuated tumor cell vaccines, and dendritic cell vaccines). The reagents can be administered with the *Listeria* or independently (before or after) the *Listeria*. For example, the reagent can be administered immediately before (or after) the *Listeria*, on the same day as, one day before (or after), one week before (or after), one month before (or after), or two months before (or after) the *Listeria*, and the like.

[0352] Biological reagents or macromolecules of the present invention encompass an agonist or antagonist of a cytokine, a nucleic acid encoding an agonist or antagonist of a cytokine, a cell expressing a cytokine, or an agonistic or antagonistic antibody. Biological reagents include, without limitation, a TH-1 cytokine, a TH-2 cytokine, IL-2, IL-12, FLT3-ligand, GM-CSF, IFN γ , a cytokine receptor, a soluble cytokine receptor, a chemokine, tumor necrosis factor (TNF), CD40 ligand, or a reagent that stimulates replacement of a proteasome subunit with an immunoproteasome subunit.

[0353] The present invention encompasses biological reagents, such cells engineered to express at least one of the following: GM-CSF, IL-2, IL-3, IL-4, IL-12, IL-18, tumor necrosis factor- α (TNF- α), or inducing protein-10. Other contemplated reagents include agonists of B7-1, B7-2, CD28, CD40 ligand, or OX40 ligand (OX40L), and novel forms engineered to be soluble or engineered to be membrane-bound (see, e.g., Karnbach, *et al.* (2001) *J. Immunol.* 167:2569-2576; Greenfield, *et al.* (1998) *Crit. Rev. Immunol.* 18:389-418; Parney and Chang (2003) *J. Biomed. Sci.* 10:37-43; Gri, *et al.* (2003) *J. Immunol.* 170:99-106; Chiodoni, *et al.* (1999) *J. Exp. Med.* 190:125-133; Enzler, *et al.* (2003) *J. Exp. Med.* 197:1213-1219; Soo Hoo, *et al.* (1999) *J. Immunol.* 162:7343-7349; Mihalyo, *et al.* (2004) *J. Immunol.* 172:5338-5345; Chapoval, *et al.* (1998) *J. Immunol.* 161:6977-6984).

[0354] Without implying any limitation, the present invention provides the following biologicals. MCP-1, MIP1- α , TNF- α , and interleukin-2, for example, are effective in treating a variety of tumors (see, e.g., Nakamoto, *et al.* (2000) *Anticancer Res.* 20(6A):4087-4096; Kamada, *et al.* (2000) *Cancer Res.* 60:6416-6420; Li, *et al.* (2002) *Cancer Res.* 62:4023-4028; Yang, *et al.* (2002) *Zhonghua Wai Ke Za Zhi* 40:789-791; Hoving, *et al.* (2005) *Cancer Res.* 65:4300-4308; Tsuchiyama, *et al.* (2003) *Cancer Gene Ther.* 10:260-269; Sakai, *et al.* (2001) *Cancer Gene Ther.* 8:695-704).

[0355] The present invention provides reagents and methods encompassing an Flt3-ligand agonist, and an Flt3-ligand agonist in combination with *Listeria*. Flt3-ligand (Fms-like tyrosine kinase 3 ligand) is a cytokine that can generate an antitumor immune response (see,

e.g., Dranoff (2002) *Immunol. Revs.* 188:147-154; Mach, *et al.* (2000) *Cancer Res.* 60:3239-3246; Furumoto, *et al.* (2004) *J. Clin. Invest.* 113:774-783; Freedman, *et al.* (2003) *Clin. Cancer Res.* 9:5228-5237; Mach, *et al.* (2000) *Cancer Res.* 60:3239-3246).

[0356] In another embodiment, the present invention contemplates administration of a dendritic cell (DC) that expresses at least one tumor antigen, or infectious disease antigen. Expression by the DC of an antigen can be mediated by way of, e.g., peptide loading, tumor cell extracts, fusion with tumor cells, transduction with mRNA, or transfected by a vector (see, e.g., Klein, *et al.* (2000) *J. Exp. Med.* 191:1699-1708; Conrad and Nestle (2003) *Curr. Opin. Mol. Ther.* 5:405-412; Gilboa and Vieweg (2004) *Immunol. Rev.* 199:251-263; Paczesny, *et al.* (2003) *Semin. Cancer Biol.* 13:439-447; Westermann, *et al.* (1998) *Gene Ther.* 5:264-271).

[0357] The methods and reagents of the present invention also encompass small molecule reagents, such as 5-fluorouracil, methotrexate, irinotecan, doxorubicin, prednisone, dolostatin-10 (D10), combretastatin A-4, mitomycin C (MMC), vincristine, colchicines, vinblastine, cyclophosphamide, fungal beta-glucans and derivatives thereof, and the like (see, e.g., Hurwitz, *et al.* (2004) *New Engl. J. Med.* 350:2335-2342; Pelaez, *et al.* (2001) *J. Immunol.* 166:6608-6615; Havas, *et al.* (1990) *J. Biol. Response Modifiers* 9:194-204; Turk, *et al.* (2004) *J. Exp. Med.* 200:771-782; Ghiringhelli, *et al.* (2004) *Eur. J. Immunol.* 34:336-344; Andrade-Mena (1994) *Int. J. Tissue React.* 16:95-103; Chrischilles, *et al.* (2003) *Cancer Control* 10:396-403). Also encompassed are compositions that are not molecules, e.g., salts and ions.

[0358] Provided are analogues of cyclophosphamide (see, e.g., Jain, *et al.* (2004) *J. Med. Chem.* 47:3843-3852; Andersson, *et al.* (1994) *Cancer Res.* 54:5394-5400; Borch and Canute (1991) *J. Med. Chem.* 34:3044-3052; Ludeman, *et al.* (1979) *J. Med. Chem.* 22:151-158; Zon (1982) *Prog. Med. Chem.* 19:205-246).

[0359] Also embraced by the invention are small molecule reagents that stimulate innate immune response, e.g., CpG oligonucleotides, imiquimod, and alphaGalCer. CpG oligonucleotides mediate immune response via TLR9 (see, e.g., Chagnon, *et al.* (2005) *Clin. Cancer Res.* 11:1302-1311; Speiser, *et al.* (2005) *J. Clin. Invest.* Feb.3 (epub ahead of print); Mason, *et al.* (2005) *Clin. Cancer Res.* 11:361-369; Suzuki, *et al.* (2004) *Cancer Res.* 64:8754-8760; Taniguchi, *et al.* (2003) *Annu. Rev. Immunol.* 21:483-513; Takeda, *et al.* (2003) *Annu. Rev. Immunol.* 21:335-376; Metelitsa, *et al.* (2001) *J. Immunol.* 167:3114-3122).

[0360] Other useful small molecule reagents include those derived from bacterial peptidoglycan, such as certain NOD2 ligands (McCaffrey, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:11386-11391).

[0361] The invention includes reagents and methods for modulating activity of T regulatory cells (Tregs; suppressor T cells). Attenuation or inhibition of Treg cell activity can enhance the immune system's killing of tumor cells. A number of reagents have been identified that inhibit Treg cell activity. These reagents include, e.g., cyclophosphamide (a.k.a. Cytoxan®; CTX), anti-CD25 antibody, modulators of GITR-L or GITR, a modulator of Forkhead-box transcription factor (Fox), a modulator of LAG-3, anti-IL-2R, and anti-CTLA4 (see, e.g., Pardoll (2003) *Annu. Rev. Immunol.* 21:807-839; Ercolini, *et al.* (2005) *J. Exp. Med.* 201:1591-1602; Haeryfar, *et al.* (2005) *J. Immunol.* 174:3344-3351; Mihalyo, *et al.* (2004) *J. Immunol.* 172:5338-5345; Stephens, *et al.* (2004) *J. Immunol.* 173:5008-5020; Schiavoni, *et al.* (2000) *Blood* 95:2024-2030; Calmels, *et al.* (2004) *Cancer Gene Ther.* Oct. 08 (epub ahead of print); Mincheff, *et al.* (2004) *Cancer Gene Ther.* Sept. 17 [epub ahead of print]; Muriglan, *et al.* (2004) *J. Exp. Med.* 200:149-157; Stephens, *et al.* (2004) *J. Immunol.* 173:5008-5020; Coffey and Burgering (2004) *Nat. Rev. Immunol.* 4:889-899; Kalinichenko, *et al.* (2004) *Genes Dev.* 18:830-850; Cobbold, *et al.* (2004) *J. Immunol.* 172:6003-6010; Huang, *et al.* (2004) *Immunity* 21:503-513). CTX shows a bimodal effect on the immune system, where low doses of CTX inhibit Tregs (see, e.g., Lutsiak, *et al.* (2005) *Blood* 105:2862-2868).

[0362] CTLA4-blocking agents, such as anti-CTLA4 blocking antibodies, can enhance immune response to cancers, tumors, pre-cancerous disorders, infections, and the like (see, e.g., Zubairi, *et al.* (2004) *Eur. J. Immunol.* 34:1433-1440; Espenschied, *et al.* (2003) *J. Immunol.* 170:3401-3407; Davila, *et al.* (2003) *Cancer Res.* 63:3281-3288; Hodi, *et al.* (2003) *Proc. Natl. Acad. Sci. USA* 100:4712-4717). Where the present invention uses anti-CTLA4 antibodies, and the like, the invention is not necessarily limited to use for inhibiting Tregs, and also does not necessarily always encompass inhibition of Tregs.

[0363] Lymphocyte activation gene-3 (LAG-3) blocking agents, such as anti-LAG-3 antibodies or soluble LAG-3 (e.g., LAG-3 Ig), can enhance immune response to cancers or infections. Anti-LAG-3 antibodies reduce the activity of Tregs (see, e.g., Huang, *et al.* (2004) *Immunity* 21:503-513; Triebel (2003) *Trends Immunol.* 24:619-622; Workman and Vignali (2003) *Eur. J. Immunol.* 33:970-979; Cappello, *et al.* (2003) *Cancer Res.* 63:2518-2525; Workman, *et al.* (2004) *J. Immunol.* 172:5450-5455; Macon-Lemaitre and Triebel (2005) *Immunology* 115:170-178).

[0364] Vaccines comprising a tumor antigen, a nucleic acid encoding a tumor antigen, a vector comprising a nucleic acid encoding a tumor antigen, a cell comprising a tumor antigen, a tumor cell, or an attenuated tumor cell, are encompassed by the invention. Provided are reagents derived from a nucleic acid encoding a tumor antigen, e.g., a codon optimized nucleic acid, or a nucleic acid encoding two or more different tumor antigens, or a nucleic acid expressing rearranged epitopes of a tumor antigen, e.g., where the natural order of epitopes is ABCD and the engineered order is ADBC, or a nucleic acid encoding a fusion protein comprising at least two different tumor antigens.

[0365] Where an administered antibody, binding compound derived from an antibody, cytokine, or other therapeutic agent produces toxicity, an appropriate dose can be one where the therapeutic effect outweighs the toxic effect. Generally, an optimal dosage of the present invention is one that maximizes therapeutic effect, while limiting any toxic effect to a level that does not threaten the life of the patient or reduce the efficacy of the therapeutic agent. Signs of toxic effect, or anti-therapeutic effect include, without limitation, e.g., anti-idiotypic response, immune response to a therapeutic antibody, allergic reaction, hematologic and platelet toxicity, elevations of aminotransferases, alkaline phosphatase, creatine kinase, neurotoxicity, nausea, and vomiting (see, e.g., Huang, *et al.* (1990) Clin. Chem. 36:431-434).

[0366] An effective amount of a therapeutic agent is one that will decrease or ameliorate the symptoms normally by at least 10%, more normally by at least 20%, most normally by at least 30%, typically by at least 40%, more typically by at least 50%, most typically by at least 60%, often by at least 70%, more often by at least 80%, and most often by at least 90%, conventionally by at least 95%, more conventionally by at least 99%, and most conventionally by at least 99.9%.

[0367] The reagents and methods of the present invention provide a vaccine comprising only one vaccination; or comprising a first vaccination; or comprising at least one booster vaccination; at least two booster vaccinations; or at least three booster vaccinations.

Guidance in parameters for booster vaccinations is available (see, e.g., Marth (1997) Biologicals 25:199-203; Ramsay, *et al.* (1997) Immunol. Cell Biol. 75:382-388; Gherardi, *et al.* (2001) Histol. Histopathol. 16:655-667; Leroux-Roels, *et al.* (2001) ActA Clin. Belg. 56:209-219; Greiner, *et al.* (2002) Cancer Res. 62:6944-6951; Smith, *et al.* (2003) J. Med. Virol. 70:Suppl.1:S38-S41; Sepulveda-Amor, *et al.* (2002) Vaccine 20:2790-2795).

[0368] Provided is a first reagent that comprises a *Listeria* bacterium (or *Listeria* vaccine), and a second reagent that comprises, e.g., a cytokine, a small molecule such as cyclophosphamide or methotrexate, or a vaccine, such as an attenuated tumor cell or

attenuated tumor cell expressing a cytokine. Provided are the following methods of administration of the first reagent and the second reagent.

[0369] The *Listeria* and the second reagent can be administered concomitantly, that is, where the administering for each of these reagents can occur at time intervals that partially or fully overlap each other. The *Listeria* and second reagent can be administered during time intervals that do not overlap each other. For example, the first reagent can be administered within the time frame of $t = 0$ to 1 hours, while the second reagent can be administered within the time frame of $t = 1$ to 2 hours. Also, the first reagent can be administered within the time frame of $t = 0$ to 1 hours, while the second reagent can be administered somewhere within the time frame of $t = 2-3$ hours, $t = 3-4$ hours, $t = 4-5$ hours, $t = 5-6$ hours, $t = 6-7$ hours, $t = 7-8$ hours, $t = 8-9$ hours, $t = 9-10$ hours, and the like. Moreover, the second reagent can be administered somewhere in the time frame of $t = \text{minus } 2-3$ hours, $t = \text{minus } 3-4$ hours, $t = \text{minus } 4-5$ hours, $t = 5-6$ minus hours, $t = \text{minus } 6-7$ hours, $t = \text{minus } 7-8$ hours, $t = \text{minus } 8-9$ hours, $t = \text{minus } 9-10$ hours, and the like.

[0370] To provide another example, the first reagent can be administered within the time frame of $t = 0$ to 1 days, while the second reagent can be administered within the time frame of $t = 1$ to 2 days. Also, the first reagent can be administered within the time frame of $t = 0$ to 1 days, while the second reagent can be administered somewhere within the time frame of $t = 2-3$ days, $t = 3-4$ days, $t = 4-5$ days, $t = 5-6$ days, $t = 6-7$ days, $t = 7-8$ days, $t = 8-9$ days, $t = 9-10$ days, and the like. Moreover, the second reagent can be administered somewhere in the time from of $t = \text{minus } 2-3$ days, $t = \text{minus } 3-4$ days, $t = \text{minus } 4-5$ days, $t = \text{minus } 5-6$ days, $t = \text{minus } 6-7$ days, $t = \text{minus } 7-8$ days, $t = \text{minus } 8-9$ days, $t = \text{minus } 9-10$ days, and the like.

[0371] In another aspect, administration of the *Listeria* can begin at $t = 0$ hours, where the administration results in a peak (or maximal plateau) in plasma concentration of the *Listeria*, and where administration of the second reagent is initiated at about the time that the concentration of plasma *Listeria* reaches said peak concentration, at about the time that the concentration of plasma *Listeria* is 95% said peak concentration, at about the time that the concentration of plasma *Listeria* is 90% said peak concentration, at about the time that the concentration of plasma *Listeria* is 85% said peak concentration, at about the time that the concentration of plasma *Listeria* is 80% said peak concentration, at about the time that the concentration of plasma *Listeria* is 75% said peak concentration, at about the time that the concentration of plasma *Listeria* is 70% said peak concentration, at about the time that the concentration of plasma *Listeria* is 65% said peak concentration, at about the time that the

concentration of plasma *Listeria* is 60% said peak concentration, at about the time that the concentration of plasma *Listeria* is 55% said peak concentration, at about the time that the concentration of plasma *Listeria* is 50% said peak concentration, at about the time that the concentration of plasma *Listeria* is 45% said peak concentration, at about the time that the concentration of plasma *Listeria* is 40% said peak concentration, at about the time that the concentration of plasma *Listeria* is 35% said peak concentration, at about the time that the concentration of plasma *Listeria* is 30% said peak concentration, at about the time that the concentration of plasma *Listeria* is 25% said peak concentration, at about the time that the concentration of plasma *Listeria* is 20% said peak concentration, at about the time that the concentration of plasma *Listeria* is 15% said peak concentration, at about the time that the concentration of plasma *Listeria* is 10% said peak concentration, at about the time that the concentration of plasma *Listeria* is 5% said peak concentration, at about the time that the concentration of plasma *Listeria* is 2.0% said peak concentration, at about the time that the concentration of plasma *Listeria* is 0.5% said peak concentration, at about the time that the concentration of plasma *Listeria* is 0.2% said peak concentration, or at about the time that the concentration of plasma *Listeria* is 0.1%, or less than, said peak concentration.

[0372] In another aspect, administration of the second reagent can begin at $t = 0$ hours, where the administration results in a peak (or maximal plateau) in plasma concentration of the second reagent and where administration of the *Listeria* is initiated at about the time that the concentration of plasma level of the second reagent reaches said peak concentration, at about the time that the concentration of plasma second reagent is 95% said peak concentration, at about the time that the concentration of plasma second reagent is 90% said peak concentration, at about the time that the concentration of plasma second reagent is 85% said peak concentration, at about the time that the concentration of plasma second reagent is 80% said peak concentration, at about the time that the concentration of plasma second reagent is 75% said peak concentration, at about the time that the concentration of plasma second reagent is 70% said peak concentration, at about the time that the concentration of plasma second reagent is 65% said peak concentration, at about the time that the concentration of plasma second reagent is 60% said peak concentration, at about the time that the concentration of plasma second reagent is 55% said peak concentration, at about the time that the concentration of plasma second reagent is 50% said peak concentration, at about the time that the concentration of plasma second reagent is 45% said peak concentration, at about the time that the concentration of plasma second reagent is 40% said peak concentration, at about the time that the concentration of plasma second reagent is 35% said peak

concentration, at about the time that the concentration of plasma second reagent is 30% said peak concentration, at about the time that the concentration of plasma second reagent is 25% said peak concentration, at about the time that the concentration of plasma second reagent is 20% said peak concentration, at about the time that the concentration of plasma second reagent is 15% said peak concentration, at about the time that the concentration of plasma second reagent is 10% said peak concentration, at about the time that the concentration of plasma second reagent is 5% said peak concentration, at about the time that the concentration of plasma second reagent is 2.0% said peak concentration, at about the time that the concentration of plasma second reagent is 0.5% said peak concentration, at about the time that the concentration of plasma second reagent is 0.2% said peak concentration, or at about the time that the concentration of plasma second reagent is 0.1%, or less than, said peak concentration. As it is recognized that alteration of the *Listeria* or second reagent may occur *in vivo*, the above concentrations can be assessed after measurement of intact reagent, or after measurement of an identifiable degradation product of the intact reagent.

[0373] Formulations of therapeutic and diagnostic agents may be prepared for storage by mixing with physiologically acceptable carriers, excipients, or stabilizers in the form of, e.g., lyophilized powders, slurries, aqueous solutions or suspensions (see, e.g., Hardman, *et al.* (2001) Goodman and Gilman's The Pharmacological Basis of Therapeutics, McGraw-Hill, New York, NY; Gennaro (2000) Remington: The Science and Practice of Pharmacy, Lippincott, Williams, and Wilkins, New York, NY; Avis, *et al.* (eds.) (1993) Pharmaceutical Dosage Forms: Parenteral Medications, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Tablets, Marcel Dekker, NY; Lieberman, *et al.* (eds.) (1990) Pharmaceutical Dosage Forms: Disperse Systems, Marcel Dekker, NY; Weiner and Kotkoskie (2000) Excipient Toxicity and Safety, Marcel Dekker, Inc., New York, NY).

[0374] The invention also provides a kit comprising a *Listeria* cell, a listerial cell culture, or a lyophilized cell preparation, and a compartment. In addition, the present invention provides a kit comprising a *Listeria* cell, listerial cell culture, or a lyophilized cell preparation and a reagent. Also provided is a kit comprising a *Listeria* cell, a listerial cell culture, or a lyophilized cell preparation and instructions for use or disposal. Moreover, the present invention provides a kit comprising a *Listeria* cell, a listerial cell culture, or lyophilized cell preparation, and compartment and a reagent. Provided is a kit comprising *Listeria* bacteria, and instructions for using the *Listeria* bacteria with a small molecule anti-cancer agent, and/or small molecule immunomodulating agent (e.g., cyclophosphamide), and/or a small molecule anti-infection agent, and the like. Also provided is a kit comprising *Listeria*

bacteria, and/or instructions for administering the *Listeria*, and/or instructions for monitoring immune response to the administered *Listeria*, and/or instructions for monitoring immune response to a heterologous antigen encoded by the administered *Listeria*.

(b). Uses.

[0375] The invention provides, in certain embodiments, a modified *Listeria* bacterium, e.g., *L. monocytogenes*, engineered to express at least one heterologous antigen. The invention is useful for enhancing immune response, stimulating immune response, enhancing immune presentation, increasing stability of an expressed mRNA or polypeptide, increasing proteolytic processing of an expressed polypeptide, increasing immune response to a mutated self antigen, increasing survival to a cancer or infection, and/or for treating a cancer or infection. The invention is also useful for enhanced expression of a heterologous antigen, e.g., for industry, agriculture, or medicine.

[0376] For methods to stimulate, enhance, or increase immune response to a cancer, tumor, or infectious agent; and for methods to stimulate, enhance, or increase survival to a cancer, tumor, or infectious agent; an increase can occur with administration of a *Listeria* containing a nucleic acid encoding a heterologous antigen. For purposes of providing an experimental control, the increase can be relative to response with administration of a *Listeria* not containing a nucleic acid encoding that particular heterologous antigen. As another alternative, the increase can be relative to response with administering a *Listeria* not containing any nucleic acid that encodes a heterologous antigen, e.g., a parental or wild type *Listeria*. As still another alternative, the increase can be relative to response without administering any *Listeria*.

[0377] For methods to stimulate, enhance, or increase immune response to a cancer, tumor, or infectious agent; and for methods to stimulate, enhance, or increase survival to a cancer, tumor, or infectious agent; an increase can occur with administration of a *Listeria* (containing or not containing a nucleic acid encoding a heterologous antigen) with an immune modulator, such as an agonist antibody, a cytokine, or an antibody that specifically binds to an antigen of the cancer, tumor, or infectious agent. For purposes of providing an experimental control, the increase can be relative to response with administration of a *Listeria* but without administering the immune modulator. As an alternative, the increase can be relative to any response with administering the immune modulator, but without administering any *Listeria*. As still another alternative, the increase can be relative to response without administering any *Listeria* and without administering the immune modulator.

[0378] In some embodiments, the invention provides methods of stimulating an immune response to an antigen in a mammal, comprising administering an effective amount of a *Listeria* bacterium described herein, or an effective amount of a composition comprising the *Listeria*, to the mammal. In some embodiments, the invention provides methods of stimulating an immune response to an antigen from, or derived from, a cancer or infectious agent, comprising administering an effective amount of a *Listeria* bacterium described herein, or an effective amount of a composition comprising the *Listeria*, to the mammal. In some embodiments, the heterologous antigen expressed by the *Listeria* shares at least one epitope with, or is immunologically cross-reactive with, the antigen from, or derived from, the cancer or infectious agent. In some embodiments, the immune response is a CD8+ T-cell immune response. In some embodiments, the immune response is a CD4+ T-cell immune response.

[0379] The invention provides a *Listeria* bacterium, or a *Listeria* strain, that is killed but metabolically active (KBMA) (see, e.g., Brockstedt, *et al* (2005) Nat. Med. 11:853-860). A KBMA *Listeria* bacterium is metabolically active, but cannot form a colony, e.g., on agar. An inactivating mutation in at least one DNA repair gene, e.g., Δ uvrAB, enables killing of *Listeria* using concentrations of a nucleic acid cross-linking agent (e.g., psoralen) at low concentrations, where these concentrations are sufficient to prevent colony formation but not sufficient to substantially impair metabolism, or to detectably impair metabolism. The result of limited treatment with psoralen/UVA light, and/or of treatment with a nucleic acid cross-linking agent that is highly specific for making interstrand genomic cross links, is that the bacterial cells are killed but remain metabolically active.

[0380] In some embodiments, the present invention results in the reduction of the number of abnormally proliferating cells, reduction in the number of cancer cells, reduction in the number of tumor cells, reduction in the tumor volume, reduction of the number of infectious organisms or pathogens per unit of biological fluid or tissue (e.g., serum), reduction in viral titer (e.g., serum), where it is normally reduced by at least 5%, more normally reduced by at least 10%, most normally reduced by at least 15%, typically reduced by at least 20%, more typically reduced by at least 25%, most typically reduced by at least 30%, usually reduced by at least 40%, more usually reduced by at least 50%, most usually reduced by at least 60%, conventionally reduced by at least 70%, more conventionally reduced by at least 80%, most conventionally reduced by at least 90%, and still most conventionally reduced by at least 99%. The unit of reduction can be, without limitation, number of tumor cells/mammalian subject; number of tumor cells/liver; number of tumor cells/spleen; mass of tumor cells/mammalian subject; mass of tumor cells/liver; mass of tumor cells/spleen; number of

viral particles or viruses or titer per gram of liver; number of viral particles or viruses or titer per cell; number of viral particles or viruses or titer per ml of blood; and the like.

[0381] The growth medium used to prepare a *Listeria* can be characterized by chemical analysis, high pressure liquid chromatography (HPLC), mass spectroscopy, gas chromatography, spectroscopic methods, and the like. The growth medium can also be characterized by way of antibodies specific for components of that medium, where the component occurs as a contaminant with the *Listeria*, e.g., a contaminant in the listerial powder, frozen preparation, or cell paste. Antibodies, specific for peptide or protein antigens, or glycolipid, glycopeptide, or lipopeptide antigens, can be used in ELISA assays formulated for detecting animal-origin contaminants. Antibodies for use in detecting antigens, or antigenic fragments, of animal origin are available (see, e.g., Fukuta, *et al.* (1977) *Jpn. Heart J.* 18:696-704; DeVay and Adler (1976) *Ann. Rev. Microbiol.* 30:147-168; Cunningham, *et al.* (1984) *Infection Immunity* 46:34-41; Kawakita, *et al.* (1979) *Jpn. Cir. J.* 43:452-457; Hanly, *et al.* (1994) *Lupus* 3:193-199; Huppi, *et al.* (1987) *Neurochem. Res.* 12:659-665; Quackenbush, *et al.* (1985) *Biochem. J.* 225:291-299). The invention supplies kits and diagnostic methods that facilitate testing the *Listeria*'s influence on the immune system. Testing can involve comparing one strain of *Listeria* with another strain of *Listeria*, or a parent *Listeria* strain with a mutated *Listeria* strain. Methods of testing comprise, e.g., phagocytosis, spreading, antigen presentation, T cell stimulation, cytokine response, host toxicity, LD₅₀, and efficacy in ameliorating a pathological condition.

[0382] The present invention provides methods to increase survival of a subject, host, patient, test subject, experimental subject, veterinary subject, and the like, to a cancer, a tumor, a precancerous disorder, an immune disorder, and/or an infectious agent. The infectious agent can be a virus, bacterium, or parasite, or any combination thereof. The method comprises administering an attenuated *Listeria*, for example, as a suspension, bolus, gel, matrix, injection, or infusion, and the like. The administered *Listeria* increases survival, as compared to an appropriate control (e.g., nothing administered or an administered placebo, and the like) by usually at least one day; more usually at least four days; most usually at least eight days, normally at least 12 days; more normally at least 16 days; most normally at least 20 days, often at least 24 days; more often at least 28 days; most often at least 32 days, conventionally at least 40 days, more conventionally at least 48 days; most conventionally at least 56 days; typically by at least 64 days; more typically by at least 72 days; most typically at least 80 days; generally at least six months; more generally at least eight months; most

generally at least ten months; commonly at least 12 months; more commonly at least 16 months; and most commonly at least 20 months, or more.

[0383] In some embodiments, the subject/host/patient to which the *Listeria* is administered is a mammal. In some embodiments, the mammal is a primate. In some embodiments, the primate is a human.

[0384] Each of the above disclosed methods contemplates administering a composition comprising a *Listeria* and an excipient, a *Listeria* and a carrier, a *Listeria* and buffer, a *Listeria* and a reagent, a *Listeria* and a pharmaceutically acceptable carrier, a *Listeria* and an agriculturally acceptable carrier, a *Listeria* and a veterinarily acceptable carrier, a *Listeria* and a stabilizer, a *Listeria* and a preservative, and the like.

[0385] The present invention provides reagents and methods for treating conditions that are both cancerous (neoplasms, malignancies, cancers, tumors, and/or precancerous disorders, dysplasias, and the like) and infectious (infections). Provided are reagents and methods for treating disorders that are both cancerous (neoplasms, malignancies, cancers, tumors, and/or precancerous disorders, dysplasias, and the like) and infectious. With infection with certain viruses, such as papillomavirus and polyoma virus, the result can be a cancerous condition, and here the condition is both cancerous and infectious. A condition that is both cancerous and infectious can be detected, as a non-limiting example, where a viral infection results in a cancerous cell, and where the cancerous cell expresses a viral-encoded antigen. As another non-limiting example, a condition that is both cancerous and infectious is one where immune response against a tumor cell involves specific recognition against a viral-encoded antigen (See, e.g., Montesano, *et al.* (1990) *Cell* 62:435-445; Ichaso and Dilworth (2001) *Oncogene* 20:7908-7916; Wilson, *et al.* (1999) *J. Immunol.* 162:3933-3941; Daemen, *et al.* (2004) *Antivir. Ther.* 9:733-742; Boudewijn, *et al.* (2004) *J. Natl. Cancer Inst.* 96:998-1006; Liu, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:14567-14571).

[0386] In some embodiments, the *Listeria* described herein that express a heterologous antigen (or compositions comprising the *Listeria*) are used to induce immune responses against cells in a subject that express the antigen. In some embodiments, the *Listeria*, vaccines, and other compositions described herein are used to treat cancerous disorders, precancerous disorders, tumors, infections, and/or angiogenesis of tumors and cancers. In some embodiments, the *Listeria*, vaccines, and other compositions described herein are used to treat cancer in a subject. In some embodiments, the *Listeria* (or compositions comprising the *Listeria*) described herein that express EphA2 antigenic peptides (or fusion proteins

comprising EphA2 antigenic peptides) are used to treat hyperproliferative disorders of EphA2-expressing cells.

[0387] The following embodiments relate to the individual embodiments disclosed herein.

[0388] The present invention, in certain embodiments, comprises a method of stimulating the immune system against an infectious disorder, where the infectious disorder is a *Listeria* infection. Also comprised, is a method of stimulating the immune system against an infectious disorder, where the infectious disorder is not a *Listeria* infection, that is, excludes *Listeria* infections.

[0389] Each of the embodiments encompasses, as an alternate or additional reagent, a *Listeria* that is not attenuated. Also, each of the embodiments encompasses, as an alternate or additional reagent, a *Listeria* that is attenuated. Each of the embodiments encompasses, as an alternate or additional method, using a *Listeria* that is not attenuated. Also, each of the embodiments encompasses, as an alternate or additional method, using a *Listeria* that is attenuated.

[0390] Each of the embodiments disclosed herein encompasses methods and reagents using a *Listeria* that comprises a nucleic acid encoding at least one tumor antigen, a *Listeria* that comprises a nucleic acid encoding at least one cancer antigen, a *Listeria* that comprises a nucleic acid encoding at least one heterologous antigen, as well as a *Listeria* that expresses at least one tumor antigen, cancer antigen, and/or heterologous antigen.

[0391] Each of the embodiments disclosed herein encompasses methods and reagents using a *Listeria* that does not comprise a nucleic acid encoding a tumor antigen, a *Listeria* that does not comprise a nucleic acid encoding a cancer antigen, a *Listeria* that does not comprise a nucleic acid encoding a heterologous antigen, as well as a *Listeria* that does not express a tumor antigen, cancer antigen, and/or a heterologous antigen.

[0392] Each of the embodiments disclosed herein encompasses methods and reagents using a *Listeria* that comprises a nucleic acid encoding an antigen from a non-listerial infectious organism. Each of the above-disclosed embodiments encompasses methods and reagents using a *Listeria* that comprises a nucleic acid encoding at least one antigen from a virus, parasite, bacterium, tumor, self-antigen derived from a tumor, or non-self antigen derived from a tumor.

[0393] Each of the embodiments disclosed herein encompasses methods and reagents using a *Listeria* that does not comprise a nucleic acid encoding an antigen from a non-listerial infectious organism. Each of the above-disclosed embodiments encompasses methods and reagents using a *Listeria* that does not comprise a nucleic acid encoding at least one antigen

from a virus, parasite, bacterium, tumor, self-antigen derived from a tumor, or non-self antigen derived from a tumor.

[0394] Each of the embodiments disclosed herein also encompasses a *Listeria* that is not prepared by growing on a medium based on animal protein, but is prepared by growing on a different type of medium. Each of the above-disclosed embodiments also encompasses a *Listeria* that is not prepared by growing on a medium containing peptides derived from animal protein, but is prepared by growing on a different type of medium. Moreover, each of the above-disclosed embodiments encompasses administration of a *Listeria* by a route that is not oral or that is not enteral. Additionally, each of the above-disclosed embodiments includes administration of a *Listeria* by a route that does not require movement from the gut lumen to the lymphatics or bloodstream.

[0395] Each of the embodiments disclosed herein further comprises a method wherein the *Listeria* are not injected directly into the tumor or are not directly injected into a site that is affected by the cancer, precancerous disorder, tumor, or infection.

[0396] Additionally, each of the embodiments disclosed herein encompasses administering the *Listeria* by direct injection into a tumor, by direct injection into a cancerous lesion, and/or by direct injection into a lesion of infection. Also, the invention includes each of the above embodiments, where administration is not by direct injection into a tumor, not by direct injection into a cancerous lesion, and/or not by direct injection into a lesion of infection.

[0397] Provided is a vaccine where the heterologous antigen, as in any of the embodiments disclosed herein, is a tumor antigen or is derived from a tumor antigen. Also provided is a vaccine where the heterologous antigen, as in any of the embodiments disclosed herein, is a cancer antigen, or is derived from a cancer antigen. Moreover, what is provided is a vaccine where the heterologous antigen, as in any of the embodiments disclosed herein, is an antigen of an infectious organism, or is derived from an antigen of an infectious organism, e.g., a virus, bacterium, or multi-cellular organism.

[0398] A further embodiment provides a nucleic acid where the heterologous antigen, as in any of the embodiments disclosed herein, is a tumor antigen or derived from a tumor antigen. Also provided is a nucleic acid where the heterologous antigen, as in any of the embodiments disclosed herein, is a cancer antigen, or is derived from a cancer antigen. Moreover, what is provided is a nucleic acid, where the heterologous antigen, as in any of the embodiments disclosed herein, is an antigen of an infectious organism, or is derived from an antigen of an infectious organism, e.g., a virus, bacterium, or multi-cellular organism.

[0399] In another embodiment, what is provided is a *Listeria* where the heterologous antigen, as in any of the embodiments disclosed herein, is a tumor antigen or derived from a tumor antigen. Also provided is a *Listeria* where the heterologous antigen, as in any of the examples disclosed herein, is a cancer antigen, or is derived from a cancer antigen.

Moreover, what is provided is a *Listeria*, where the heterologous antigen, as in any of the embodiments disclosed herein, is an antigen from an infectious organism or derived from an antigen of an infectious organism, e.g., a virus, bacterium, parasite, or multi-cellular organism.

[0400] Each of the above-disclosed embodiments also encompasses an attenuated *Listeria* that is not prepared by growing on a medium based on animal or meat protein, but is prepared by growing on a different type of medium. Provided is an attenuated *Listeria* not prepared by growing on a medium based on meat or animal protein, but is prepared by growing on a medium based on yeast and/or vegetable derived protein.

[0401] Unless specified otherwise, each of the embodiments disclosed herein encompasses a bacterium that does not contain a nucleic acid encoding a heterologous antigen. Also, unless specified otherwise, each of the embodiments disclosed herein encompasses a bacterium that does not contain a nucleic acid encoding a heterologous regulatory sequences. Optionally, every one of the embodiments disclosed herein encompasses a bacterium that contains a nucleic acid encoding a heterologous antigen and/or encoding a heterologous regulatory sequence.

[0402] The following concerns bacterial embodiments, e.g., of *Listeria*, *Bacillus anthracis*, or another bacterium, that encode secreted antigens, non-secreted antigens, secreted antigens that are releasable from the bacterium by a mechanism other than secretion, and non-secreted antigens that are releasable by a mechanism other than secretion. What is embraced is a bacterium containing a polynucleotide comprising a nucleic acid, where the nucleic acid encodes a polypeptide that contains a secretory sequence and is secreted under appropriate conditions; where the nucleic acid encodes a polypeptide that does not contain a secretory sequence; where the nucleic acid does contain a secretory sequence and where the polypeptide is releasable by some other mechanism such as enzymatic damage or perforation to the cell membrane or cell wall; and where the nucleic acid encodes a polypeptide that does not contain any secretory sequence but where the polypeptide is releasable by some other mechanism, such as enzymatic damage or perforation to the cell membrane and/or cell wall.

[0403] Without implying any limitation, as to narrowness or breadth, of the present invention, the invention can be modified by the skilled artisan to comprise any one of the following embodiments, or to consist of any one of the following embodiments (Table 10).

Table 10. Spread of the bacterium of the present invention, i.e., transmission of a bacterium from a first host cell to a second host cell.		
Without implying any limitation to the bacterium of the present invention, e.g., with regard to its ability to spread from cell to cell, the spread of the bacterium of the present invention can encompass one or more of the following. Without implying any lack of limitation to the bacterium of the present invention, e.g., with regard to its ability to spread from cell to cell, the spread of the bacterium of the present invention can encompass one or more of the following.		
The spread of the bacterium of the present invention can be	at most 1%; at most 5%; at most 10%; at most 20%; at most 30%; at most 40%; at most 50%; at most 60%; at most 70%; at most 80%; at most 90%; at most 95%; at most 100%; at most 200%; at most 300%; at most 400%; at most 500%,	as compared to the spread of a suitable control or parent bacterium.
The spread of the bacterium of the present invention can be	at least 1%; at least 5%; at least 10%; at least 20%; at least 30%; at least 40%; at least 50%; at least 60%; at least 70%; at least 80%; at least 90%; at least 95%; at least 100%; at least 200%; at least 300%; at least 400%; at least 500%,	as compared to the spread of a suitable control or parent bacterium.
The spread of the bacterium of the present invention can be	0 to 1%; 1% to 5%; 5% to 10%; 10% to 20%; 20% to 30%; 30% to 40%; 40% to 50%; 50% to 60%; 60% to 70%; 70% to 80%; 80% to 90%; 90% to 95%; 90% to 100%; 100% to 200%; 200% to 300%; 300% to 400%; 400% to 500%, or greater than 500%,	as compared to the spread of a suitable control or parent bacterium.

Table 10 (continued). Growth of the <i>Listeria</i> strain of the present invention. Without implying any limitation to the present invention, e.g., as to narrowness or to breadth, the present invention can encompass any one, or any of combination, of the following embodiments. Without implying any lack of limitation to the present invention, the present invention can encompass any one, or any combination, of the following embodiments.		
Growth of the <i>Listeria</i> strain of the present invention is at least	0.1%; 0.5%; 1.0%; 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; 95%; 99%; 99.5%; 99.5%, 2-fold; 5-fold; 10-fold; or greater than 10-fold,	as compared with the parent <i>Listeria</i> or with a suitable control <i>Listeria</i> .
Growth of the <i>Listeria</i> strain of the present invention is at most	not detectable, 0.1%; 0.5%; 1.0%; 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; 95%; 99%; 99.5%; 99.5%, 2-fold; 5-fold; 10-fold; or greater than 10-fold,	as compared with the parent <i>Listeria</i> or with a suitable control <i>Listeria</i> .
Growth of the <i>Listeria</i> strain of the present invention is less than	0.1%; 0.5%; 1.0%; 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; 95%; 99%; 99.5%; or 99.5%; 2-fold; 5-fold; 10-fold; or greater than 10-fold,	as compared with the parent <i>Listeria</i> or with a suitable control <i>Listeria</i> .
Growth of the <i>Listeria</i> strain of the present invention is more than	0.1%; 0.5%; 1.0%; 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; 95%; 99%; 99.5%; 99.5%, 2-fold; 5-fold; 10-fold; or greater than 10-fold,	as compared with the parent <i>Listeria</i> or with a suitable control <i>Listeria</i> .
Growth of the <i>Listeria</i> of the present invention is between	0-0.1%; 0.1-0.5%; 0.5-1.0%; 1.0-5%; 5-10%; 10-15%; 15-20%; 20-25%; 25-30%; 30-35%; 35-40%; 40-45%; 45-50%; 50-55%; 55-60%; 60-65%; 65-70%; 70-75%; 75-80%; 80-85%; 85-90%; 90-95%; 95-99%; 99-99.5%; 99.5-99.5%, 99.5%-greater, 100% to 2-fold; 2-fold to 10-fold; 10-fold to greater than 10-fold,	as compared with the parent <i>Listeria</i> or with a suitable control <i>Listeria</i> .

Table 10 (continued). Growth of the <i>Listeria</i> strain of the present invention.		
Extracellular growth of the <i>Listeria</i> strain of the present invention is at least	0.1%; 0.5%; 1.0%; 5%; 10%; 15%; 20%; 25%; 30%; 35%; 40%; 45%; 50%; 55%; 60%; 65%; 70%; 75%; 80%; 85%; 90%; 95%; 99%; 99.5%; or greater than 99.5%, 100%, 2-fold greater; 5-fold greater; or 10-fold greater,	as compared with intracellular growth of the same <i>Listeria</i> strain.
Extracellular growth of the <i>Listeria</i> strain of the present invention is	0-0.1%; 0.1-0.5%; 0.5-1.0%; 1.0-5%; 5-10%; 10-15%; 15-20%; 20-25%; 25-30%; 30-35%; 35-40%; 40-45%; 45-50%; 50-55%; 55-60%; 60-65%; 65-70%; 70-75%; 75-80%; 80-85%; 85-90%; 90-95%; 95-99%; 99-99.5%; 99.5-100%, 100-200%; 200-500%; 500-1000%; or greater than 1000%,	as compared with intracellular growth of the same <i>Listeria</i> strain.
Growth related genes. A growth related gene of the present invention can include, but is not necessarily limited in narrowness or in breadth, by the following.		
A growth related gene embraces one that stimulates the rate of intracellular growth by	the same amount, by at least 10% greater; by at least 20% greater; by at least 30% greater; by at least 40% greater; by at least 50% greater; by at least 60% greater; by at least 70% greater; by at least 80% greater; by at least 90% greater; by at least 100% (2-fold) greater; by at least 3-fold greater; by at least 4-fold greater; by at least 10-fold greater; by at least 20-fold greater; by at least 40-fold greater,	than the rate the gene stimulates extracellular growth.
Growth of a <i>Listeria</i> strain of the present invention can be compared with a parent, or suitable control, <i>Listeria</i> strain, where only intracellular growth is compared. Growth of a <i>Listeria</i> strain of the present invention can be compared with a parent, or suitable control, <i>Listeria</i> strain, where only extracellular growth is compared. Growth of a <i>Listeria</i> strain of the present invention can be compared with a parent, or suitable control, <i>Listeria</i> strain, where intracellular growth of the present invention strain is compared with extracellular growth of a parent or suitable control strain.		
Growth of a <i>Listeria</i> strain of the present invention can be compared with a parent, or suitable control, <i>Listeria</i> strain, where extracellular growth of the present invention strain is compared with intracellular growth of a parent or suitable control strain.		

Table 10 (continued). Metabolically active bacteria. Without implying any limitation to the present invention, e.g., as to narrowness or to breadth, the present invention can encompass any one, or any of combination, of the following embodiments. Without implying any lack of limitation to the present invention, e.g., as to narrowness or to breadth, the present invention can encompass any one, or any of combination, of the following embodiments.		
A metabolically active but colony formation impaired (and/or cell division or replication impaired) <i>Listeria</i> bacterium of the present invention encompasses a <i>Listeria</i> bacterium where the rate of colony formation, cell division, and/or replication is under 40% that of a parent or control <i>Listeria</i> bacterium,	and where metabolism is greater than 10-fold; 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%,	that of the control or parent <i>Listeria</i> bacterium.
A metabolically active but colony formation impaired (and/or cell division or replication impaired) <i>Listeria</i> bacterium of the present invention encompasses a <i>Listeria</i> bacterium where the rate of colony formation, cell division, and/or replication is under 30% that of a parent or control <i>Listeria</i> bacterium,	and where metabolism is greater than 10-fold; 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; or 40% to 30,	that of the control or parent <i>Listeria</i> bacterium.
A metabolically active but colony formation impaired (and/or cell division or replication impaired) <i>Listeria</i> bacterium of the present invention encompasses a <i>Listeria</i> bacterium where the rate of colony formation, cell division, and/or replication is under 20% that of a parent or control <i>Listeria</i> bacterium,	and where metabolism is greater than 10-fold; 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; 40% to 30; or 30 to 20%,	that of the control or parent <i>Listeria</i> bacterium.
A metabolically active but colony formation impaired (and/or cell division or replication impaired) <i>Listeria</i> bacterium of the present invention encompasses a <i>Listeria</i> bacterium where the rate of colony formation, cell division, and/or replication is under 10% that of a parent or control <i>Listeria</i> bacterium,	and where metabolism is greater than 10-fold; 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; 40% to 30; 30 to 20%; or 20 to 10%,	that of the control or parent <i>Listeria</i> bacterium.
A metabolically active but colony formation impaired (and/or cell division or replication impaired) <i>Listeria</i> bacterium of the present invention encompasses a <i>Listeria</i> bacterium where the rate of colony formation, cell division, and/or replication is under 5% that of a parent or control <i>Listeria</i> bacterium,	and where metabolism is greater than 10-fold; 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; 40% to 30; 30 to 20%; 20 to 10%; or 10 to 5%,	that of the control or parent <i>Listeria</i> bacterium.

Table 10 (continued). Metabolically active bacteria.		
A metabolically active but colony formation impaired (and/or cell division or replication impaired) <i>Listeria</i> bacterium of the present invention encompasses a <i>Listeria</i> bacterium where the rate of colony formation, cell division, and/or replication is under 1% that of a parent or control <i>Listeria</i> bacterium,	and where metabolism is greater than 10-fold; 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; 40% to 30; 30 to 20%; 20-10%; 10-5%; or 5% to 1%,	that of the control or parent <i>Listeria</i> bacterium.
A "killed but metabolically active" (KMBA) bacterium, is a <i>Listeria</i> bacterium that is unable to form colonies and where metabolism is, e.g., 10-fold to 5-fold (an indicator of metabolism occurring at a level higher than normally found); 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; 40% to 30; 30 to 20%; 20 to 10%; or 10 to 5%, that of a control or parent <i>Listeria</i> bacterium. In another aspect, a KBMA bacterium is a <i>Listeria</i> bacterium where the rate of colony formation is under 1% that of a control or parent <i>Listeria</i> bacterium, and where metabolism is, e.g., 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; 40% to 30; 30 to 20%; 20 to 10%; or 10 to 5%, that of the control or parent <i>Listeria</i> bacterium. In yet another aspect, a KBMA bacterium is a <i>Listeria</i> bacterium where the rate of colony formation is under 2% that of a control or parent <i>Listeria</i> bacterium, and where metabolism is, e.g., 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; 40% to 30; 30 to 20%; 20 to 10%; or 10 to 5%, that of the control or parent <i>Listeria</i> bacterium. In another embodiment, a KBMA bacterium is a <i>Listeria</i> bacterium where the rate of colony formation is under 5% that of a control or parent <i>Listeria</i> bacterium, and where metabolism is, e.g., 10-fold to 5-fold; 5-fold to 4-fold; 4-fold to 2-fold; 2-fold to 100%; essentially 100%; 100% to 95%; 95% to 90%; 90% to 80%; 80% to 70%; 70% to 60%; 60% to 50%; 50% to 40%; 40% to 30; 30 to 20%; 20 to 10%; or 10 to 5%, that of the control or parent <i>Listeria</i> bacterium.		

[0404] The rate of metabolism can be measured by various indicia, e.g., translation, respiration, secretion, transport, fermentation, glycolysis, amino acid metabolism, or the Krebs cycle. Various indicia of metabolism for *L. monocytogenes* are disclosed (see, e.g., Karlin, *et al.* (2004) Proc. Natl. Acad. Sci. USA 101:6182-6187; Gilbreth, *et al.* (2004) Curr. Microbiol. 49:95-98). Often, metabolism is assessed with intact bacteria by way of radioactive, heavy isotope, or fluorescent tagged metabolites. The skilled artisan can choose a suitable gene for measuring translation, or a suitable enzyme for measuring glycolysis, amino acid metabolism, or the Krebs cycle. A heat-killed bacterium generally is essentially or totally metabolically inactive. Residual apparent metabolic activity of an essentially or totally metabolically inactive bacterium can be due, e.g., to oxidation of lipids, oxidation of sulfhydryls, reactions catalyzed by heavy metals, or to enzymes that are stable to heat-treatment.

(c) Methods for assessing immune response; methods of diagnosis.

[0405] Reagents and methods useful for determining, assessing, monitoring, and/or diagnosing immune response are available. The present invention, in some situations, provides the following methods for diagnosing a mammalian subject administered with the compositions of the present invention. In other aspects, what is provided are the following methods for assessing immune response to one or more of the administered compositions of the present invention. These methods, which can be applied, e.g., *in vivo*, *in vitro*, *ex vivo*, *in utero*; to living or deceased mammals; to cells; to recombinant, chimeric, or hybrid cells; to biological fluids, to isolated nucleic acids, and the like, include:

- i. Methods for measuring cellular parameters. What can be measured includes effector T cells; central memory T cells (T_{CM}); effector memory T cells (T_{EM}), and constituents thereof. What can be measured are biological functions of these cells including cytotoxic function, expression of markers, affinity for antigen, number of cells in a biological compartment such as serum, preferred location in the body such as in lymph node or spleen, and rate of response when exposed or re-exposed to antigen.
- ii. Methods for measuring antibodies. What can be measured is affinity maturation of antibodies (see, e.g., McHeyzer-Williams and McHeyzer-Williams (2005) Ann. Rev. Immunol. 23:487-513), antibody titer or isotype, including IgG (IgG₁; IgG₂; IgG₃; IgG₄); IgA (IgA₁; IgA₂); IgM; IgD; IgE; isotype switching of antibodies, for example, decreases in IgM and increases in IgG (see, e.g., Hasbold, *et al.* (2004) Nature Immunol. 5:55-63; Ryffel, *et al.* (1997) J. Immunol. 158:2126-2133; Lund, *et al.* (2002) J. Immunol. 169:5236-5243;

Palladino, *et al.* (1995) *J. Virol.* 69:2075-2081; Karrer, *et al.* (2000) *J. Immunol.* 164:768-778); isotype switching that is a function of Th1-type or Th2-type response (Delale, *et al.* (2005) *J. Immunol.* 175:6723-6732; McKenzie, *et al.* (1999) *J. Exp. Med.* 189:1565-1572; Fayette, *et al.* (1997) *J. Exp. Med.* 185:1909-1918).

iii. Parameters of B cells. What can be measured includes naive B cells (high in membrane IgD and low in CD27), memory B cells (low in IgD and high in CD27), and constituents of these cells (see, e.g., Fecteau and Neron (2003) *J. Immunol.* 171:4621-4629). What can be measured is formation of memory B cells within germinal centers (see, e.g., Ohkubo, *et al.* (2005) *J. Immunol.* 174:7703-7710). What can be measured includes terminally differentiated B cells, for example, cell's ability to respond to CXCL12 (see, e.g., Roy, *et al.* (2002) *J. Immunol.* 169:1676-1682). What can be measured includes commitment antibody-secreting cells (ASCs) (see, e.g., Hasbold, *et al.* (2004) *Nature Immunol.* 5:55-63).

iv. Parameters of T cells. What can be measured is affinity of a peptide for T cell receptor, affinity maturation of T cell receptor (see, e.g., Rees, *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:9781-9786; McKinney, *et al.* (2004) *J. Immunol.* 173:1941-1950). What can be measured is affinity of a cytotoxic T cell for a target cell (see, e.g., Montoya and Del Val (1999) *J. Immunol.* 163:1914-1922). What can be measured includes markers, for example, effector memory T cells (T_{EM}) can be identified as $CD62L^{LOW}$ and $CCR7^{LOW}$, where these cells show immediate effector function with antigen re-encounter. Central memory T cells (T_{CM}) can be identified by relatively high expression of CD62L and CCR7, where the cells show a relatively slow activation kinetics. Other available markers include, e.g., CCL4, CCL5, XCL1, granulysin, granzyme A, granzyme B, and so on (see, e.g., Chtanova, *et al.* (2005) *J. Immunol.* 175:7837-7847; Kondrack, *et al.* (2003) *J. Exp. Med.* 198:1797-1806; Huster, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:5610-5615; Ahmadzadeh, *et al.* (2001) *J. Immunol.* 166:926-935; Goldrath, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:16885-16890; Wherry, *et al.* (2003) *Nature Immunol.* 4:225-234; Sallusto, *et al.* (2004) *Ann. Rev. Immunol.* 22:745-763). Different types of immune cells, as well as different stages of maturation of a particular cell, or different stages of activation of a cell, can be distinguished by titrating with a reagent specific to any given marker (see, e.g., Ahmadzah, *et al.* (2001) *J. Immunol.* 166:926-935).

v. Parameters of antigen presenting cells (APCs), including dendritic cells (DCs). What can be measured is mmoles of peptide presented (or bound) per mmole MHC Class I. Moreover, what can be measured is mmoles peptide presented or bound per mmol of MHC Class II. Also, what can be measured is the amino acid sequence of the bound

peptides (see, e.g., Velazquez, *et al.* (2001) *J. Immunol.* 166:5488-5494). In addition, what can be measured is relative ability of the APC to present epitopes derived from peptides versus epitopes derived from proteins, as well as ability to present epitopes acquired from low levels of peptides versus high levels of peptides and, in other aspects, the identity of the APC suitable for presentation (see, e.g., Constant, *et al.* (1995) *J. Immunol.* 154:4915-4923).

[0406] A number of specific examples of generally applicable methods for assessing expression, secretion, presentation, immunogenicity, and/or therapeutic efficacy of candidate expression constructs and candidate *Listeria*-based vaccines are exemplified in the Examples below. See, e.g., Example IX, below. Assays for assessing immunogenicity of a particular candidate *Listeria*-based vaccine are further provided, e.g., in U.S. Patent Publication Nos. 2005/0249748, 2005/0281783, and 2004/0228877, each of which is incorporated by reference herein in its entirety.

[0407] Guidance is available for the skilled artisan in designing diagnostic appropriate controls (see, e.g., Wilson (1991) *An Introduction to Scientific Research*, Dover Publications, Mineola, NY).

[0408] The broad scope of this invention is best understood with reference to the following examples, which are not intended to limit the invention to any specific embodiments.

EXAMPLES

I. General methods.

[0409] Standard methods of biochemistry and molecular biology are described (see, e.g., Maniatis, *et al.* (1982) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY; Sambrook and Russell (2001) *Molecular Cloning*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Wu (1993) *Recombinant DNA*, Vol. 217, Academic Press, San Diego, CA; Innis, *et al.* (eds.) (1990) *PCR Protocols: A Guide to Methods and Applications*, Academic Press, N.Y. Standard methods are also found in Ausbel, *et al.* (2001) *Curr. Protocols in Mol. Biol.*, Vols. 1-4, John Wiley and Sons, Inc. New York, NY, which describes cloning in bacterial cells and DNA mutagenesis (Vol. 1), cloning in mammalian cells and yeast (Vol. 2), glycoconjugates and protein expression (Vol. 3), and bioinformatics (Vol. 4)). Methods for producing fusion proteins are described (see, e.g., Invitrogen (2005) *Catalogue*, Carlsbad, CA; Amersham Pharmacia Biotech. (2005) *Catalogue*, Piscataway, NJ; Liu, *et al.* (2001) *Curr. Protein Pept. Sci.* 2:107-121; Graddis, *et al.* (2002) *Curr. Pharm. Biotechnol.* 3:285-297).

[0410] Splice overlap extension PCR, and other methods, for creating mutations, restriction sites, loxP sites, and the like, are described (see, e.g., Horton, *et al.* (1990) *Biotechniques* 8:528-535; Horton, *et al.* (1989) *Gene* 77:61-68; Horton (1995) *Mol Biotechnol.* 3:93-99; Cutrone and Langer (2001) *J. Biol. Chem.* 276:17140-17148; Cox, *et al.* (2002) *Nucleic Acids Res.* 30:e108; Warrens, *et al.* (1997) *Gene* 186:29-35; Guo and Bi (2002) *Methods Mol. Biol.* 192:111-119; Johnson (2000) *J. Microbiol. Methods* 41:201-209; Lantz, *et al.* (2000) *Biotechnol. Annu. Rev.* 5:87-130; Gustin and Burk (2000) *Methods Mol. Biol.* 130:85-90; QuikChange® Mutagenesis Kit, Stratagene, La Jolla, CA). Engineering codon preferences of signal peptides, secretory proteins, and heterologous antigens, to fit the optimal codons of a host are described (Sharp, *et al.* (1987) *Nucl. Acids Res.* 15:1281-1295; Uchijima, *et al.* (1998) *J. Immunol.* 161:5594-5599). Engineering codon preferences of signal peptides, secretory proteins, and heterologous antigens, to fit the optimal codons of a host are described (Sharp, *et al.* (1987) *Nucl. Acids Res.* 15:1281-1295; Uchijima, *et al.* (1998) *J. Immunol.* 161:5594-5599). Polynucleotides and nucleic acids are available, e.g., from Blue Heron Biotechnology, Bothell, WA).

[0411] Methods for effecting homologous recombination in, e.g., bacteria, phages, and plasmids, are available (see, e.g., Kuzminov (1999) *Microb. Mol. Biol. Rev.* 63:751-813; Camerini-Otero and Hsieh (1995) *Annu. Rev. Genet.* 29:509-552; Amundsen and Smith (2003) *Cell* 112:741-744; Cox (2001) *Annu. Rev. Genet.* 35:53-82; Quiberoni, *et al.* (2001) *Res. Microbiol.* 152:131-139; Fernandez, *et al.* (2000) *Res. Microbiol.* 151:481-486; Wedland (2003) *Curr. Genet.* 44:115-123; Muttucumaru and Parish (2004) *Curr. Issues Mol. Biol.* 6:145-157; Bhattacharyya, *et al.* (2004) *Infect. Genet. Evol.* 4:91-98).

[0412] A number of transducing listeriophages, as well as techniques for infecting *L. monocytogenes* with listeriophages are available. These listeriophages include, e.g., P35, U153, and derivatives thereof (see, e.g., Lauer, *et al.* (2002) *J. Bact.* 184:4177-4186; Hodgson (2000) *Mol. Microbiol.* 35:312-323; Mee-Marquet, *et al.* (1997) *Appl. Environ. Microbiol.* 63:3374-3377; Zink and Loessner (1992) *Appl. Environ. Microbiol.* 58:296-302; Loessner, *et al.* (1994) *Interviol.* 37:31-35; Loessner, *et al.* (1994) *J. Gen. Virol.* 75:701-710; Loessner, *et al.* (2000) *Mol. Microbiol.* 35:324-340).

[0413] Methods for using electroporation and *E.coli*-mediated conjugation for introducing nucleic acids into *Listeria* are described. Plasmids suitable for introducing a nucleic acid into a bacterium include, e.g., pPL1 (GenBank accession no:AJ417488), pPL2 (Acc. No. AJ417449), pLUCH80, pLUCH88, and derivatives thereof (see, e.g., Lauer, *et al.* (2002) *J. Bact.* 184:4177-4186; Wilson, *et al.* (2001) *Infect. Immunity* 69:5016-5024; Chesneau, *et al.*

(1999) FEMS Microbiol. Lett. 177:93-100; Park and Stewart (1990) Gene 94:129-132; Luchansky, *et al.* (1988) Mol. Microbiol. 2:537-646; He and Luchansky (1997) Appl. Environ. Microbiol. 63:3480-3487).

[0414] Methods for protein purification such as immunoprecipitation, column chromatography, electrophoresis, isoelectric focusing, centrifugation, and crystallization, are described (Coligan, *et al.* (2000) Current Protocols in Protein Science, Vol. 1, John Wiley and Sons, Inc., New York). Chemical analysis, chemical modification, post-translational modification, and glycosylation of proteins is described. See, e.g., Coligan, *et al.* (2000) Current Protocols in Protein Science, Vol. 2, John Wiley and Sons, Inc., New York; Walker (ed.) (2002) Protein Protocols Handbook, Humana Press, Towota, NJ; Lundblad (1995) Techniques in Protein Modification, CRC Press, Boca Raton, FL. Techniques for characterizing binding interactions are described (Coligan, *et al.* (2001) Current Protocols in Immunology, Vol. 4, John Wiley and Sons, Inc., New York; Parker, *et al.* (2000) J. Biomol. Screen. 5: 77-88; Karlsson, *et al.* (1991) J. Immunol. Methods 145:229-240; Neri, *et al.* (1997) Nat. Biotechnol. 15:1271-1275; Jonsson, *et al.* (1991) Biotechniques 11:620-627; Friguet, *et al.* (1985) J. Immunol. Methods 77: 305-319; Hubble (1997) Immunol. Today 18:305-306; Shen, *et al.* (2001) J. Biol. Chem. 276:47311-47319).

[0415] Software packages for determining, e.g., antigenic fragments, leader sequences, protein folding, functional domains, glycosylation sites, and sequence alignments, are available (see, e.g., Vector NTI® Suite (Informax, Inc, Bethesda, MD); GCG Wisconsin Package (Accelrys, Inc., San Diego, CA); DeCypher® (TimeLogic Corp., Crystal Bay, Nevada); Menne, *et al.* (2000) Bioinformatics 16: 741-742; Menne, *et al.* (2000) Bioinformatics Applications Note 16:741-742; Wren, *et al.* (2002) Comput. Methods Programs Biomed. 68:177-181; von Heijne (1983) Eur. J. Biochem. 133:17-21; von Heijne (1986) Nucleic Acids Res. 14:4683-4690). Methods for determining coding sequences (CDS) are available (Furono, *et al.* (2003) Genome Res. 13:1478-1487).

[0416] Computer algorithms (e.g., BIMAS; SYFPEITHI) for identifying peptides that bind to MHC Class I and/or MHC Class II are available (Thomas, *et al.* (2004) J. Exp. Med. 200:297-306). These algorithms can provide nucleic acids of the present invention that encode proteins comprising the identified peptides.

[0417] Sequences of listerial proteins and nucleic acids can be found on the world wide web at: (1) ncbi.nlm.nih.gov; (2) genolist.pasteur.fr (with clicking on "listilist"); and (3) tigr.org (with clicking on "databases," then on "comprehensive microbial resource").

- [0418] Methods are available for assessing internalization of a *Listeria* by an APC, and for assessing presentation of listerial-encoded antigens by the APC. Methods are also available for presentation of these antigens to T cell, and for assessing antigen-dependent priming of the T cell. A suitable APC is murine DC 2.4 cell line, while suitable T cell is the B3Z T cell hybridoma (see, e.g., U.S. Provisional Pat. Appl. Ser. No. 60/490,089 filed July 24, 2003; Shen, *et al.* (1997) *J. Immunol.* 158:2723-2730; Kawamura, *et al.* (2002) *J. Immunol.* 168:5709-5715; Geginat, *et al.* (2001) *J. Immunol.* 166:1877-1884; Skoberne, *et al.* (2001) *J. Immunol.* 167:2209-2218; Wang, *et al.* (1998) *J. Immunol.* 160:1091-1097; Bullock, *et al.* (2000) *J. Immunol.* 164:2354-2361; Lippolis, *et al.* (2002) *J. Immunol.* 169:5089-5097). Methods for preparing dendritic cells (DCs), *ex vivo* modification of the DCs, and administration of the modified DCs, e.g., for the treatment of a cancer, pathogen, or infective agent, are available (see, e.g., Ribas, *et al.* (2004) *J. Immunother.* 27:354-367; Gilboa and Vieweg (2004) *Immunol. Rev.* 199:251-263; Dees, *et al.* (2004) *Cancer Immunol. Immunother.* 53:777-785; Eriksson, *et al.* (2004) *Eur. J. Immunol.* 34:1272-1281; Goldszmid, *et al.* (2003) *J. Immunol.* 171:5940-5947; Coughlin and Vonderheide (2003) *Cancer Biol. Ther.* 2:466-470; Colino and Snapper (2003) *Microbes Infect.* 5:311-319).
- [0419] Assays for *Listeria* plaque size, LD₅₀, and motility are described. Plaque diameter is a function of a bacterium's ability to grow, to move from cell to cell, and to escape from a secondary vesicle formed in an adjacent cell (see, e.g., Lauer, *et al.* (2001) *Mol. Microbiol.* 42:1163-1177; Theriot, *et al.* (1994) *Cell* 76:505-517; Theriot, *et al.* (1998) *Meth. Enzymol.* 298:114-122; Portnoy, *et al.* (1988) *J. Exp. Med.* 167:1459-1471).
- [0420] Elispot assays and intracellular cytokine staining (ICS) for characterizing immune cells are available (see, e.g., Lalvani, *et al.* (1997) *J. Exp. Med.* 186:859-865; Waldrop, *et al.* (1997) *J. Clin. Invest.* 99:1739-1750; Hudgens, *et al.* (2004) *J. Immunol. Methods* 288:19-34; Goulder, *et al.* (2001) *J. Virol.* 75:1339-1347; Goulder, *et al.* (2000) *J. Exp. Med.* 192:1819-1831; Anthony and Lehman (2003) *Methods* 29:260-269; Badovinac and Harty (2000) *J. Immunol. Methods* 238:107-117). The "tetramer staining" method is also available (see, e.g., Serbina and Pamer (2003) *Curr. Opin. Immunol.* 15:436-442; Skinner and Haase (2002) *J. Immunol. Methods* 268:29-34; Pittet, *et al.* (2001) *Int. Immunopharmacol.* 1:1235-1237).
- [0421] Methods are available for determining if an antigen or epitope is presented via direct presentation or by cross-presentation. These methods include use of TAP-deficient mice with administration of cells (from another source) that contain an antigen of interest. Another method involves preparing a mouse genetically deficient in an MHC Class I or Class II molecule that is required for presenting a specific epitope, e.g., MHC Class I H-2^b, and

administering H-2^b-expressing antigen presenting cells (APCs) (from another source) that contain the antigen of interest (or that were pulsed with an epitope of interest) (see, e.g., van Mierlo, *et al.* (2004) *J. Immunol.* 173:6753-6759; Pozzi, *et al.* (2005) *J. Immunol.* 175:2071-2081).

[0422] Methods for determining binding affinities, binding specificities, and affinity maturation are available. The present invention provides methods for stimulating and/or diagnosing affinity maturation, as it applies to, e.g., maturation of antibodies and/or of T cells (see, e.g., Chen, *et al.* (2004) *J. Immunol.* 173:5021-5027; Rees, *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:9781-9786; Busch and Pamer (1999) *J. Exp. Med.* 189:701-709; Ploss, *et al.* (2005) *J. Immunol.* 175:5998-6005; Brams, *et al.* (1998) *J. Immunol.* 160:2051-2058; Choi, *et al.* (2003) *J. Immunol.* 171:5116-5123).

[0423] Methods for using animals in the study of cancer, metastasis, and angiogenesis, and for using animal tumor data for extrapolating human treatments are available (see, e.g., Hirst and Balmain (2004) *Eur J Cancer* 40:1974-1980; Griswold, *et al.* (1991) *Cancer Metastasis Rev.* 10:255-261; Hoffman (1999) *Invest. New Drugs* 17:343-359; Boone, *et al.* (1990) *Cancer Res.* 50:2-9; Moulder, *et al.* (1988) *Int. J. Radiat. Oncol. Biol. Phys.* 14:913-927; Tuveson and Jacks (2002) *Curr. Opin. Genet. Dev.* 12:105-110; Jackson-Grusby (2002) *Oncogene* 21:5504-5514; Teicher, B.A. (2001) *Tumor Models in Cancer Research*, Humana Press, Totowa, NJ; Hasan, *et al.* (2004) *Angiogenesis* 7:1-16; Radovanovic, *et al.* (2004) *Cancer Treat. Res.* 117:97-114; Khanna and Hunter (2004) *Carcinogenesis* Sept.9 [epub ahead of print]; Crnic and Christofori (2004) *Int. J. Dev. Biol.* 48:573-581).

[0424] Colorectal cancer hepatic metastases can be generated using primary hepatic injection, portal vein injection, or whole spleen injection of tumor cells (see, e.g., Suh, *et al.* (1999) *J. Surgical Oncology* 72:218-224; Dent and Finley-Jones (1985) *Br. J. Cancer* 51:533-541; Young, *et al.* (1986) *J. Natl. Cancer Inst.* 76:745-750; Watson, *et al.* (1991) *J. Leukoc. Biol.* 49:126-138).

EXAMPLE II. Vectors for use in mediating site-specific recombination and homologous recombination.

[0425] The *Listeria monocytogenes* strains used in the present work are described (see, Brockstedt, *et al.* (2004) *Proc. Natl. Acad. Sci. USA* 101:13832-13837). *L. monocytogenes* ΔActAΔinlB (also known as DP-L4029inlB) was deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, United States

of America, on October 3, 2003, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and designated with accession number PTA-5562. *L. monocytogenes* Δ ActA Δ uvrAB (also known as DP-L4029uvrAB) was deposited with the ATCC, on October 3, 2003, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and designated with accession number PTA-5563. Yeast medium without glucose contained 25 grams/L yeast extract (Bacto® yeast extract) (BD Biosciences, Sparks, MD); 9 grams/L potassium phosphate monobasic, pH 7.2.

[0426] Homologous recombination can be mediated by pKSV7 (SEQ ID NO:3) (see also, Smith and Youngman (1992) *Biochimie* 74:705-711; Camilli, *et al.* (1993) *Mol. Microbiol.* 8:143-157; Camilli (1992) *Genetic analysis of Listeria monocytogenes Determinants of Pathogenesis*, Univ. of Pennsylvania, Doctoral thesis).

(SEQ ID NO:28, pKSV7)

CTCGCGGATTGTTGATGATTACGAAAATATTAAGAGCACAGACTATTACACAGA
 AAATCAAGAATTAACAAAAACGTAGAGAGAGTTTGAAAGAAGTAGTGAATACATG
 GAAAGAGGGGTATCACGAAAAAAGTAAAGAGGTTAATAAATTAAGCGAGAGA
 ATGATAGTTTGAAATGAGCAGTTGAATGTATCAGAGAAATTTCAAGATAGTACAGT
 GACTTTATATCGTGCTGCGAGGGCGAATTTCCCTGGGTTTGAGAAAGGGTTTAAT
 AGGCTTAAAGAGAAATTCTTTAATGATTCCAAATTCGAGCGTGTGGGACAGTTTA
 TGGATGTTGTACAGGATAATGTCCAGAAGGTCGATAGAAAGCGTGAGAAACAGC
 GTACAGACGATTTAGAGATGTAGAGGTACTTTTATGCCGAGAAAACCTTTTTGCGT
 GTGACAGTCCTTAAATATACTTAGAGCGTAAGCGAAAGTAGTAGCGACAGCTA
 TTAACTTTCGGTTGCAAAGCTCTAGGATTTTAAATGGACGCAGCGCATCACACGC
 AAAAAGGAAATTGGAATAAATGCGAAATTTGAGATGTTAATTAAAGACCTTTTT
 GAGGTCTTTTTTTCTTAGATTTTTTGGGGTTATTTAGGGGAGAAAACATAGGGGGG
 TACTACGACCTCCCCCCTAGGTGTCCATTGTCCATTGTCCAAACAAATAAATAAA
 TATTGGGTTTTTAATGTTAAAAGGTTGTTTTTATGTTAAAGTGAAAAAACAGA
 TGTGAGGAGGTACAGTGATGGTTGTAGATAGAAAAGAAGAGAAAAAAGTTGCTG
 TTAACTTAAGACTTACACAGAAGAAAATGAGATATTAATAGAAATCCAAGAAAA
 ATATAATATTAGCAAATCAGATGCACCGGTATTCTAATAAAAAAATATGYRMAGG
 AGGAATACSGTGCAATTTTAACAAAAAAGATAGACAGCACTGGCATGCTGCCTA

TCTATGACTAAATTTTGTAAATGTATTAGCACCGTTATTATATCATGAGCGAAA
ATGTAATAAAAGAAACTGAAAACAAGAAAAATTCAAGAGGACGTAATTGGACAT
TTGTTTTATATCCAGAATCAGCAAAAGCCGAGTGGTTAGAGTATTTAAAAGAGTT
ACACATTCAATTTGTAGTGTCTCCATTACATGATAGGGATACTGATACAGAAGAT
AGGATGAAAAAAGAGCATTATCATATTCTAGTGATGTATGAGGGTAATAAATCTT
ATGAACAGATAAAAATAATTACAGAAGAATTGAATGCGACTATTCCGCAGATTG
CAGGAAGTGTGAAAGGTCTTGTGAGATATATGCTTCACATGGACGATCCTAATAA
ATTTAAATATCAAAAAGAAGATATGATAGTTTATGGCGGTGTAGATGTTGATGAA
TTATTAAGAAAAACAACAGATAGATATAAATTAATTAAGAAATGATTGAG
TTTATTGATGAACAAGGAATCGTAGAATTTAAGAGTTTAATGGATTATGCAATGA
AGTTTAAATTTGATGATTGGTTCCCGCTTTTATGTGATAACTCGGCGTATGTTATT
CAAGAATATATAAAATCAAATCGGTATAAATCTGACCGATAGATTTTGAATTTAA
GAGTGTCACAAGACACTCTTTTTTCGCACCAACGAAAACCTGGTTTAAGCCGACTG
CGCAAAAGACATAATCGATTACAAAAAATAGGCACACGAAAAACAAGTTAAG
GGATGCAGTTTATGCATCCCTTANCTTACTTATTAATAATTTATAGCTATTGAAA
AGAGATAAGAATTGTTCAAGCTAATATTGTTTAAATCGTCCATTCCTGCATGTTTT
ANGGAAWTGTTAANTTGATTTTTTGTAAATTTTTCTKGTATYCTTTGTTAMCCCA
TTTCATAACGAAATAATTATACTTTTGTITATCTTTGTGTGATATTCTTGATTTTTT
TCTACTTAATCTGATAAGTGAGCTATTCACTTTAGGTTTAGGATGAAAATATTCTC
TTGGAACCATACTTAATATAGAAATATCAACTTCTGCCATTAAAAGTAATGCCAA
TGAGCGTTTTGTATTTAATAATCTTTTAGCAAACCCGTATTCCACGATTAAATAAA
TCTCATTAGCTATACTATCAAAAACAATTTTGC GTATTATATCCGTACTTATGTTA
TAAGGTATATTACCATATATTTTATAGGATTGGTTTTTAGGAAATTTAACTGCA
ATATATCCTTGTTTAAAACCTTGGAATTATCGTGATCTTCCTTCAGGTTATGACCA
TCTGTGCCAGTTCGTAATGTCTGGTCAACTTTCCGACTCTGAGAACTTCTGGAAT
CGCTAGAGAATTTCTGGAATGGGATTCAGGAGTGGACAGAACGACACGGATATA
TAGTGGATGTGTCAAAACGCATACCATTTTGAACGATGACCTCTAATAATTGTTA
ATCATGTTGGTTACGTATTTATTAACCTTCTCCTAGTATTAGTAATTATCATGGCTG
TCATGGCGCATTAAACGGAATAAAGGGTGTGCTTAAATCGGGCCATTTTGCGTAAT
AAGAAAAAGGATTAATTATGAGCGAATTGAATTAATAATAAGGTAATAGATTTA
CATTAGAAAATGAAAGGGGATTTTATGCGTGAGAATGTTACAGTCTATCCCGGCA
ATAGTTACCTTATTATYWSGATAAGAANGAAAGGATTTTTCGCTACGCTCAATC
CTTTAAAAAAACACAAAAGACCACATTTTTTAATGTGGTCTTTTATTCTTCAACTA
AAGCACCCATTAGTTCAACAAACGAAAATTGGATAARGTGGGATATTTTWAAWA

TAATWTATKTATGTTACAGTAATATTGACTTTTAAAAAAGGATTGATTCTAATGA
AGAAAGCAGACAAGTAAGCCTCCTAAATTCACTTTAGATAAAAAATTTAGGAGGC
ATATCAAATGAACTTTAATAAAATTGATTTAGACAATTGGAAGAGAAAAGAGAT
ATTTAATCATTATTTGAACCAACAAACGACTTTTAGTATAACCACAGAAATTGAT
ATTAGTGTTTTATACCGAAACATAAAACAAGAAGGATATAAATTTTACCCTGCAT
TTATTTTCTTAGTGACAAGGGTGATAAACTCAAATACAGCTTTTAGAACTGGTTA
CAATAGCGACGGAGAGTTAGGTTATTGGGATAAGTTAGAGCCACTTTATACAATT
TTTGATGGTGTATCTAAAACATTCTCTGGTATTTGGACTCCTGTAAAGAATGACTT
CAAAGAGTTTTATGATTTATACCTTTCTGATGTAGAGAAATATAATGGTTCGGGG
AAATTGTTTCCCAAAACACCTATACCTGAAAATGCTTTTTCTCTTTCTATTATTCC
ATGGACTTCATTTACTGGGTTTAACTTAAATATCAATAATAATAGTAATTACCTTC
TACCATTATTACNGCAGGAAANTTCATTAATAANGGTAATTCAATATATTTACC
GCTATCTTTACAGGTACATCATTCTGTTTGTGATGGTTATCATGCNGGATTGTTTA
TGAAGTCTATTCAGGAATTGTCAGATAGGCCTAATGACTGGCTTTTATATATGAG
ATAATGCCGACTGTACTTTTTACRGTCGGTTTTCTAACGATMCATTAATAGGTMC
GAAAAAGCMACTTTTTTKSCGCTTAAAACCAGTCATACCAATAACTTAAGGGTAA
CTAGCCTCGCCGAAAGAGCGAAAATGCCTCACATTTGTGCCACCTAAAAAGGA
GCGATTTACATATGAGTTATGCAGTTTGTAGAATGCAAAAAGTGAAATCAGCTGC
ATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTC
CGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTTCGGCTGCGGCGAGCGGTA
TCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCA
GGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGC
CGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAAT
CGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCG
TTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGG
ATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCT
GTAGGTATCTCAGTTCGGTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCACGA
ACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCC
AACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATT
AGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTRSSY
ACKSSKMYCCTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTAC
CTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAAMAAACCACCGCTGGTAG
CGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAA
GAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGAACGAAAACCTCAC

GTAAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTT
AAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCT
GACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTT
GTTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGG
CTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCT
CCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGT
CCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAG
TAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCAT
CGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTACAGCTCCGGTTCCCAACGAT
CAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGG
TCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATG
GCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGAC
TGGTGRKKASTCWCMCMAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTG
CTCTTGCCCNNGSGTCAATACGGGATAATACCGCSCCACAATAGCARAACTTTAAA
AGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCG
CTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCAT
CTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC
AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTT
CAATATTATTGAAGCATTATCAGGGTTATTGTCTCATGAGCGGATACATATTG
AATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAG
GCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTC
TGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGG
AGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGG
CTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACMATATGCGGTGT
GAAATACCGCACAGATGCGTAAGGAGAAAATAACCGCATCAGGCGCCATTGCGCA
TTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTAC
GCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAG
GGTTTTYCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTTGTCATGCCT
GCAGGTCGACTCTAGAGGATCCCCNNGGTACCGAGCTCGAATTCGTAATCATGTC
ATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCACACAACATACGA
GCCGGAAGCATAAAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACA
TTAATTGCGTTGCGCTCACTGCCCGCTTTCAGTCGGGAAACCTGTCGTGCCAGC
TGGACTAAAAGGCATGCAATTCATAATCAAAGAGAGCGAAAAAGTAGAACGAA

TGATGATATTGACCATGAGCGAACACGTGAAAATTATGATTTGAAAAATGATAA
 AAATATTGATTACAACGAACGTGTCAAAGAAATTATTGAATCACAAAAACAGG
 TACAAGAAAAACGAGGAAAGATGCTGTTCTTGTAATGAGTTGCTAGTAACATC
 TGACCGAGATTTTTTTTGAGCAACTGGATCAGTACAAGAAAGATACTGTATTTTCAT
 AAACAGGAACTGCAAGAAGTTAAGGATGAGTTACAGAAGGCAAATAAGCAGTT
 ACAGAGTGGAATAGAGCATATGAGGTCTACGAAACCCTTTGATTATGAAAATGA
 GCGTACAGGTTTGTCTCTGGACGTGAAGAGACTGGTAGAAAGATATTAAGTCTGCT
 GATGAATTTGAACGCCTGCAAGAAACAATCTCTTCGAACGGATTGTTGATGATTA
 CGAAATATAAGAGCCCGACTATTCCCAGAAATCAGAATTAACAAACGTTAGAGAGA
 G (SEQ ID NO:28, pKSV7).

[0427] Site-specific integration can be mediated by pPL1, pPL2, pINT, or variants thereof (see, e.g., Lauer, *et al.* (2002) J. Bacteriol. 184:4177-4186; Int. Appl. No. PCT/US03/13492 (Int. Publ. No. WO 03/092600) of Portnoy, Calendar, and Lauer).

[0428] The pINT plasmid has loxP sites that allow the specific removal of most of the plasmid from the listerial chromosome, leaving behind the attP and MCS (multiple cloning site), and the contents of the multi-cloning site (MCS) (e.g., an antigen cassette). pINT can work differently from pPL2 as follows. Up to a 100 microliters aliquot of a 10:1 dilution of a pPL2 conjugation can be plated on double selection plates. Plating up to a 100 microliters aliquot of a 10:1 dilution of a pPL2 conjugation generally results in 50-100 colonies. Plating more than 100 microliters of a 10:1 dilution of pPL2 conjugation gives little or no colonies due to a background growth from the *E. coli* donor. pINT, on the other hand, can be plated without diluting and even concentrating the conjugation mix because erythromycin (Erm) is more selective than chloramphenicol against *E. coli*. The use of pINT broadens the dynamic range for successful integration by approximately 2 logs.

pINT vector.

AGATCTCCAAAAATAAACAGGTGGTGGTATTAATGAAGATAAAAAAATTAGCA
 AACGGTAAATATTGTGTTTCGCCTACGTATAAAAGTCGATGGTGAATGGAAAGA
 AAAGCGTTTGACAGATACAAGTGAAACAACTTAATGTATAAAGCATCTAAAT
 TATTAAACAAGTTCAGCATGATAGTAGTTCTCTGAAAGAATGGAAGTTCAAA
 GAATTTTATACGCTATTCATGAAAACATTTAAAGATGGGAAAAGTAGTCAATC
 TACTATTAATTTATACGATCTTGCTTATAATCAATTCGTTGATTATTTTCGATG
 AAAAAATTAAATTTAATTCGATTGATGCGGTTCAATATCAACAATTTATTAAT
 CATTTATCTGTAGACTATGCAATATCCACTGTAGACACCAGACACCGCAAAAT
 TAGAGCGATTTTAAACAAGGCTGTTTATTTAGGTTACATGAAGAAAAACCCCA
 CTATAGGGGCTCATATAAGCGGACAGGACGTAGCGAAAAATAAGCACAAATTT
 ATGGAAACAGACAAAGTTCATTTACTATTAGAAGAACTTGCAAAATTTTCATTC

TATATCACGAGCAGTTATCTTTCTAGCTGTCCAGACAGGCATGAGGTTCTGAAG
AAATTATTGCACTAACAAAGAAGGATATTAATTTCACTAAACGTTCAATAACT
GTGAATAAAGCTTGGGATTACAAGTACACTAATACATTTCATTGATACCAAAC
AAAAAATCACGAGTGATCTATATTGATAACTCTACCGCTCAATATTTACATT
CGTATTTAAATTGGCATACTGAATATATGAAGGAACATGCTATTAAGAATCCA
TTGATGTTATTATTTCATCACTTACCACAATAAGCCAGTAGACAACGCGTCTTG
TAATAAAGCTTTGAAGAAGATATGTAGTACAATCAATTCTGAACCAGTGACAT
TACACAAGCTACGACATACGCATACAGGCTTATGTGTAGAAGCGGGTATGGAT
ATTATTTATGTAGCTGATAGGCTTGGTCATGATGACATTAATACAACATTAAA
ATACTATAGTCATCTAAGCTCTAATTTAAGACAACATAATCAGTCCAAAGTAG
ATGCTTTTTTTCACACTAAAAACAGATGAAAATACCACAAATTTTACCACAAAT
GCCACAAAACAACGGAATAACCTAGGATAACTTCGTATAATGTATGCTATAC
GAAGTTATATGCATGGGTATTATACGATATAAAAAAACTCCAAAACATTCAT
CCGCCCTTTAATATCAAGGCTTTTCAACGTTTGTAGAGATTTCTTTACATTACT
ATTTAACGCTCCTGAGAGGGATTAAACACACACTGATATAAAGCCATTTAGGATA
TATATACCACAAATAATACCACAAACATTTTATGTAATAATAAATATTATTTA
TTATTACATTGAAATAAATATTTCGTTATAAATAGTTTTTATATCAAGATGTTT
TTTCTCAAGGTTTTTATAAATGACTTTAATTCCTTTGTTTCAAGTAGTCCAG
AGAAGATTTTTTCAACAGCGTCTTCTTTCCCTCCACGCATGCGACGTCAATA
CGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCTCGAGGTCGACGGTA
TCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGA
GCGGCCGCCACCGCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAA
TTAAATAACTTCGTATAATGTATGCTATACGAAGTTATGCGATCGCCTCTCGC
CTGTCCCCTCAGTTCAGTAATTCCTGCATTTGCCTGTTTCCAGTCGGTAGAT
ATTCCACAAAACAGCAGGGAAGCAGCGCTTTTCCGCTGCATAACCCTGCTTCG
GGGTCAATTATAGCGATTTTTTCGGTATATCCATCCTTTTTTCGCACGATATACA
GGATTTTGCCAAAGGGTTCGTGTAGACTTTCCTTGGTGTATCCAACGGCGTCA
GCGGGCGAGGATAGGTGAAGTAGGCCACCCGCGAGCGGGTGTCTCTTCTCA
CTGTCCCTTATTTCGCACCTGGCGGTGCTCAACGGGAATCCTGCTCTGCGAGGC
TGGCCGGCTACCGCCGGCGTAACAGATGAGGGCAAGCGGCGGAGAATTACAAC
TTATATCGTATGGGGCTGACTTCAGGTGCTACATTTGAAGAGATAAATTGCAC
TGAAATCTAGAAATATTTTATCTGATTAATAAGATGATCTTCTTGAGATCGTT
TTGGTCTGCGCGTAATCTCTTGCTCTGAAAACGAAAAACCGCCTTGACAGGGC
GGTTTTTCGAAGGTTCTCTGAGCTACCAACTCTTTGAACCGAGGTAACCTGGCT
TGGAGGAGCGCAGTCAACAAAACCTTGCTCTTTCAGTTTAGCCTTAACCGGCGC
ATGACTTCAAGACTAACTCCTCTAAATCAATTACCAGTGGCTGCTGCCAGTGG
TGCTTTTGATGTCTTTCCGGGTTGGACTCAAGACGATAGTTACCGGATAAGG
CGCAGCGGTGCGACTGAACGGGGGGTTCGTGCATACAGTCCAGCTTGGAGCGA
ACTGCCTACCCGGAAGTGAAGTGTAGGCGTGAATGAGACAAACGCGGCCATA
ACAGCGGAATGACACCGGTAAACCGAAAGGCAGGAACAGGAGAGCGCACGAGG
GAGCCGCCAGGGGAAACGCCTGGTATCTTTATAGTCTGTGCGGTTTCGCCA
CCACTGATTTGAGCGTCAGATTTCTGTATGCTTGTGAGGGGGCGGAGCCTAT
GGAAAAACGGCTTTGCCGCGGCCCTCTCACTTCCCTGTTAAGTATCTTCCTGG
CATCTTCCAGGAAATCTCCGCCCCGTTCTGTAAGCCATTTCCGCTCGCCGAGT
CGAACGACCGAGCGTAGCGAGTCACTGAGCGAGGAAGCGGAATATATCCTGTA
TCACATATTCTGCTGACGCACCGGTGCAGCCTTTTTTCTCCTGCCACATGAAG
CACTTCACTGACACCTCATCAGTGCCAACATAGTAAGCCAGTATACACTCCG
CTAGCGCTGATGTCCGGCGGTGCTTTTGCCGTTACGCACCAACCCCGTCAGTAG
CTGAACAGGAGGGACAGCTGATAGAAACAGAAGCCACTGGAGCACCTCAAAAA
CACCATCATACACTAAATCAGTAAGTTGGCAGCATACCCGACGCACTTTGCG
CCGAATAAATACCTGTGACGGAAGATCACTTCGCAGAATAAATAAATCCTGGT
GTCCCTGTTGATACCGGGAAGCCCTGGGCCAACTTTTGCGGAAAATGAGACGT
TGATCGGCACGTAAGAGGTTCCAACCTTACCATAATGAAATAAGATCACTAC
CGGGCGTATTTTTTGTGTTATCGAGATTTTTCAGGAGCTAAGGAAGCTAAAATG
GAGAAAAAATCACTGGATATACCACCGTTGATATATCCCAATGGCATCGTAA

AGAACATTTTGAGGCATTTTCAGTCAGTTGCTCAATGTACCTATAACCAGACCG
 TTCAGCTGGATATTACGGCCTTTTTAAAGACCGTAAAGAAAAATAAGCACAAG
 TTTTATCCGGCCTTTATTCACATTCTTGCCCGCCTGATGAATGCTCATCCGGA
 ATTCCGTATGGCAATGAAAGACGGTGAGCTGGTGATATGGGATAGTGTTCACC
 CTTGTTACACCGTTTTTCCATGAGCAAACGTAAACGTTTTTCATCGCTCTGGAGT
 GAATACCACGACGATTTCCGGCAGTTTCTACACATATATTCGCAAGATGTGGC
 GTGTTACGGTGAAAACCTGGCCTATTTCCCTAAAGGGTTTATTGAGAATATGT
 TTTTCGTCTCAGCCAATCCCTGGGTGAGTTTCACCAGTTTTGATTTAAACGTG
 GCCAATATGGACAACCTCTTCGCCCCCGTTTTTACCATGGGCAAATATTATAC
 GCAAGGCGACAAGGTGCTGATGCCGCTGGCGATTTCAGGTTTCATCATGCCGTTT
 GTGATGGCTTCCATGTTCGGCAGAATGCTTAATGAATTACAACAGTACTGCGAT
 GAGTGGCAGGGCGGGGCGTAATTTTTTTAAAGGCAGTTATTGGTGCCCTTAAAC
 GCCTGGTTGCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAATT
 CGAAAGCAAATTCGACCCGGTCGTTCGGTTTCAGGGCAGGGTCGTAAATAGCGA
 CGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAAATAGGCGTATCA
 CGAGGCCCTTTTCGTCTCGCGCGTTTTTCGGTGATGACGGTGAAAACCTCTGACAC
 ATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAG
 ACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTA
 ACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGCACAATCGCATCCGATTG
 CAGTATAAATTTAACGATCACTCATCATGTTTCATATTTATCAGAGCTCGTGCT
 ATAATTATACTAATTTTATAAGGAGGAAAAAATATGGGCATTTTTAGTATTTT
 TGTAATCAGCACAGTTCATTATCAACCAAACAAAAAATAAGTGGTTATAATGA
 ATCGTTAATAAGCAAATTCATATAACCAAATTAAGAGGGTTATAATGAACG
 AGAAAAATATAAAACACAGTCAAAACTTTATTACTTCAAACATAATATAGAT
 AAAATAATGACAAATATAAGATTAAATGAACATGATAATATCTTTGAAATCGG
 CTCAGGAAAAGGCCATTTTACCCTTGAATTAGTAAAGAGGTGTAATTTTCGTAA
 CTGCCATTGAAATAGACCATAAATTATGCAAACTACAGAAAATAAACTTGTT
 GATCACGATAATTTCCAAGTTTTTAAACAAGGATATATTGCAGTTTAAATTTCC
 TAAAAACCAATCCTATAAAATATATGGTAATATACCTTATAACATAAGTACGG
 ATATAATACGCAAATTTGTTTTTGATAGTATAGCTAATGAGATTTATTTAATC
 GTGGAATACGGGTTTGCTAAAAGATTATTAATAACAAACGCTCATTGGCATT
 ACTTTTAATGGCAGAAGTTGATATTTCTATATTAAGTATGGTTCCAAGAGAAT
 ATTTTCATCCTAAACCTAAAGTGAATAGCTCACTTATCAGATTAAGTAGAAAA
 AAATCAAGAATATCACACAAAGATAAACAAAAGTATAATTATTTTCGTTATGAA
 ATGGGTAAACAAAGAAATACAAGAAAATATTACAAAAAATCAATTTAACAATT
 CCTTAAACATCAGGAATTGACGATTTAAACAATATTAGCTTTGAACAATTC
 TTATCTCTTTTTCAGCTATAAATTATTTAATAAGTAAGTTAAGGGATGCAT
 AAATCGCATCCCTTAACTTGTTTTTCGTGTGCCCGATCGGTGCGGGCCTCTTC
 GCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGG
 TAACGCCAGGGTTTTCCAGTCACGACGTTGTAAACGACGGCCAGTGCCAAG
 CTAGCTTTCGATCATCATAATTCTGTCTCATTATATAACATCCTCCATACCTT
 CTATTATAGAATAACCATAAACTCATCTGGCAATTCATTTTCGAGTCACGAAGAA
 CGGAAAAACTGCCGGTTTTTATATTACAAATGTATTAAGTTTTTCTATTAACA
 AAAACAATAGGTTTCCCATAGCGAAAGTTGTTGATTAACGTTTACATCCCAC
 TTACACTATAAAGGTTTACCCAGCAATACATCTCAAGCCCTAAGAATACACGT
 TCGCTTTTCAACTGTTACAGAATTATTACAAATAGTTGGTATAGTCCTCTTTA
 GCCTTTGGAGCTATTATCTCATCATTTGTTTTTTAGGTGAAAACCTGGGTAAAC
 TTAGTATTAATCAATATAAAATTAATTCTCAAATACTTAATTACGTACTGGGA
 TTTTCTGAAAAAA
 (SEQ ID NO:29)

EXAMPLE III. ActA-based fusion protein partners, including ActA derivatives that are truncated or deleted in one or more motifs.

[0429] The present invention, in some embodiments, provides reagents and methods comprising a first nucleic acid encoding an ActA-based fusion protein partner operably linked to and in frame with a second nucleic acid encoding at least one heterologous antigen. Provided is a nucleic acid that can hybridize under stringent conditions to any of the disclosed nucleic acids.

[0430] What is encompassed is a first nucleic acid and second nucleic acid that are operably linked with each other, and in frame with each other. In this context, "operably linked with each other" means that any construct comprising the first and second nucleic acids encode a fusion protein. In another embodiment, the second nucleic acid can be embedded in the first nucleic acid.

[0431] The ActA-based fusion protein partner can comprise one or more of the following. "Consisting" embodiments are also available, and here the ActA-based fusion protein partner can consist of one or more of the following embodiments:

- (1) ActA-N100 (amino acids 1-100 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence).
- (2) Full length ActA, where a nucleic acid encoding at least one heterologous antigen is connected to (and in frame with) the C-terminus of full length ActA, residing at an internal position of ActA, or both connected to the C-terminus of the full length ActA and also residing at an internal position of ActA.
- (3) A truncated ActA that normally supports less than 90% the activity of nucleating the Arp2/3 complex, as compared with the activity of full length ActA; conventionally supports less than 80% the nucleating activity of full length ActA; characteristically supports less than 70% the nucleating activity of full length ActA; typically supports less than 60% the nucleating activity of full length ActA; more typically supports less than 50% the nucleating activity of full length ActA; most typically supports less than 40% the nucleating activity of full length ActA; often supports less than 30% the nucleating activity of full length ActA; more often supports less than 20% the nucleating activity of full length ActA; most often supports less than 10% the nucleating activity of full length ActA; usually supports less than 5% the nucleating activity of full length ActA; more usually supports less than 2% the nucleating activity of full length ActA; and most usually is undetectable in any ability to nucleate the Arp2/3 complex.

The reduced, or eliminated, nucleation activity of progressively truncated ActA was demonstrated by Skoble (Skoble, *et al.* (2000) *J. Cell Biol.* 150:527-537). It was demonstrated that ActA truncated at amino acid-101, and ActA truncated at amino acid-135,

have little or no nucleating activity, while ActA truncated at amino acids 165, 201, and 263, are as potent as full length ActA in nucleating the Arp2/3 complex.

(4) A truncated ActA, wherein the ActA is truncated at about amino acid-40; truncated at about amino acid-45; truncated at about amino acid-50; truncated at about amino acid-55; truncated at about amino acid-60; truncated at about amino acid-65; truncated at about amino acid-70; truncated at about amino acid-75; truncated at about amino acid-80; truncated at about amino acid-85; truncated at about amino acid-90; truncated at about amino acid-95; truncated at about amino acid-100; truncated at about amino acid-105; truncated at about amino acid-110; truncated at about amino acid-115; truncated at about amino acid-120; truncated at about amino acid-125; truncated at about amino acid-130; truncated at about amino acid-135; truncated at about amino acid-140; truncated at about amino acid-145; truncated at about amino acid-150; truncated at about amino acid-155; and truncated at about amino acid-160. The term "about" in this context means plus or minus one amino acid, plus or minus two amino acids, plus or minus three amino acids, plus or minus four amino acids, or plus or minus five amino acids.

(5) ActA secretory sequence (amino acids 1-29 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence).

(6) Does not comprise an ActA secretory sequence (amino acids 1-29 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence).

(7) ActA secretory sequence and the mature N-terminal domain (amino acids 1-263 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence).

(8) Mature N-terminal domain without the secretory sequence (amino acids 30-263 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence).

(9) ActA sequence with reduced ability to directly stimulate actin polymerization. The reduced ability can be, e.g., normally at most 90% maximal, more normally at most 80% maximal, most normally at most 70% maximal, usually at most 60% maximal, more usually at most 50% maximal, most usually at most 40% maximal, often at most 30% maximal, more often at most 20% maximal, most often at most 10% maximal, and typically at most 5% maximal.

(10) ActA sequence with a reduced ability to bind to a member of the Ena/VASP family of proteins (mammalian Enabled (Mena); Ena/VASP-like protein (Evl); vasodilator-stimulated phosphoprotein (VASP) (see, e.g., Machner, *et al.* (2001) J. Biol. Chem. 276:40096-40103). The reduced ability can be, e.g., normally at most 90% maximal, more normally at most 80% maximal, most normally at most 70% maximal, usually at most 60% maximal, more usually

at most 50% maximal, most usually at most 40% maximal, often at most 30% maximal, more often at most 20% maximal, most often at most 10% maximal, and typically at most 5% maximal.

(11) ActA that is truncated at the point of, deleted in, or mutated in amino acids 93-98 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (LKEKAE (SEQ ID NO:124)) (homologous to actin binding domain of caldesmon (see, e.g., Pistor, *et al.* (2000) *J. Cell Science* 113:3277-3287; Lasa, *et al.* (1997) *EMBO J.* 16:1531-1540).

(12) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 126-155 (PAIQ, etc.) of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence, that are critical for ActA dimer formation (see, e.g., Mourrain, *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:10034-10039).

(13) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 121-170 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (minimal ARP2/3 activating domain) (see, e.g., Zalevsky, *et al.* (2001) *J. Biol. Chem.* 276:3468-3475).

(14) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 146-150 KKRRK (SEQ ID NO:30)) of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (a region essential for recruiting Arp2/3 complex) (Lasa, *et al.* (1997) *EMBO J.* 16:1531-1540; Pistor, *et al.* (2000) *J. Cell Science* 113:3277-3287).

(15) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 41-46 DEWEEE (SEQ ID NO:31) of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (a region involved in Arp2/3 complex binding) (see, e.g., Boujemaa-Paterski, *et al.* (2001) *Biochemistry* 40:11390-11404).

(16) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 481-492 (DRLADLRDRGTG (SEQ ID NO:32)), which is a vinculin homology region. Vinculin mediates cell-to-cell spread of *S. flexneri* (see, e.g., Kocks, *et al.* (1992) *Cell* 68:521-531).

(17) ActA that is truncated at the point of, deleted in, or mutated in, the cofilin homology domain (IKKKRRKAIASSD (SEQ ID NO:33)) (amino acids 145-156 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence) (see, e.g., Skoble, *et al.* (2000) *J. Cell Biol.* 150:527-537).

(18) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 50-125 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (continuity of filament elongation region) (see, e.g., Lasa, *et al.* (1997) *EMBO J.* 16:1531-1540).

(16) ActA that is truncated at the point of, deleted in, or mutated in, the first FP₄ motif (amino acids 265-269. or 264-269. and the like), second FP₄ motif (amino acids 300-304, or 299-304,

- and the like), third FP₄ motif (amino acids 335-339, or 334-339, and the like), fourth FP₄ motif (amino acids 380-384, or 379-384, and the like), all four FP₄ motifs, or any combination of the above, where the amino acids refer to GenBank Acc. No. X59723, or a similar or homologous ActA sequence (see, e.g., Machner, *et al.* (2001) *J. Biol. Chem.* 276:40096-40103). The FP₄ motifs enhance actin polymerization and bacterial motility by recruiting focal contact proteins (e.g., VASP and Mena) and profilin, which promote elongation of filaments nucleated by interactions between motifs at the N-terminal region of ActA and Arp2/3 complex (see, e.g., Welch, *et al.* (1998) *Science* 281:105-108; Skoble, *et al.* (2000) *J. Cell Biol.* 150:527-537; Pistor, *et al.* (2000) *J. Cell Science* 113:3277-3287).
- (17) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 136-165 of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (cofilin homology region, a region that stimulates Arp2/3 complex) (see, e.g., Lauer, *et al.* (2001) *Mol. Microbiol.* 42:1163-1177).
- (18) ActA that is truncated at the point of, deleted in, or mutated in, the "acidic stretch," that is, amino acids 31-58 (TDSED (SEQ ID NO:34), etc.) of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence. The acidic stretch contributes to actin polymerization, movement of *Listeria* in the host cell cytoplasm, cell to cell spreading, and to plaque size (see, e.g., Skoble, *et al.* (2000) *J. Cell Biol.* 150:527-537; Lauer, *et al.* (2001) *Mol. Microbiol.* 42:1163-1177).
- (19) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 60-101 (AB region, an actin binding domain) of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (see, e.g., Lauer, *et al.* (2001) *Mol. Microbiol.* 42:1163-1177).
- (20) ActA that is truncated at the point of, deleted in, or containing the mutation of mutant 34 (no movement; no plaque) amino acids 117-121 (KKRRK (SEQ ID NO:30)) of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (Lauer, *et al.* (2001) *Mol. Microbiol.* 42:1163-1177).
- (21) ActA that is truncated at the point of, deleted in, or containing the mutation of mutant 34 (no movement; no plaque) amino acids 244-249 (DKSAGLID (SEQ ID NO:123)) of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence. The mutation can be, e.g., replacement of the D, K, and D by alanines (Lauer, *et al.* (2001) *Mol. Microbiol.* 42:1163-1177).
- (22) ActA that is truncated at the point of, deleted in, or containing the mutation of mutants 39, 47-52, 54 and/or 48 (reduced movement) (Lauer, *et al.* (2001) *Mol. Microbiol.* 42:1163-1177).

(23) ActA that is truncated at the point of, deleted in, or mutated in, amino acids 264-390 (central repeat region) of GenBank Acc. No. X59723, or of a similar or homologous ActA sequence (see, e.g., Lauer, *et al.* (2001) *Mol. Microbiol.* 42:1163-1177; Skoble, *et al.* (2000) *J. Cell Biol.* 150:527-537; Skoble, *et al.* (2001) *J. Cell Biol.* 155:89-100).

[0432] The present invention provides an ActA-based fusion protein partner that can comprise any one, or any combination of, the above-disclosed embodiments. "Consisting" embodiments are also available, and here the ActA-based fusion protein partner can consist of one or more of the above-disclosed embodiments.

[0433] When provided with the present disclosure, the skilled artisan can envision and prepare embodiments containing conservative modifications, or modifications where one or more amino acids is deleted, or where one or more amino acids is replaced with alanine, and the like.

[0434] In the present context, "fusion protein partner" encompasses, but is not limited to, a nucleic acid encoding a polypeptide, or the polypeptide itself, that occurs as a fusion protein with a heterologous antigen, where the fusion protein partner enhances, e.g., transcription, translation, stability, processing by an antigen presenting cell (APC), presentation by an APC, immune presentation, cytotoxic T cell response, CD8⁺ T cell response, CD4⁺ T cell response, reduction in tumor size, number, or metastasis, increase in survival to a tumor or infective agent, and the like.

[0435] The present invention provides nucleic acids and polypeptides of ActA-N100, and fusion proteins thereof, including fusion proteins that comprise at least one antigen. Without implying any limitation on the invention, the at least one antigen can comprise mesothelin, H-ras, a mesothelin derivative, a H-ras derivative, or any combination thereof. The nucleic acid encoding at least one antigen can be operably linked to, and in frame with, the N-terminus of an ActA-based fusion protein partner. Alternatively, the nucleic acid encoding the at least one antigen can be operably linked to, and in frame with, the C-terminus of the ActA fusion protein partner. Or the nucleic acid encoding the at least one antigen can be operably linked with, and reside within a nucleic acid encoding an ActA-based fusion protein partner.

EXAMPLE IV. Building blocks used for assembling nucleic acids encoding ActA fusion proteins.

[0436] The following discloses nucleic acids and polypeptides used for making constructs that contain ActA-N100 as a fusion protein partner. Sequences codon optimized for expression in *L. monocytogenes*, and non-codon optimized sequences, are identified.

Nucleic acid encoding ActA-N100 native sequence (not codon optimized), including Shine-Dalgarno sequence. (SEQ ID NO:122)	GTGGGATTAAATAGATTTATGCGTGCGATGATGGTAGT TTTCATTACTGCCAACTGCATTACGATTAAACCCGACA TAATATTTGCAGCGACAGATAGCGAAGATTCCAGTCTA AACACAGATGAATGGGAAGAAGAAAAACAGAAGAGCA GCCAAGCGAGGTAAATACGGGACCAAGATACGAACTG CACGTGAAGTAAGTTCACGTGATATTGAGGAACTAGAA AAATCGAATAAAGTGAAAAATACGAACAAAGCAGACCT AATAGCAATGTTGAAAGCAAAGCAGAGAAAGGT
ActA promoter <i>L. monocytogenes</i> 10403S. (SEQ ID NO:35)	AAGCTTGGGAAGCAGTTGGGGTAACTGATTAAACAAATGTTAGAGAA AAATTAATTCTCCAAGTGATATTCTTAAATAATTCATGAATATTTT TTCTTATATTAGCTAATTAAGAAGATAATTAAGTCTAATCCAATTT TTAACGGAATAAATTAGTGAAAATGAAGGCCGAATTTTCCTTGTTCT AAAAAGGTTGTATTAGCGTATCACGAGGAGGGAGTATAA
Native actA promoter together with the 5' UTR (including the Shine Dalgarno sequence), and GUG translation site. (SEQ ID NO:157) Underlined and lowercase is a KpnI site added for cloning. PrfA box is in italics and bold. +1 for transcription is shown as "<u>T</u>" Shine-Dalgarno sequence near end is underlined. Lowercase "gtg" at end is start site for ActA in	<u>ggtacc</u>GGGAAGCAGTTGGGGTAACTGA<i>TTAACAAAT</i> <i>GTTAGAGAAAAATTAATTCTCCAAGTGATATTCTTAAA</i> <i>A</i><u>T</u>AATTCATGAATATTTTTTCTTATATTAGCTAATTAA GAAGATAATTAAGTCTAATCCAATTTTAAACGGAATA AATTAGTGAAAATGAAGGCCGAATTTTCCTTGTTCTAA AAAGGTTGTATTAGCGTATCACGAGGAGGGAGTATAAgtg

<i>Listeria.</i>	
ActA-N100 native sequence (not codon optimized), including Shine-Dalgarno sequence, with human mesothelin (codon optimized) with SS deleted and GPI deleted. The <i>Bam</i>HI (GGATCC) and <i>Sac</i>I (GAGCTC) sites are shown in BOLD. (SEQ ID NO:36)	GTGGGATTAAATAGATTTATGCGTGCGATGATGGTAGTTTTTCAT TACTGCCAACTGCATTACGATTAACCCCGACATAATATTTGCAG CGACAGATAGCGAAGATTCCAGTCTAAACACAGATGAATGGGAA GAAGAAAAACAGAAGAGCAGCCAAGCGAGGTAAATACGGGACC AAGATACGAAACTGCACGTGAAGTAAGTTCACGTGATATTGAGG AACTAGAAAAATCGAATAAAGTGAAAAATACGAACAAAGCAGAC CTAATAGCAATGTTGAAAGCAAAAGCAGAGAAAGGTGGATCCCG TACATTAGCAGGTGAAACAGGTCAAGAAGCAGCACCACCTTGACG GTGTATTAAACGAATCCACCAATATATCAAGTTTAAGTCCACGT CAATTATTAGGTTTTCCATGTGCAGAAGTTTCAGGTTTAAGTAC AGAACGTGTCCGTGAGTTAGCAGTTGCATTAGCACAAAAAACG TTAAATTATCTACAGAACAGTTACGTTGTTTAGCCCATAGATTA AGCGAACCACCAGAAGACTTAGATGCACCTTCCTTTAGACCTTCT TTTATTCTTAAATCCAGATGCATTTTTCAGGACCACAAGCATGTA CACGTTTTTTTTAGTCTGAATTACAAAAGCCAATGTTGATTTATTA CCTCGTGGGGCTCCTGAAAGACAACGTTTATTACCTGCTGCATT AGCATGCTGGGGTGTTCGCGGTAGCTTATTAAGTGAAGCCGATG TTCGTGCTTTAGGGGGTTTAGCATGTGATTTACCTGGTCGTTTC GTTGCAGAATCAGCAGAAAGTGTATTACCGAGATTAGTTTCATG CCCAGGACCTTTAGATCAAGATCAACAAGAGGCAGCTAGAGCAG CTCTTCAAGGAGGAGGCCCCACCATATGGCCCCACCAAGTACATGG AGTGTTCCTACAATGGATGCGTTAAGAGGTTTATTACCGGTTTTT AGGACAACCAATTATTCGTAGTATTCCACAAGGCATTGTAGCAG CATGGCGTCAACGTAGTTCTCGTGATCCGTCTTGCGCACAACCA GAACGTACAATTCTACGTCCAAGATTTCGTAGAGAAGTAGAAAA AACGGCGTGTCTAGTGGCAAAAAGCACGTGAAATTGATGAAA GTTTAATTTTTTTATAAAAAATGGGAATTAGAAGCATGTGTCGAT GCAGCATTACTAGCTACACAAATGGATCGTGTTAATGCTATTCC ATTCACATATGAACAATTAGATGTTTTAAAGCATAAATTAGACG AATTATATCCACAAGGTTATCCAGAATCAGTTATTCAACATTTA GGTTACTTATTTTTTAAAAATGAGTCCAGAAGACATACGCAATG GAATGTTACAAGTTTAGAAACATTAAAAGCGCTTTTAGAAGTTA ACAAAGGTCATGAAATGAGTCCACAAGTTGCTACGTTAATTGAT AGATTCGTTAAAGGCCGTGGTCAATTAGATAAAGATACTTTAGA TACATTAACAGCATTTTATCCTGGCTACTTATGCAGTTTATCAC CAGAAGAATTAAAGTTCCGTTCCACCGAGTAGTATCTGGGCAGTT CGTCCGCAAGATTTAGATACATGCGACCCACGTCAATTAGATGT TTTATATCCAAAAGCAAGATTAGCTTTCCAAAATATGAACGGTA GTGAATATTTCTGTAATAAATCAATCCTTTTTAGGTGGTGCACCA ACTGAAGATCTAAAAGCATTAAAGCCAACAAAATGTAAGTATGGA TTTAGCTACGTTTATGAAATTACGTACAGATGCAGTTCTACCAT TAACAGTTGCAGAAGTTCAAAAATTATTAGGTCCACACGTAGAA GGATTAAAAGCAGAAGAAGCTCACCGTCCAGTTTCGCGATTGGAT TTTACGTCAACGTCAAGATGATTTAGATACATTAGGTTTAGGTT TACAAGGCTAAGGAGCTC
Nucleic acid encoding full-length ActA <i>L. monocytogenes</i> 10403S. (SEQ ID NO:37)	GTGGGATTAAATAGATTTATGCGTGCGATGATGGTAGTTTTT CATTACTGCCAACTGCATTACGATTAACCCCGACATAATAT TTGCAGCGACAGATAGCGAAGATTCCAGTCTAAACACAGA TGAATGGGAAGAAGAAAAACAGAAGAGCAGCCAAGCGA GGTAAATACGGGACCAAGATACGAAACTGCACGTGAAGTA AGTTCACGTGATATTGAGGAACTAGAAAAATCGAATAAAG TGAAAAATACGAACAAAGCAGACCTAATAGCAATGTTGAA AGCAAAAGCAGAGAAAAGGTCCGAATAACAATAATAACAAC GGTGAGCAAAACAGGAAATGTGGCTATAAATGAAGAGGCTTC AGGAGTCGACCGACCAACTCTGCAAGTGGAGCGTCGTCATC CAGGTCGTTCATCGGATAGCGCAGCGGAAATTAATAAAGA AGAAAAGCCATAGCGTCGTCGGATAGTGAGCTTGAAAGCCT

	TACTTATCCAGATAAACCAACAAAAGCAAATAAGAGAAAAG TGGCGAAAGAGTCAGTTGTGGATGCTTCTGAAAGTGACTTAG ATTCTAGCATGCAGTCAGCAGACGAGTCTACACCACAACCTT TAAAAGCAAATCAAAAACCATTTTTCCTAAAGTATTTAAAA AAATAAAAGATGCGGGGAAATGGGTACGTGATAAAATCGAC GAAAATCCTGAAGTAAAGAAAGCGATTGTTGATAAAAGTGC AGGGTTAATTGACCAATTATTAACCAAAAAGAAAAGTGAAG AGGTAAATGCTTCGGACTTCCCGCCACCACCTACGGATGAAG AGTTAAGACTTGCTTTTGCCAGAGACACCGATGCTTCTCGGTTT TAATGCTCCTACTCCATCGGAACCGAGCTCATTCGAATTTCCG CCGCCACCTACGGATGAAGAGTTAAGACTTGCTTTGCCAGAG ACGCCAATGCTTCTTGGTTTAAATGCTCCTGCTACATCGGAAC CGAGCTCATTCGAATTTCCACCGCCTCCAACAGAAGATGAAC TAGAAATTATGCGGGAAACAGCACCTTCGCTAGATTCTAGTT TTACAAGCGGGGATTTAGCTAGTTTGAGAAGTGCTATTAATC GCCATAGCGAAAATTTCTCTGATTTCCCACTAATCCCAACAG AAGAAGAGTTGAACGGGAGAGCGGTAGACCAACATCTGAA GAATTTAGTTCGCTGAATAGTGGTGATTTTACAGATGACGAA AACAGCGAGACAACAGAAGAAGAAATTGATCGCCTAGCTGA TTTAAGAGATAGAGGAACAGGAAAACACTCAAGAAATGCGG GTTTTTTTACCATTAAATCCATTTATTAGTAGCCCTGTTCCCTCA TTAACTCCAAAGGTACCGAAAATAAGCGCGCCGCTCTGATA AGTGACATAACTAAAAAAGCGCCATTTAAGAATCCATCACAG CCATTAAATGTGTTTAAATAAAAAAACTACAACGAAAACAGTG ACTAAAAAACCAACCCCTGTAAAGACCGCACCAAGCTAGCA GAACTTCCTGCCACAAAACCAAGAAACCGTACTTAGGGAA AATAAAACACCCTTTATAGAAAAACAAGCAGAAACAAACAAG CAGTCAATCAATATGCCGAGCCTACCAGTAATCCAAAAAGAA GCTACAGAGAGCGATAAAGAGGAAATGAAACCACAAACCGA GGAAAAAATGGTAGAGGAAAGCGAATCAGCTAATAACGCAA ACGGAAAAAATCGTTCTGCTGGCATTGAAGAAGGAAAACATA TTGCTAAAAGTGACAGAAGACGAAAAAGCGAAGGAAGAACCA GGGAACCATACGACGTTAATTCTTGCAATGTTAGCTA TTGGCGTGTTCTCTTTAGGGGCGTTTATCAAAATTATT CAATTAAGAAAAAATAATTAA
ActA polypeptide from <i>L. monocytogenes</i> 10403S. (SEQ ID NO:38) (Predicted amino acid sequence if not at N-terminus when expressed in <i>Listeria</i>.)	VGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEKT EEQPSEVNTGPRYETAREVSSRDIEELEKSNKVKNNTKADLIAMLKAK AEKGPNNNNNNGEQTNVAINEEASGVDRPTLQVERRHPGLSSDSAAE IKKRRKAIASSDSELESLTYPDKPTKANKRKVAKESVVDASESDLSS MQSADESTPQPLKANQKPFPPKVFKKIKDAGKWVRDKIDENPEVKKAI VDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFN APTPSEPSSFEPPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEPF PPPTDELEIMRETAPSLDSSFTSGDLASLRSAINRHSENFSDFLIP TEEELNGRGGRPTESEFSSLNSGDFDENSETTEEEIDRLADLRDRG TGKHSRNAGFLPLNPFISSPVPSLTPKVPKISAPALISDITKKAPFKN PSQPLNVFNKKTITTKVTKKPTPVKTAPKLAELPATKPQETVLRENKT PFIEKQAEITNKQSIINMPSLPVIQKEATESDKEEMKPQTECKMVEESES ANNANGKNRSAGIEEGKLIKSAEDEKAKEEFGNHTTLILAMLAIGVF SLGAFIKIIQLRKNN
ActA polypeptide from <i>L. monocytogenes</i> 10403S. (SEQ ID NO:152) (Actual amino acid sequence when expressed in <i>Listeria</i>. Although the	MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEKT EEQPSEVNTGPRYETAREVSSRDIEELEKSNKVKNNTKADLIAMLKAK AEKGPNNNNNNGEQTNVAINEEASGVDRPTLQVERRHPGLSSDSAAE IKKRRKAIASSDSELESLTYPDKPTKANKRKVAKESVVDASESDLSS MQSADESTPQPLKANQKPFPPKVFKKIKDAGKWVRDKIDENPEVKKAI VDKSAGLIDQLLTKKKSEEVNASDFPPPPTDEELRLALPETPMLLGFN APTPSEPSSFEPPPPPTDEELRLALPETPMLLGFNAPATSEPSSFEPF PPPTDELEIMRETAPSLDSSFTSGDLASLRSAINRHSENFSDFLIP TEEELNGRGGRPTESEFSSLNSGDFDENSETTEEEIDRLADLRDRG TGKHSRNAGFLPLNPFISSPVPSLTPKVPKISAPALISDITKKAPFKN PSQPLNVFNKKTITTKVTKKPTPVKTAPKLAELPATKPQETVLRENKT PFIEKQAEITNKQSIINMPSLPVIQKEATESDKEEMKPQTECKMVEESES ANNANGKNRSAGIEEGKLIKSAEDEKAKEEFGNHTTLILAMLAIGVF

coding sequence contains a valine codon at the N-terminus, the <i>Listeria</i> actually biosynthesizes a polypeptide starting with methionine, not valine.)	SLGAFIKIIQLRKNN
Nucleic acid encoding ActA-N100 fragment used in our constructs, including promoter and restriction enzyme sites (<i>KpnI</i> site and <i>BamHI</i> site underlined, promoter sequence lowercase, N100 ORF sequence in UPPERCASE). (SEQ ID NO:39)	<u>Ggtaccgggaagcagttgggggttaactgattaacaaatgtagagaaa</u> <u>Aattaattctccaagtgatattcttaaaataattcatgaatatttttt</u> <u>Cttatattagctaattaagaagataattaactgctaattccaattttta</u> <u>Acggaataaattagtgaaaatgaaggccgaattttccttggttctaaaa</u> <u>AggttgattagcgtatcacgaggagggagtataaGTGGGATTAAATA</u> <u>GATTTATGCGTGCGATGATGGTAGTTTTTCATTACTGCCAACTGCATTA</u> <u>CGATTAACCCCGACATAATATTTGCAGCGACAGATAGCGAAGATTCCA</u> <u>GTCTAAACACAGATGAATGGGAAGAAGAAAAAACAGAAGAGCAGCCAA</u> <u>GCGAGGTAAATACGGGACCAAGATACGAAACTGCACGTGAAGTAAGTT</u> <u>CACGTGATATTGAGGAAC TAGAAAAATCGAATAAAGTGA AAAATACGA</u> <u>ACAAAGCAGACCTAATAGCAATGTTGAAAGCAAAAGCAGAGAAAGGT</u> <u>ggatcc</u>
Amino acid sequence of ActA-N100. (SEQ ID NO:40) (Predicted amino acid sequence if not at N-terminus when expressed in <i>Listeria</i> .)	VGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSS LNTDEWEEEEKTEEQPSEVNTGPRYETAREVSSRDIEE LEKSNKVKNTNKADLIAMLKAKAEKG
Amino acid sequence of ActA-N100. (SEQ ID NO:153) Actual amino acid sequence when expressed in <i>Listeria</i> . The nucleic acid encoding ActA-N100 contains a valine codon at the	MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSS LNTDEWEEEEKTEEQPSEVNTGPRYETAREVSSRDIEE LEKSNKVKNTNKADLIAMLKAKAEKG

N-terminus, but the <i>Listeria</i> actually biosynthesizes a polypeptide starting with methionine, not valine.)	
Amino acid sequence of fusion protein of ActA-N100 with human mesothelin (ss deleted; GPI deleted). The <i>Bam</i>HI site adds two amino acids (GS). (SEQ ID NO:41)	VGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEE EKTEEQPSEVNTGPRYETAREVSSRDIEELEKSNKVKNTNKADLI AMLKAKAEKGGSR⁴¹TLAGETGQEAAPLDGVLTNPPNISSLSRQLL GFPCAIEVSGLSTERVRELAVALAQKNVKLSTEQLRCLAHRLSEPP EDLDALPLDLLLFLNPDAFSGPQACTRFFSRITKANVDLLPRGA PERQRLPAALACWGVRSLLSEADVRLGGLACDLPGRFVAES AEVLLPRLVSCPGPLDQDQQAARAALQGGGPPYGPPSTWSVST MDALRGLLPVLGQPIIRSIPQGIVAARQRSSRDPSWRQPRTI LRPRFRREVEKTACPSGKKAREIDESLIIFYKKWELEACVDAALL ATQMDRVNAIPFTYEQLDVLKHKLDELYPQGYPESVIQHLGYLF LKMSPEDIRKWNVTSLKALLEVNKGHEMSPQVATLIDRFVK GRGQLDKDTLDTLTAIFYPGYLCSLSPEELSSVPPSSIWAVRPQD LDTCDPRQLDVLYPKARLAFQNMNGSEYFVKIQSFLGGAPTEDL KALSQQNVSMDLATFMKLRTDAVLPLTVAEVQKLLGPHVEGLKA EERHRPVRDWILRQRQDDLDLTLGLGLQG
Amino acid sequence of fusion protein of ActA-N100 with human mesothelin (ss deleted; GPI deleted). (SEQ ID NO: 154) (Actual amino acid sequence when expressed in <i>Listeria</i>. The nucleic acid encoding ActA-N100, or a fusion protein thereof, contains a valine codon at the N-terminus, but the <i>Listeria</i> actually biosynthesizes a polypeptide	MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEE EKTEEQPSEVNTGPRYETAREVSSRDIEELEKSNKVKNTNKADLI AMLKAKAEKGGSR¹⁵⁴TLAGETGQEAAPLDGVLTNPPNISSLSRQLL GFPCAIEVSGLSTERVRELAVALAQKNVKLSTEQLRCLAHRLSEPP EDLDALPLDLLLFLNPDAFSGPQACTRFFSRITKANVDLLPRGA PERQRLPAALACWGVRSLLSEADVRLGGLACDLPGRFVAES AEVLLPRLVSCPGPLDQDQQAARAALQGGGPPYGPPSTWSVST MDALRGLLPVLGQPIIRSIPQGIVAARQRSSRDPSWRQPRTI LRPRFRREVEKTACPSGKKAREIDESLIIFYKKWELEACVDAALL ATQMDRVNAIPFTYEQLDVLKHKLDELYPQGYPESVIQHLGYLF LKMSPEDIRKWNVTSLKALLEVNKGHEMSPQVATLIDRFVK GRGQLDKDTLDTLTAIFYPGYLCSLSPEELSSVPPSSIWAVRPQD LDTCDPRQLDVLYPKARLAFQNMNGSEYFVKIQSFLGGAPTEDL KALSQQNVSMDLATFMKLRTDAVLPLTVAEVQKLLGPHVEGLKA EERHRPVRDWILRQRQDDLDLTLGLGLQG

starting with methionine, not valine.) The <i>Bam</i> HI site adds two amino acids (GS).	
Nucleic acid sequence of 12ras. (SEQ ID NO:42)	GCCATGACAGAATATAAATTAGTTGTAGTTGGTGCAGA TGGTGTGGTAAAAGTGCATTAACAATTCAATTAATTC AATAA
Amino acid sequence of 12ras. (SEQ ID NO:43)	AMTEYKLVVVVGADGVGKSALTIQLIQ
Nucleic acid of fusion protein of ActA-N100 with codon optimized human mesothelin (deleted SS; deleted GPI) and 12ras. 12ras is fused to the 3'-end of mesothelin (deleted in SS; deleted in GPI). The mesothelin-ras fusion construct is codon optimized and cloned (as a <i>Bam</i> HI- <i>Sac</i> I fragment) downstream of the ActA-N100-fusion protein partner. The BOLD nucleotides indicate restriction sites. <i>Bam</i> HI is GGATCC. <i>Sac</i> I is GAGCTG. (SEQ ID NO:44)	GTGGGATTAAATAGATTTATGCGTGCGATGATGGTAGTTTTTCATTA CTGCCAACTGCATTACGATTAACCCCGACATAATATTTGCAGCGAC AGATAGCGAAGATTCAGTCTAAACACAGATGAATGGGAAGAAGAA AAAACAGAAGAGCAGCCAAGCGAGGTAAATACGGGACCAAGATACG AAACTGCACGTGAAGTAAGTTCACGTGATATTGAGGAAC'TAGAAAA ATCGAATAAAGTGAAAAATACGAACAAAGCAGACCTAATAGCAATG TTGAAAGCAAAAGCAGAGAAAAGGT GGATCC CGTACATTAGCAGGTG AAACAGGTCAAGAAGCAGCACCAC'TTGACGGTGTATTAACGAATCC ACCAAATATATCAAGTTTAAGTCCACGTCAATTATTAGGTTTTTCCA TGTGCAGAAGTTTTAGGTTTAAGTACAGAACGTGTCCGTGAGTTAG CAGTTGCATTAGCACAAAAAACGTTAAATTATCTACAGAACAGTT ACGTTGTTTAGCCCATAGATTAAGCGAACCACCAGAAGACTTAGAT GCACTTCCTTTAGACCTTCTTTTATTCTTAAATCCAGATGCATTTT CAGGACCACAAGCATGTACACGTTTTTTTTAGTGAATTACAAAAGC CAATGTTGATTTATTACCTCGTGGGGCTCCTGAAAGACAACGTTTA TTACCTGCTGCATTAGCATGCTGGGGTGTTTCGCGGTAGCTTATTAA GTGAAGCCGATGTTTCGTGCTTTAGGGGGTTTAGCATGTGATTTACC TGGTCGTTTCGTTGCAGAATCAGCAGAAAGTGTTATTACCGAGATTA GTTTCATGCCCAGGACCTTTAGATCAAGATCAACAAGAGGCAGCTA GAGCAGCTCTTCAAGGAGGAGGCCACCATATGGCCACCAAGTAC ATGGAGTGTTTCTACAATGGATGCGTTAAGAGGTTTATTACCGGTT TTAGGACAACCAATTATTTCGTAGTATTCACAAAGGCATTGTAGCAG CATGGCGTCAACGTAGTTCTCGTGATCCGCTTTGGCGACAACACAGA ACGTACAATTCTACGTCCAAGATTTTCGTAGAGAAGTAGAAAAACG GCGTGTCCTAGTGGCAAAAAAGCACGTGAAATTGATGAAAGTTTAA TTTTTTTATAAAAAATGGGAATTAGAAGCATGTGTGATGCAGCATT ACTAGCTACACAAATGGATCGTGTTAATGCTATTCCATTACATAT GAACAATTAGATGTTTTTAAAGCATAAATTAGACGAATTATATCCAC AAGGTTATCCAGAATCAGTTATTCAACATTTAGGTTACTTATTTTT AAAAATGAGTCCAGAAGACATACGCAAAATGGAATGTTACAAGTTTA GAAACATTAAAAGCGCTTTTAGAAGTTAACAAAGGTCATGAAATGA GTCCACAAGTTGCTACGTTAATTGATAGATTTCGTTAAAGGCCGTGG TCAATTAGATAAAGATACTTTAGATACATTAACAGCATTTTATCCT GGCTACTTATGCAGTTTATCACCAGAAGAATTAAGTTCCGTTCCAC CGAGTAGTATCTGGGCAGTTTCGTCCGCAAGATTTAGATACATGCGA CCCACGTCAATTAGATGTTTTATATCCAAAAGCAAGATTAGCTTTC CAAAATATGAACGGTAGTGAATATTTTCGTAAAAATTCATCCTTTT TAGGTGGTGCACCAACTGAAGATCTAAAAGCATTAAGCCAACAAAA TGTAAGTATGGATTTAGCTACGTTTATGAAATTACGTACAGATGCA GTTCTACCATTAACAGTTGCAGAAGTTCAAAAAATTATTAGGTCCAC ACGTAGAAGGATTAAAAGCAGAAGAAGTCACCGTCCAGTTTCGCGA TTGGATTTTACGTCAACGTCAAGATGATTTAGATACATTAGGTTTA GGTTTACAAGGCGCCATGACAGAATATAAATTAGTTGTAGTTGGTG CAGATGGTGTGGTAAAAGTGCATTAACAATTCAATTAATTCAATA

	ATTAATTAAGAGCTC
Fusion protein of ActA-N100 with human mesothelin (deleted SS; deleted GPI) and 12ras. The <i>Bam</i> HI site adds two amino acids (GS). (SEQ ID NO:45)	VGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSL NTDEWEEEEKTEEQPSEVNTGPRYETAREVSSRDIEELE KSNKVKNTNKADLIAMLKAKAEKGGSRTLAGETGQEEA PLDGVLTNPPNISSLSRQLLGFPACAEVSGLSTERVRE LAVALAQKNVKLSTEQLRCLAHRLSEPPEDLDALPLDL LLFLNPDAFSGPQACTRFFSRITKANVDLLPRGAPERQ RLLPAALACWGVRSLLSEADVRLGGLACDLPGRFVA ESAEVLLPRLVSCPGPLDQDQQAARAALQGGGPPYGP PSTWSVSTMDALRGLLPVLGQPIIRSIPOGIVAARQR SSRDPSWRQPRTLILRPRFRREVEKTACPSGKKAREID ESLIFYKKWELEACVDAALLATQMDRVNAIPFTYEQLD VLKHKLDELYPQGYPEVSIQHLGYLFLKMSPEDIRKWN VTSLETLKALLEVNKGHEMSPQVATLIDRFVKGRGQLD KDTLDTLTAFYPGYLCSLSPEELSSVPPSSIWAVRPQD LDTCDPRQLDVLYPKARLAFQNMNGSEYFVKIQSFLGG APTEDLKALSQQNVSMDLATFMKLRTDAVLPLTVAEVQ KLLGPHVEGLKAEERHRPVRDWILRQRQDDLDLGLGL QGAMTEYKLVVVGADGVGKSALTIQLIQ
Fusion protein of ActA-N100 with human mesothelin (deleted SS; deleted GPI) and 12ras. (SEQ ID NO: 155) (Actual amino acid sequence when expressed in <i>Listeria</i> . The nucleic acid encoding ActA-N100, or a fusion protein thereof, contains a valine codon at the N-terminus, but the <i>Listeria</i> actually biosynthesizes a polypeptide starting with methionine, not valine.) The <i>Bam</i> HI site adds two amino acids (GS).	MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSL NTDEWEEEEKTEEQPSEVNTGPRYETAREVSSRDIEELE KSNKVKNTNKADLIAMLKAKAEKGGSRTLAGETGQEEA PLDGVLTNPPNISSLSRQLLGFPACAEVSGLSTERVRE LAVALAQKNVKLSTEQLRCLAHRLSEPPEDLDALPLDL LLFLNPDAFSGPQACTRFFSRITKANVDLLPRGAPERQ RLLPAALACWGVRSLLSEADVRLGGLACDLPGRFVA ESAEVLLPRLVSCPGPLDQDQQAARAALQGGGPPYGP PSTWSVSTMDALRGLLPVLGQPIIRSIPOGIVAARQR SSRDPSWRQPRTLILRPRFRREVEKTACPSGKKAREID ESLIFYKKWELEACVDAALLATQMDRVNAIPFTYEQLD VLKHKLDELYPQGYPEVSIQHLGYLFLKMSPEDIRKWN VTSLETLKALLEVNKGHEMSPQVATLIDRFVKGRGQLD KDTLDTLTAFYPGYLCSLSPEELSSVPPSSIWAVRPQD LDTCDPRQLDVLYPKARLAFQNMNGSEYFVKIQSFLGG APTEDLKALSQQNVSMDLATFMKLRTDAVLPLTVAEVQ KLLGPHVEGLKAEERHRPVRDWILRQRQDDLDLGLGL QGAMTEYKLVVVGADGVGKSALTIQLIQ
ActA promoter and ActA-N100:	AAGCTTGGGAAGCAGTTGGGGTTAACTGATTAAACAAATGTTAGAGAAAAA TTAATTCTCCAAGTGATATTCTTAAATAATTATGAATATTTTTCTTA

N100 coding sequence is native. Tumor antigens are inserted at the BamHI site (GGATCC). (SEQ ID NO:46)	TATTAGCTAATTAAGAAGATAATTAAGTCTAATCCAATTTTAAACGGAA TAAATTAGTGAAAATGAAGGCCGAATTTTCCTTGTTCTAAAAAGGTTGTA TTAGCGTATCACGAGGAGGGAGTATAAGTGGGATTAAATAGATTATGCG TGCGATGATGGTAGTTTTTCATTACTGCCAACTGCATTACGATTAACCCCG ACATAATATTTGCAGCGACAGATAGCGAAGATTCCAGTCTAAACACAGAT GAATGGGAAGAAGAAAAAACAGAAGAGCAGCCAAGCGAGGTAAATACGGG ACCAAGATACGAACTGCACGTGAAGTAAGTTCACGTGATATTGAGGAAC TAGAAAAATCGAATAAAGTGAAAAATACGAACAAAGCAGACCTAATAGCA ATGTTGAAAGCAAAGCAGAGAAAGGT<u>GGATCC</u>
Amino acid sequence of ActAN100: the BamHI site adds two amino acids (GS). (SEQ ID NO:47) (Predicted amino acid sequence if not at N-terminus when expressed in <i>Listeria</i>.)	VGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEK TEEQPSEVNTGPRYETAREVSSRDIEELEKSNKVKNNTNKADLIAMLK AKAEKGGS
Amino acid sequence of ActAN100: the BamHI site adds two amino acids (GS). (SEQ ID NO:156) (Actual amino acid sequence when expressed in <i>Listeria</i>, since the <i>Listeria</i> actually biosynthesizes a polypeptide starting with methionine, not valine.)	MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEK TEEQPSEVNTGPRYETAREVSSRDIEELEKSNKVKNNTNKADLIAMLK AKAEKGGS
Nucleic acid sequence of fusion protein of ActA-N100 with human mesothelin (codon optimized, deleted SS). The BamHI site adds two amino acids (GS). (SEQ ID NO: 160)	GTGGGATTAAATAGATTTATGCGTGCGATGATGGTAGTTTTTCATTACTGC CAACTGCATTACGATTAACCCCGACATAATATTTGCAGCGACAGATAGCG AAGATTCCAGTCTAAACACAGATGAATGGGAAGAAGAAAAACAGAAGAG CAGCCAAGCGAGGTAAATACGGGACCAAGATACGAACTGCACGTGAAGT AAGTTCACGTGATATTGAGGAAGTAGAAAAATCGAATAAAGTGAAAAATA CGAACAAAGCAGACCTAATAGCAATGTTGAAAGCAAAAGCAGAGAAAGGT GGATCCCGTACATTAGCAGGTGAAACAGGTCAAGAAGCAGCACCCTTGA CGGTGTATTAAACGAATCCACCAAAATATATCAAGTTTAAAGTCCACGTCAAT TATTAGGTTTTCCATGTGCAGAAGTTTCAGGTTTAAAGTACAGAACGTGTC CGTGAGTTAGCAGTTGCATTAGCACAAAAAACGTTAAATTATCTACAGA ACAGTTACGTTGTTTAGCCCATAGATTAAAGCGAACCACCAGAAGACTTAG ATGCACTTCCTTTAGACCTTCTTTTATTCTTAAATCCAGATGCATTTTCA GGACCACAAGCATGTACACGTTTTTTTAGTCGAATTACAAAAGCCAATGT

	TGATTTATTACCTCGTGGGGCTCCTGAAAGACAACGTTTATTACCTGCTG CATTAGCATGCTGGGGTGTTCGCGGTAGCTTATTAAGTGAAGCCGATGTT CGTGCTTTAGGGGGTTTAGCATGTGATTTACCTGGTCTGTTTCGTTGCAGA ATCAGCAGAAAGTGTTATTACCGAGATTAGTTTCATGCCCAGGACCTTTAG ATCAAGATCAACAAGAGGCAGCTAGAGCAGCTCTTCAAGGAGGAGGCCCA CCATATGGCCCAAGTACATGGAGTGTTTCTACAATGGATGCGTTAAG AGGTTTATTACCGGTTTTAGGACAACCAATTATTTCGTAGTATTCCACAAG GCATTGTAGCAGCATGGCGTCAACGTAGTTCTCGTGATCCGTCTTGGCGA CAACCAGAACGTACAATTCTACGTCCAAGATTTTCGTAGAGAAGTAGAAAA AACGGCGTGCTCTAGTGGCAAAAAAGCACGTGAAATTGATGAAAGTTTAA TTTTTTATAAAAAATGGGAATTAGAAGCATGTGTGTCGATGCAGCATTACTA GCTACACAAATGGATCGTGTTAATGCTATTCCATTACATATGAACAATT AGATGTTTTAAAGCATAAATTAGACGAATTATATCCACAAGGTTATCCAG AATCAGTTATTCAACATTTAGGTTACTTATTTTTAAAAATGAGTCCAGAA GACATACGCAAAATGGAATGTTACAAGTTTAGAAACATTTAAAGCGCTTTT AGAAGTTAACAAAGGTCATGAAATGAGTCCACAAGTTGCTACGTTAATTG ATAGATTCTGTTAAAGGCCGTGGTCAATTAGATAAAGATACTTTAGATACA TTAACAGCATTTTATCCTGGCTACTTATGCAGTTTATCACCAGAAGAATT AAGTTCCGTTCCACCGAGTAGTATCTGGGCAGTTTCGTCCGCAAGATTTAG ATACATGCGACCCACGTCAATTAGATGTTTTATATCCAAAAGCAAGATTA GCTTTCCAAAATATGAACGGTAGTGAATATTTTCGTAAAAATTCATACCTT TTTAGGTGGTGCAACCACTGAAGATCTAAAAGCATTAAAGCAACAAAATG TAAGTATGGATTTAGCTACGTTTATGAAATTACGTACAGATGCAGTTCTA CCATTAACAGTTGCAGAAGTTCAAAAATTATTAGGTCCACACGTAGAAGG ATTAAAAGCAGAAGAACGTACCGTCCAGTTTCGCGATTGGATTTTACGTC AACGTCAAGATGATTTAGATACATTAGGTTTTCAGGTTTACAAGCGGTATT CCGAATGGATATTTAGTGTTAGATTTATCGGTTCAAGAAGCATTAAAGTGG TACACCGTGTTTATTAGGTCCAGGTTTAAACAGTGTTAGCATTAT TATTAGCCAGTACATTAGCT
Amino acid sequence of fusion protein of ActA-N100 with human mesothelin (codon optimized, deleted SS). The <i>Bam</i>HI site adds two amino acids (GS). (SEQ ID NO: 161) (Actual amino acid sequence when expressed in <i>Listeria</i>, since the <i>Listeria</i> actually biosynthesizes a polypeptide starting with methionine, not valine.)	MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEKTEE QPSEVNTGPRYETAREVSSRDIEELEKSNKVKNNTNKADLIAMLKAKAEKG GSRTLGETGQEAAPLDGVLTPNPNISSLSPRQLLGFPCAIVSGLSTERV RELAVALAQKNVKLSTEQLRCLAHRLSEPPEDLDALPLDLLFLNPDAFS GPQACTRFFSRITKANVDLLPRGAPERQRLPAALACWGVRSLLSEADV RALGGLACDLPGRFVAESAIEVLLPRLVSCPGPLDQDQQAARAALQGGGP PYGPPSTWSVSTMDALRGLLPVLGQPIIRSIPQGIVAAWRQRSSRDPSWR QPERTILRPRFRREVEKTACPSGKKAREIDESLIFYKKWELEACVDAALL ATQMDRVNAIPFTYEQLDVLKHKLDELTPQGYPESVIQHLGYLFLKMSPE DIRKWNVTSLKALLEVNKGHEMSPQVATLIDRFVKGGRGLDKDTLDT LTAFYPGYLCSLSPHEELSSVPPSSIWAVRPQDLDTCDPRQLDVLYPKARL AFQNMNGSEYFVKIQSFLGGAPTEDLKALSQQNVSMDLATFMKLRTDAVL PLTVAEVQKLLGPHVEGLKAEERHRPVRDWILRQRQDDLDTLGLGLQGGI PNGYLVLDSLVSQEALSGTPCLLGPVLTVLALLLASTLA

EXAMPLE V. Building blocks used for assembling listeriolysin (LLO; hly gene) fusion proteins.

Nucleic acid of LLO open reading frame (ORF) from wild type <i>Listeria</i> 10403S. (SEQ ID NO:48)	Atgaaaaaaaaataatgctagttttttattacacttatattagttagtcta Ccaattgcgcaacaaactgaagcaaaggatgcatctgcattcaataaa Gaaaattcaattttcatccatggcaccaccagcatctccgcctgcaagt Cctaagacgccaatcgaaaagaaacacgcggatgaaatcgataagtat Atacaaggattggattacaataaaaaacaatgtattagtataaccacgg Agatgcagtgacaaatgtgcccgaagaaaagggttacaagatggaa Atgaatatattgttgtggagaaaaagaagaatccatcaatcaaaat Aatgcagacattcaagttgtgaatgcaatttcgagcctaacctatcc Aggtgctctcgtaaaaagcgaattcgggaattagtagaaaaatcaaccag Atgttctccctgtaaaacgtgattcattaacactcagcattgatttg CcaggatgActAatcaagacaataaaaatcggttgtaaaaaatgccac Taaatcaaacggttaacaacgcagtaaaatcattagtggaagatgga Atgaaaaatattgctcaagcttatccaaatgtaagtgcaaaaattgat Tatgatgacgaaatggcttacagtgaatcacaattaattgcgaaatt Tggtacagcattttaagctgtaaataatagcttgaaatgtaaaacttcg Gcgcaatcagtgaagggaaaaatgcaagaagaagtcattagttttaa CaaatttActAtaacgtgaatgttaatgaacctacaagacctccag AtttttcggcaaagctgttActAaagagcagttgcaagcgcttgga Tgaatgcagaaaaatcctcctgcatatatctcaagtggtggcgatggc CgtcaagtttatttgaaattatcaActAattcccatagttActAaagt Aaaagctgcttttgatgctgccgtaagcggaaaaatctgtctcaggtg AtgtagaActAacaaatatcatcaaaaattcttcccttcaaagccgta Atttacggagggttcgcgaaaagatgaagttcaaatcatcgacggcaa Cctcgggagacttacgcgatattttgaaaaaaggcgctacttttaac Gagaaacaccaggagttcccatgtcttatacaacaaacttcctaaaa Gacaatgaattagctgttattaaaaacaactcagaatatattgaaac Aacttcaaaagcttatacagatggaaaaattaacatcgatcactctg Gaggatacgttgctcaattcaacatttcttgggatgaagtaaattat Gatcctgaaggtaacgaaattgttcaacataaaaaactggagcgaaaa Caataaaagcaagctagctcatttcacatcgctccatctatttgctg Gtaacgcgagaaatattaatgtttacgctaaagaatgcactgggtta Gcttgggaatggtggagaacggttaattgatgaccggaacttaccact Tgtgaaaaatagaaatatctccatctggggcaccacgctttatccga Aatatagtaataaagtagataatccaatcgaataa
Codon optimized LLO (GGATCC is a BamHI site added at the 3' end for in-frame fusions). (SEQ ID NO:49)	Atgaaaaaaaaataatgctagtcctttattacattaatttttagtaagtctaccaatt gca Caacaaaccgaagctaaagatgcatcagcgttcaacaaagaaaattcaattagt tca Atggccccaccagcttctccaccagcatctccaaaaacaccaattgaaaaaaaa cat Gcagacgaaattgataaatatattcaaggttttagattacaataagaataacggtt tta Gtataccacggcgatgcagtaacaaatgtacctccaagaaaaggctataaagac gga Aatgaatatattgttgttgaaaaaaaaaagaaatctattaatcaaaacaatgcc gac Atccaagtagttaacgcgattagctcattgacgtatccaggcgcccttgtaaaa gct Aactctgaattagtggaatcaaccagacgtacttccagtcacacgtgatagt cta Accttaagtattgatttaccaggaatgacaaatcaagataacaaaattgttgtt aaa

	AatgcaActAaatccaatgtaaataatgcagttaacacattagtagaacgatgg aac Gaaaaatacgcacaggcatatcccaaattgtatcagctaaaattgattacgacgac gaa Atggcctactcagaaagtcaattaattgctaaatttggtacagcattcaaagca gtc Aataatagttttaaattgtaaattttggagcgatctctgaaggaaagatgcaggaa gaa Gtaatttcattcaaacaaatttattataatgttaacgtaaattgaaccaaccgt cct TcccgtttctttggcaaagcagttActAaagaacaattacaagcActAggtgtg aat Gcagaaaaccaccggcatatatttcaagcgtcgcttacggacgacaagtttac tta Aaattatctacaaacagtcatagtacaaaagtaaaagcagcattcgatgcagct gtg Tcaggaaaatcagtttagtgagatgtagaattaaccaatattattaaaaattcg agt Tttaaagctgttattttatggaggttctgcaaaagatgaagtacaaattattgac gga Aacttaggcgatttacgtgacattttaaaaaaaggcgcaacatttaatagagaa aca CcaggggttccaattgcttatacaActAattttcttaaagataatgaacttgca gta Attaaaaacaattcagaatacattgaaacaacttcgaaagcatatacagacgga aaa Attaatatgatcactcaggagggtagcttgacaaatttaattagttgggat gaa GtaaActAtgatccagaaggcaatgaaattgtacaacataaaaattggtctgaa aat AacaaatctaaActAgcacactttaccagttctatctatttaccaggaaatgct cgc AatattaatgtttacgcaaaagaatgtaccggattagcatgggaaTGGTGGcgc aca Gttattgacgaccgcaatcttctctagtaaaaaacagaaacatcagcatttg gga acaacgctttatccgaaatacagtaataaagttgataatccaattgaa GGATCC
One mutant variation on codon optimized LLO (as a translational fusion – GGATCC is a BamHI site added at the 3' end for in- frame fusions; mutant variation is in	Atgaaaaaaataatgctagtctttattacattaatttttagtaagtctaccaatt gc Acaacaaaccgaagctaaagatgcatcagcgttcaacaaagaaaattcaattag tt Caatggccccaccagcttctccaccagcatctccaaaaacaccaattgaaaaaa aa Catgcagacgaaattgataaatatattcaagggttagattacaataagaataac gt Tttagtataccacggcgatgcagtaacaaatgtacctccaagaaaaggctataa ag Acggaaatgaatatattggttggtgaaaaaaaaaagaaatctattaatcaaaaca at Gccgacatccaagtagttaacgcgattagctcattgacgtatccaggcgccctt gt Aaaagctaactctgaattagtggaataaaccagacgtacttccagtcacacg tg

CAPS, changes TGGTGG to TTTTTT amino acid changes WW to FF). (SEQ ID NO:50)	Atagtctaaccttaagtattgatttaccaggaatgacaaatcaagataacaaaa tt GttgttaaaaatgcaActAaatccaatgtaaataatgcagttaacacattagta ga Acgatggaacgaaaaatacgcacaggcatacccaaagtatcagctaaaattga tt Acgacgacgaaatggcctactcagaaagtcaattaattgctaaatttggtacag ca Ttcaaagcagtcaataatagttttaaatgtaaattttggagcgatctctgaagga aa Gatgcaggaagaagtaatttcattcaaacaattttattataatgttaacgtaaa tg AaccaaccgctccttcccggtttctttggcaaagcagttActAaagaacaattac aa GcActAggtgtgaatgcagaaaacccaccggcatatatttcaagcgctcgttac gg Acgacaagtttactttaaaattatctacaaacagtcatagtacaaaagtaaaagc ag Cattcgatgcagctgtgtcaggaaaatcagttagtggagatgtagaattaacca at Attattaaaaattcgagttttaagctgttattttatggaggttctgcaaagat ga Agtacaaattattgacggaaacttaggcgatttacgtgacattttaaaaaaagg cg CaacatttaatagagaaacaccaggggttccaattgcttatacaActAattttc tt Aaagataatgaacttgcagtaattaaaaacaattcagaatacattgaaacaact tc Gaaagcatatacagacggaaaaattaatattgatcactcaggaggggtacgttgc ac AatttaatatattagttgggatgaagtaaActAtgatccagaaggcaatgaaattg ta CaacataaaaattgggtctgaaaataacaaatctaaActAgcacactttaccagt tc Tatctatttaccaggaaatgctcgcaatattaatgtttacgcaaaagaatgtac cg GattagcatgggaaTTTTTcgcacagttattgacgaccgcaatcttcctctag ta Aaaaacagaaacatcagcatttggggaacaacgctttatccgaaatacagtaat aa agttgataatccaattgaa GGATCC
Nucleic acid of LLO59 (not codon optimized). (SEQ ID NO:51)	ATGAAAAAATAATGCTAGTTTTTATTACACTTATATT AGTTAGTCTACCAATTGCGCAACAACTGAAGCAAAGG ATGCATCTGCATTCAATAAAGAAAATTCAATTCATCC ATGGCACCACCAGCATCTCCGCCGTGCAAGTCCTAAGAC GCCAATCGAAAAGAAACACGCGGAT

Nucleic acid of LLO59, codon optimized for expression in <i>Listeria</i> . (SEQ ID NO:52)	ATGAAAAAAAAATTATGTTAGTTTTTTATTACATTAATTTT AGTTAGTTTACCAATTGCACAACAAACAGAAGCAAAAG ATGCAAGTGCATTTAATAAAGAAAATAGTATTAGTAGT ATGGCACCACCAGCAAGTCCACCAGCAAGTCCAAAAAC ACCAATTGAAAAAAAAACATGCAGAT
Amino acids of LLO59. (SEQ ID NO:53)	MKKIMLVFITLILVSLPIAQOTEAKDASAFNKENSIS MAPPASPPASPKTPIEKKHAD
Nucleic acid of LLO59, codon optimized for expression in <i>Listeria</i> , with codon optimized human mesothelin (deleted SS; deleted GPI), cloned in frame with LLO as a BamHI/SacI fragment. The BamHI (GGATCC) and SacI (GAGCTC) sites are indicated in BOLD . This construct can be called: LLOpt59-hMesothelin(deleted SS; deleted gpi) fusion. (SEQ ID NO:54)	ATGAAAAAAAAATTATGTTAGTTTTTTATTACATTAATTTT CCAATTGCACAACAAACAGAAGCAAAAGATGCAAGTGCATTTAATAAA GAAAATAGTATTAGTAGTATGGCACCACCAGCAAGTCCACCAGCAAGT CCAAAAACACCAATTGAAAAAAAAACATGCAGAT GGATCC CGTACATTA GCAGGTGAAACAGGTCAAGAAGCAGCACCCTTGACGGTGTATTAAACG AATCCACCAATATATCAAGTTTAAGTCCACGTCAATTATTAGGTTTT CCATGTGCAGAAGTTTCAGGTTTAAGTACAGAACGTGTCCGTGAGTTA GCAGTTGCATTAGCACAAAAAACGTTAAATTATCTACAGAACAGTTA CGTTGTTTAGCCCATAGATTAAGCGAACCACCAGAAGACTTAGATGCA CTTCCTTTAGACCTTCTTTTATTCCTAAATCCAGATGCATTTTCAGGA CCACAAGCATGTACACGTTTTTTTAGTCGAATTACAAAAGCCAATGTT GATTTATTACCTCGTGGGGCTCCTGAAAGACAACGTTTATTACCTGCT GCATTAGCATGCTGGGGTGTTCGCGGTAGCTTATTAAGTGAAGCCGAT GTTCTGTGCTTTAGGGGGTTTAGCATGTGATTTACCTGGTCTGTTCTGTT GCAGAATCAGCAGAAGTGTATTACCGAGATTAGTTTCATGCCCAGGA CCTTTAGATCAAGATCAACAAGAGGCAGCTAGAGCAGCTCTTCAAGGA GGAGGCCCAACATATGGCCCACCAAGTACATGGAGTGTCTTCTACAATG GATGCGTTAAGAGGTTTATTACCGGTTTTAGGACAACCAATTATTTCGT AGTATTCCACAAGGCATTGTAGCAGCATGGCGTCAACGTAGTTCTCGT GATCCGCTCTGGCGACAACCAGAACGTACAATTCACGTCCAAGATTT CGTAGAGAAGTAGAAAAACGGCGTGTCTAGTGGCAAAAAGCACGT GAAATTGATGAAAGTTAATTTTATAAAAAATGGGAATTAGAAGCA TGTGTGATGCAGCATTACTAGCTACACAAATGGATCGTGTAAATGCT ATTCCATTACATATGAACAATTAGATGTTTTAAAGCATAAATTAGAC GAATTATATCCACAAGGTTATCCAGAATCAGTTATTCAACATTTAGGT TACTTATTTTTAAAAATGAGTCCAGAAGACATACGCAATGGAATGTT ACAAGTTTAGAAACATTAAAAGCGCTTTTAGAAGTTAACAAAGGTCAT GAAATGAGTCCACAAGTTGCTACGTTAATTGATAGATTCTGTTAAAGGC CGTGGTCAATTAGATAAAGATACCTTTAGATACATTAACAGCATTTTAT CCTGGCTACTTATGCAGTTTATCACCAGAAGAATTAAGTTCCGTTCCA CCGAGTAGTATCTGGGCAGTTCGTCCGCAAGATTTAGATACATGCGAC CCACGTCAATTAGATGTTTTATATCCAAAAGCAAGATTAGCTTTCCAA AATATGAACGGTAGTGAATATTCGTAAAAATCAATCCTTTTTAGGT GGTGCACCAACTGAAGATCTAAAAGCATTAAAGCCAACAAATGTAAGT ATTGGATTTAGCTACGTTTATGAAATTACGTACAGATGCAGTTCTACCA TTAACAGTTGCAGAAGTTCAAAAATTATTAGGTCCACACGTAGAAGGA TTAAAAGCAGAAGAACGTCACCGTCCAGTTTCGCGATTGGATTTTACGT CAACGTCAAGATGATTTAGATACATTAGGTTTAGGTTTACAAGGCTA AGAGCTC
Amino acids of fusion protein of	MKKIMLVFITLILVSLPIAQOTEAKDASAFNKENSIS MAPPASPPASPKTPIEKKHAD GS RTLGETGQEAAPLDGVLNPPNSSL SPRQLLGFPCEVSGSLSTERVRELAVALAQKNVLSLEQLRCLA

LLO59, codon optimized, with codon optimized human mesothelin (deleted SS; deleted GPI). (SEQ ID NO:55)	HRLSEPPEDLDALPLDLLLFLNPDAFSGPQACTRFFSRITKANV DLLPRGAPERQRLPLAALACWGVRSLLSEADVRLGGLACDLP GRFVAESAIEVLLPRLVSCPGPLDQDQQAARAALQGGGPPYGGP STWSVSTMDALRGLLPVLGQPIIRSIPQGIWAARQRSSRDPSW RQPRTLRLPRFRREVEKTACPSGKKAREIDESLI FYKKWELEA CVDAALLATQMDRVNAIPFTYEQLDVLKHKLDLYPQGYVESVI QHLGYLFLKMSPEDIRKWNVTSLLETLKALLEVNKGHEMSPQVAT LIDRFVKGRGQLDKDTLDTLTA FYPGYLC SLSPHEELSSVPPSSI WAVRPQDLDTCDPRQLDVLYPKARLAFQNMNGSEYFVKIQSFLG GAPTEDLKALSQQNVSMDLATFMKLRTDAVLPLTVAEVQKLLGP HVEGLKAEERHRPVRDWILRQRQDDLDLTLGLGLQG
Nucleic acid of fusion protein of LLO59 (not codon optimized) with human mesothelin (codon optimized) with deleted SS and deleted GPI, as BamHI-SacI fragment. The BamHI site (GGATCC) and the SacI site (GAGCTC) are shown in BOLD. This sequence can be called: LLOnat59 hMesothelin (deleted SS; deleted gpi) fusion. "nat" means natural, not codon optimized. Regarding the amino acid sequences, the amino acid encoded by this sequence is the same as that encoded by the corresponding sequence	ATGAAAAAATAATGCTAGT TTTTATTACACTTATATTAGTTA GTCTACCAAT TGCGCAACAACTGAAGCAAAGGATGCATCTGC ATTCAATAAAGAAAATTCAATTCATCCATGGCACCACCAGCA TCTCCGCCTGCAAGTCCTAAGACGCCAATCGAAAAAGAAACACG CGGATGGATCCCGTACATTAGCAGGTGAAACAGGTCAAGAAGC AGCACCACCTTGACGGTGTATTAAACGAATCCACCAAATATATCA AGTTTAAGTCCACGTCAATTATTAGGTTTTTCATGTGCAGAAG TTTCAGGT TTAAGTACAGAACGTGTCCGTGAGTTAGCAGTTGC ATTAGCACAAAAAACGTTAAATTATCTACAGAACAGTTACGT TGTTTTAGCCCATAGATTAAGCGAACCACCAGAAGACTTAGATG CACTTCCTTTAGACCTTCTTTTATTCTTAAATCCAGATGCATT TTCAGGACCACAAGCATGTACACGTTTTTTTTAGTCGAATTACA AAAGCCAATGTTGATTTATTACCTCGTGGGGCTCCTGAAAGAC AACGTTTATTACCTGCTGCATTAGCATGCTGGGGTGTTTCGCGG TAGCTTATTAAAGTGAAGCCGATGTTTCGTGCTTTAGGGGGTTTA GCATGTGATTTACCTGGTCTGTTTCGTGTCAGAATCAGCAGAAG TGTTATTACCGAGATTAGTTTCATGCCCAGGACCTTTAGATCA AGATCAACAAGAGGCAGCTAGAGCAGCTCTTCAAGGAGGAGGC CCACCATATGGCCCACCAAGTACATGGAGTGT TTTCTACAATGG ATGCGTTAAGAGGTTTTATTACCGGTTTTTAGGACAACCAATTAT TCGTAGTATTTCCACAAGGCATTTGTAGCAGCATGGCGTCAACGT AGTTCTCGTGATCCGTCTTGCGGACAACCAGAACGTACAATTC TACGTCCAAGATTTTCGTAGAGAAGTAGAAAAACGGCGTGTCC TAGTGGCAAAAAAGCACGTGAAATTGATGAAAGTTTAATTTTT TATAAAAAATGGGAATTAGAAGCATGTGTCGATGCAGCATTAC TAGCTACACAAATGGATCGTGTTAATGCTATTCCATTACATA TGAACAATTAGATGTTTTAAAGCATAAATTAGACGAATTATAT CCACAAGGTTATCCAGAATCAGTTATTCAACATTTAGGTTACT TATTTTTTAAAAATGAGTCCAGAAGACATACGCAAAATGGAATGT TACAAGTTTAGAAACATTAAAGCGCTTTTAGAAGTTAACAAA GGTCATGAAATGAGTCCACAAAGTTGCTACGTTAATTGATAGAT TCGTTAAAGGCCGTTGGTCAATTAGATAAAGATACTTTAGATAC ATTAACAGCATTTTATCCTGGCTACTTATGCAGTTTATCACCA GAAGAATTAAGTTCGTTCCACCGAGTAGTATCTGGGCAGTTC GTCCGCAAGATTTAGATACATGCGACCCACGTCAATTAGATGT TTTATATCCAAAAGCAAGATTAGCTTTTCCAAAATATGAACGGT AGTGAATATTTTCGTAAAAATTCATCCTTTTTAGGTGGTGCAC CAACTGAAGATCTAAAAGCATTAAGCCAACAAAATGTAAGTAT GGATTTAGCTACGTTTATGAAATTACGTACAGATGCAGTTCTA CCATTAAACAGTTGCAGAAGTTCAAAAATTATTAGGTCCACAG TAGAAGGATTAAAAGCAGAAGAACGTCACCGTCCAGTTTCGCGA TTGGATTTTACGTCAACGTCAAGATGATTAGATACATTAGGT TTAGGTTTACAAGGCTAAGGAGCTC

where mesothelin is codon optimized. (SEQ ID NO:56)	
<i>hly</i> promoter. (SEQ ID NO:57)	GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACT TTTATGTGGAGGCATTAACATTTGTTAATGACGTCAAAGG ATAGCAAGACTAGAATAAAGCTATAAAGCAAGCATATAATA TTGCGTTTCATCTTTAGAAGCGAATTTGCGCAATATTATAA TTATCAAAGAGAGGGGTGGCAAACGGTATTTGGCATTATT AGGTTAAAAAATGTAGAAGGAGAGTGAAACCC
Nucleic acid for codon-optimized BaPA signal peptide. (SEQ ID NO:58)	ATGAAAAAACGTAAAGTTTTAATTCCATTAATGGCATTAAGTACAA TTTTAGTTAGTAGTACAGGTAATTTAGAAGTTATTCAAGCAGAAGT TGGATCC
Amino acids of BaPA signal peptide. (SEQ ID NO:59)	MKKRKVLIPLMALSTILVSSTGNLEVIQAEVGS
The <i>hly</i> promoter and BaPA signal peptide are fused seamlessly together. The <i>hly</i> promoter and BaPA signal peptide are fused seamlessly together (no restriction sites) and the promoter-signal peptide assembly is inserted into plasmids as a <i>KpnI</i> (GGTACC)- <i>BamHI</i> (GGATCC) fragment. The tumor	GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGG AGGCATTAACATTTGTTAATGACGTCAAAGGATAGCAAGACTAGAATAA AGCTATAAAGCAAGCATATAATATTGCGTTTCATCTTTAGAAGCGAATTT CGCCAATATTATAATTATCAAAGAGAGGGGTGGCAAACGGTATTTGGCA TTATTAGGTTAAAAAATGTAGAAGGAGAGTGAAACCCATGAAAAACGTA AAGTTTTAATTCCATTAATGGCATTAAGTACAATTTTAGTTAGTAGTACA GGTAATTTAGAAGTTATTCAAGCAGAAGTT GGATCC

antigen is inserted at the <i>Bam</i> HI site. (SEQ ID NO:60)	
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EXAMPLE VI. Building blocks used for assembling p60 fusion proteins and fusion proteins other polypeptides that mediate SecA2-dependent secretion.

[0437] The present invention provides a polynucleotide comprising a first nucleic acid encoding a protein secreted by a SecA2-dependent pathway and a second nucleic acid encoding a heterologous antigen. Autolysins, such as p60 and NamA (N-acetyl-muramidase), are proteins secreted from *Listeria* by the SecA2-dependent pathway (Lenz, *et al.* (2003) Proc. Natl. Acad. Sci. USA 100:12432-12437). In one embodiment, the fusion protein partner (e.g., p60 or NamA) retains its enzymatic or structural activity. In another embodiment, the fusion protein partner lacks its enzymatic or structural activity. Yet another embodiment places or inserts a nucleic acid encoding a heterologous protein between the signal sequence (SS) and nucleic acids encoding the cell wall binding domains (LysSM) and catalytic domains Lyz-2 (NamA) and p60-dom (p60).

[0438] The following discloses, as a non-limiting example, nucleic acids encoding fusion proteins comprising p60 and human mesothelin (hMeso). Mesothelin was inserted into *Listeria*'s p60 protein as follows. A nucleic acid encoding mesothelin was inserted into a nucleic acid encoding p60, so that when expressed, mesothelin would be inserted into p60 at amino acid 70. A polynucleotide encoding the resulting fusion protein was prepared for use in expression by a *Listeria* bacterium.

[0439] In another embodiment, protein chimera contained optimal codons for expression in *Listeria* in the p60 amino acids 1-70 as well as in the entire mesothelin coding sequence. In yet another embodiment, the p60-human mesothelin protein chimera was functionally linked to the *L. monocytogenes* hly promoter, incorporated into the pPL2 vector, which was used subsequently to generate recombinant *L. monocytogenes* strains expressing and secreting human mesothelin.

[0440] The sequence of the first 70 amino acids of p60 from *L. monocytogenes*, strain 10403S is disclosed.

M N M K K A T I A A T A G I A V T A F A A P T I A S A S T V V V
E A G D T L W G I A Q S K G T T V D A I K K A N N L T T D K I V
P G Q K L Q (SEQ ID NO:61)

[0441] The synthesized DNA sequence corresponding to the *hly* promoter-70 N-terminal p60 amino acids is shown below. The codons encoding p60 amino acid residues 69 (L) and 70 (Q), were modified to contain a unique *Pst I* enzyme recognition sequence, to facilitate functional insertion of a heterologous sequence (e.g., a nucleic acid encoding mesothelin). Moreover, the 5' end of the synthesized sub-fragment contains a unique *KpnI* enzyme recognition sequence.

[0442] At this point in the commentary on vector synthesis, the nucleic acid sequence corresponds to the following:

***hly* promoter-p60 (70 N-terminal amino acids of p60).**

[0443] The unique *PstI* site (CTGCAG) is visible at the 3'-end.

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCAT
TAACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAG
CAAGCATATAATATTGCGTTTCATCTTTAGAAGCGAATTTTCGCCAATATTATAATT
ATCAAAAGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTA
GAAGGAGAGTGAAACCCATGAATATGAAAAAAGCTACGATTGCAGCTACAGCCG
GCATTGCCGTAACAGCTTTTGCAGCACCAACTATTGCCTCAGCCTCTACAGTTGTT
GTCGAAGCAGGAGACACATTATGGGGAATCGCACAAATCAAAAGGTACAACGGTT
GATGCTATTAAAAAAGCGAATAATTTAACAACAGATAAAATCGTGCCAGGTCAA
AAACTGCAG (SEQ ID NO:62).

[0444] The 447 bp *KpnI* and *PstI* digested sub-fragment is ligated into the corresponding *KpnI* and *PstI* sites of the pPL2 vector, treated by digestion with *KpnI* and *PstI* enzymes and digestion with calf intestinal alkaline phosphatase (CIAP). This plasmid is known as pPL2-hlyP-Np60 CodOp. Subsequently, the remainder of the native p60 gene was cloned into the pPL2-hlyP-Np60 CodOp plasmid, between the unique *Pst I* and *BamHI* sites. The remainder of the p60 gene was cloned by PCR, using a proof-reading containing thermostable polymerase, and the following primer pair:

Forward primer:

5'- CGC CTGCAGGTAAATAATGAGGTTGCTG (SEQ ID NO:63)

Reverse primer:

5'-CGCGGATCCTTAATTATACGCGACCGAAG (SEQ ID NO:64)

[0445] The 1241 bp amplicon is digested with *PstI* and *BamHI*, and the purified 1235 bp is ligated into the pPL2-hlyP-Np60 CodOp plasmid, digested with *PstI* and *BamHI*, and treated with CIAP. The resulting plasmid contains the full p60 gene with optimal codons corresponding to amino acids 1-77, and native codons corresponding to amino acids 78-478. The full p60 gene is linked functional to the *L. monocytogenes hly* promoter.

[0446] At this point in the commentary on vector synthesis, the nucleic acid sequence corresponds to the following:

hly promoter-p60-[70 N-terminal amino acids 1-77 of p60 (codon optimized)]-[*PstI*]-[C-terminal amino acids 78-478 of p60 (non-codon optimized)].

[0447] At this point, the construct has not yet received a nucleic acid encoding a heterologous antigen. In commentary to follow, the unique *PstI* site will receive a nucleic acid encoding a heterologous antigen (mesothelin). This plasmid, which contains full length p60, but with the N-terminal region codon optimized, and the C-terminal region non-codon optimized, is known as: pPL2-hlyP-Np60 CodOp (1-77). The sequence of the *KpnI-BamHI* sub-fragment that contains the hlyP linked functionally to the p60 encoding sequence is shown below (SEQ ID NO:65). The expected sequence of the pPL2-hlyP-Np60 CodOp(1-77) plasmid was confirmed by sequencing.

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GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCAT
TAACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAG
CAAGCATATAATATTGCGTTTCATCTTTAGAAGCGAATTTCGCCAATATTATAATT
ATCAAAAGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTA
GAAGGAGAGTGAAACCCATGAATATGAAAAAAGCTACGATTGCAGCTACAGCCG
GCATTGCCGTAACAGCTTTTGCAGCACCAACTATTGCCTCAGCCTCTACAGTTGTT
GTCGAAGCAGGAGACACATTATGGGGAATCGCACAAATCAAAAGGTACAACGGTT
GATGCTATTAAAAAAGCGAATAATTTAACAACAGATAAAATCGTGCCAGGTCAA
AAACTGCAGGTAAATAATGAGGTTGCTGCTGCTGAAAAAACAGAGAAATCTGTT
AGCGCAACTTGGTTAAACGTCCGTA CTGGCGCTGGTGTTGATAACAGTATTATTA
CGTCCATCAAAGGTGGAACAAAAGTAACTGTTGAAACAACCGAATCTAACGGCT
GGCACAAAATTACTTACAACGATGGAAAAACTGGTTTCGTTAACGGTAAATACTT
AACTGACAAAGCAGTAAGCACTCCAGTTGCACCAACACAAGAAGTGAAAAAAG
AAACTACTACTCAACAAGCTGCACCTGTTGCAGAAACAAAAACTGAAGTAAAC
AAACTACACAAGCAACTACACCTGCGCCTAAAGTAGCAGAAACGAAAGAACTC
CAGTAATAGATCAAAATGCTACTACACACGCTGTCAAAAGCGGTGACACTATTTG
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GGCTTTATCCGTAAAATACGGTGTTTCTGTTCAAGACATTATGTCATGGAATAAT
 TTATCTTCTTCTTCTATTTATGTAGGTCAAAAGCTTGCTATTAAACAAACTGCTAA
 CACAGCTACTCCAAAAGCAGAAGTGAAAACGGAAGCTCCAGCAGCTGAAAAAC
 AAGCAGCTCCAGTAGTTAAAGAAAATACTAACACAAATACTGCTACTACAGAGA
 AAAAAGAAACAGCAACGCAACAACAAACAGCACCTAAAGCACCAACAGAAGCT
 GCAAAACCAGCTCCTGCACCATCTACAAACACAAATGCTAATAAAACGAATACA
 AATACAAATACAAACAATACTAATAACACCATCTAAAAATACTAATACAAACCTCA
 AATACTAATACGAATACAAACTCAAATACGAATGCTAATCAAGGTTCTTCCAACA
 ATAACAGCAATTCAAGTGCAAGTGCTATTATTGCTGAAGCTCAAAAACACCTTGG
 AAAAGCTTATTCATGGGGTGTTAACGGACCAACTACATTTGATTGCTCTGGTTAC
 ACTAAATATGTATTTGCTAAAGCGGGTATCTCCCTTCCACGTACATCTGGCGCAC
 AATATGCTAGCACTACAAGAATTTCTGAATCTCAAGCAAAACCTGGTGATTTAGT
 ATTCTTCGACTATGGTAGCGGAATTTCTCACATTGGTATTTATGTTGGTAATGGTC
 AAATGATTAACGCGCAAGACAATGGCGTTAAATACGATAACATCCACGGCTCTG
 GCTGGGGTAAATATCTAGTTGGCTTCGGTCGCGTATAATAAGGATCC (SEQ ID
 NO:65).

[0448] The next step in the construction is the functional insertion of a heterologous protein encoding sequence at the unique *PstI* site of plasmid as pPL2-hlyP-Np60 CodOp(1-77).

[0449] A nucleic acid encoding human mesothelin that was codon-optimized for optimal expression in *L. monocytogenes* was inserted into the unique *PstI* site of plasmid as pPL2-hlyP-Np60 CodOp (1-77). Specifically, full-length mesothelin, or mesothelin that was deleted of the signal peptide and GPI linker domains (mesothelin Δ SP/ Δ GPI) was cloned from a plasmid that contains the full-length human mesothelin, containing optimal codons for expression in *L. monocytogenes*, using a thermostable polymerase with proof-reading activity, and the primer pair shown below. The present invention provides for other nucleic acids encoding antigens other than mesothelin or in addition to mesothelin, for use in the present protocol. Moreover, the present invention provides for codon-optimization of nucleic acids encoding an antigen, codon-optimization of nucleic acids encoding a fusion protein partner, and/or codon-optimization of nucleic acids encoding a fusion protein partner.

[0450] The skilled artisan will understand that expressions that recite "an antigen was inserted into a polypeptide," or expressions to that effect, can encompass "a first nucleic acid

encoding an antigen was inserted into a second nucleic acid encoding a polypeptide,” and the like.

[0451] PCR Primers used to amplify full length human mesothelin:

Forward Primer (huMeso 3F):

5'-AAACTGCAGGCATTGCCAACTGCACGTCC (SEQ ID NO:66)

Reverse Primer (hMeso 1935R):

5'-AAACTGCAGAGCTAATGTACTGGCTAATAATAATGCTAAC (SEQ ID NO:67)

PCR primers used to amplify human mesothelin (Δ SS Δ GPI anchor).

Forward Primer (huMeso 133F):

5'- CGCCTGCAGCGTACATTAGCAGGTGAAACAGG (SEQ ID NO:68)

Reverse Primer (huMeso 1770R):

5'-CGCCTGCAGGCCTTGTAACCTAAACCTAATGTATC (SEQ ID NO:69)

[0452] In viewing the following embodiments of mesothelin, the skilled artisan will recognize that the disclosed nucleic acids and polypeptides of mesothelin can be inserted or used in into a variety of polypeptide constructs including fusion proteins, nucleic acids encoding fusion proteins and the like, multicistronic constructs, plasmids, vectors, fusion proteins, bacterial vaccines, and the like.

Nucleic acid of the signal peptide of human mesothelin. (SEQ ID NO:70)	GCATTGCCAACTGCACGTCCATTACTAGGTAGTTGC GGTACACCAGCACTAGGTTCTTTATTATTTTGTGA TTTTCTCTAGGTTGGGTCAACCAAGT
Nucleic acid of the GPI anchor of human mesothelin. (SEQ ID NO:71)	GGTATTCCGAATGGATATTTAGTGTTAGATTT ATCTGTTCAAGAAGCATTAAAGTGGTACACCGT GTTTATTAGGTCCAGGTCCAGTTTAAACAGTGT TAGCATTATTATTAGCCAGTACATTAGCT
Human mesothelin	GGATCCGCATTGCCAACTGCACGTCCATTACTAGGTAGTTGCG

nucleic acid cassette, codon optimized for expression in <i>Listeria</i>, with 5'-<i>Bam</i>HI (GGATCC) and 3'-<i>Sac</i>I (GAGCTC) cloning sites. As this is full length mesothelin, it contains the C-terminal gpi anchor domain. (SEQ ID NO:72)	GTACACCAGCACTAGGTTCTTTATTATTTTGTATTCTCT AGGTTGGGTTCAACCAAGTCGTACATTAGCAGGTGAAACAGGT CAAGAAGCAGCACCCTTGACGGTGTATTAACGAATCCACCAA ATATATCAAGTTTAAGTCCACGTCAATTATTAGGTTTTCCATG TGCAGAAGTTTCAGGTTTAAGTACAGAACGTGTCCGTGAGTTA GCAGTTGCATTAGCACAAAAAACGTTAAATTATCTACAGAAC AGTTACGTTGTTTAGCCCATAGATTAAGCGAACCACCAGAAGA CTTAGATGCACCTTCCTTTAGACCTTCTTTTATTCTTAAATCCA GATGCATTTTCAGGACCACAAGCATGTACACGTTTTTTTAGTC GAATTACAAAAGCCAATGTTGATTTATTACCTCGTGGGGCTCC TGAAAGACAACGTTTATTACCTGCTGCATTAGCATGCTGGGT GTTTCGCGGTAGCTTATTAAAGTGAAGCCGATGTTTCGTGCTTAG GGGGTTTAGCATGTGATTTACCTGGTTCGTTTCGTTGCAGAATC AGCAGAAGTGTTATTACCGAGATTAGTTTCATGCCCAGGACCT TTAGATCAAGATCAACAAGAGGCAGCTAGAGCAGCTCTTCAAG GAGGAGGCCACCATATGGCCACCAAGTACATGGAGTGTTTC TACAATGGATGCGTTAAGAGGTTTATTACCGGTTTTAGGACAA CCAATTATTCGTAGTATTCCACAAGGCATTGTAGCAGCATGGC GTCAACGTAGTTCTCGTGATCCGTCTTGGCGACAACCAGAACG TACAATTCTACGTCCAAGATTTTCGTAGAGAAGTAGAAAAACG GCGTGTCCTAGTGGCAAAAAAGCACGTGAAATTGATGAAAGTT TAATTTTTTATAAAAAATGGGAATTAGAAGCATGTGTGATGC AGCATTACTAGCTACACAAATGGATCGTGTTAATGCTATTCCA TTCACATATGAACAATTAGATGTTTTAAAGCATAAATTAGACG AATTATATCCACAAGGTTATCCAGAATCAGTTATTCAACATTT AGGTTACTTATTTTTTAAAAATGAGTCCAGAAGACATACGCAA TGGAATGTTACAAGTTTAGAAACATTAAAAAGCGCTTTTAGAAG TTAACAAAGGTCATGAAATGAGTCCACAAGTTGCTACGTTAAT TGATAGATTTCGTTAAAGGCCGTGGTCAATTAGATAAAGATACT TTAGATACATTAAACAGCATTTTATCCTGGCTACTTATGCAGTT TATCACCAGAAGAATTAAGTTCGTTCCACCGAGTAGTATCTG GGCAGTTCGTCCGCAAGATTTAGATACATGCGACCCACGTCAA TTAGATGTTTTATATCCAAAAGCAAGATTAGCTTTCCAAAATA TGAACGGTAGTGAATATTTTCGTAAAAATTCAATCCTTTTTAGG TGGTGCACCAACTGAAGATCTAAAAGCATTAAAGCCAACAAAT GTAAGTATGGATTTAGCTACGTTTATGAAATTACGTACAGATG CAGTTCTACCATTAAACAGTTGCAGAAGTTCAAAAATTATTAGG TCCACACGTAGAAGGATTAAAAGCAGAAGAACGTCACCGTCCA GTTTCGCGATTGGATTTTACGTCAACGTCAAGATGATTTAGATA CATTAGGTTTAGGTTTACAAGGCGGTATTCCGAATGGATATTT AGTGTTAGATTTATCTGTTCAAGAAGCATTAAAGTGGTACACCG TGTTTATTAGGTCCAGGTCCAGTTTTAACAGTGTTAGCATTAT TATTAGCCAGTACATTAGCTTAAGAGCTC
Amino acids of full length human mesothelin. (SEQ ID NO:73) (If sequence is fused at the N-terminus to a heterologous sequence, the initial methionine is optionally deleted)	MALPTARPLLGSCGTPALGSLLFLLFSLGWVQPSRTL ETGQEAAPLDGVLNPPNIISSLSRQLLGFPCAIEVSG STERVRELAVALAQKNVKLSTEQLRCLAHRLSEPPEDL DALPLDLLLFLNPDAFSGPQACTRFFSRITKANVDLLP RGAPERQRLPAALACWGVRSLLSEADVRLGGLACD LPGRFVAESAIEVLLPRLVSCPGPLDQDQQAARAALQG GGPPYGPSTWSVSTMDALRGLLPVLGQPIIRSIPQGI VAAWRQRSSRDPSWRQPERTILRPRFRREVEKTACPSG KKAREIDESLIIFYKKWELEACVDAALLATQMDRVNAIP FTYEQLDVLKHKLDELYPQGYPESVIQHLGYLFLKMSP EDIRKWNVTSLLETLKALLEVNKGHEMSQVATLIDRFV

(by deletion of the corresponding codon.)	KGRGQLDKDTLDTLTAIFYPGYLCSLSPPELSSVPPSSI WAVRPQDLDTCDPRQLDVLYPKARLAFQNMNGSEYFVK IQSFLGGAPTEDLKALSQQNVSMDLATFMKLRTDAVLP LTVAEVQKLLGPHVEGLKAEERHRPVRDWILRQRQDDL DTLGLGLQGGIPNGYLVLDLSVQEALSGTPCLLGPGPV LTVLALLLASTLA
Human mesothelin nucleic acid (codon optimized), deleted SS, deleted GPI anchor. This is a cassette encoding human mesothelin, where the cassette contains the restriction sites 5'- <i>Bam</i> HI and 3'- <i>Sac</i> I. (SEQ ID NO:74)	GGATCCCGTACATTAGCAGGTGAAACAGGTCAAGAAGCAGCACC ACTTGACGGTGTATTAACGAATCCACCAAATATATCAAGTTTAA GTCCACGTCAATTATTAGGTTTTCCATGTGCAGAAAGTTTCAGGT TTAAGTACAGAACGTGTCCGTGAGTTAGCAGTTGCATTAGCACA AAAAAACGTTAAATTATCTACAGAACAGTTACGTTGTTTAGCCC ATAGATTAAGCGAACCACAGAAGACTTAGATGCACTTCCTTTA GACCTTCTTTTATTCTTAAATCCAGATGCATTTTCAGGACCACA AGCATGTACACGTTTTTTTAGTCTGAATTACAAAAGCCAATGTTG ATTTATTACCTCGTGGGGCTCCTGAAAGACAACGTTTATTACCT GCTGCATTAGCATGTCTGGGGTGTTCGCGGTAGCTTATTAAGTGA AGCCGATGTTTCGTGCTTTAGGGGGTTTAGCATGTGATTTACCTG GTCGTTTCGTTGCAGAATCAGCAGAAGTGTTATTACCGAGATTA GTTTCATGCCCAGGACCTTTAGATCAAGATCAACAAGAGGCAGC TAGAGCAGCTCTTCAAGGAGGAGGCCACCATATGGCCACCAA GTACATGGAGTGTCTTACAATGGATGCGTTAAGAGGTTTATTA CCGGTTTTAGGACAACCAATTATTCGTAGTATTCACAAAGGCAT TGTAGCAGCATGGCGTCAACGTAGTTCTCGTGATCCGTCTTGCG GACAACCAGAACGTACAATTCTACGTCCAAGATTTTCGTAGAGAA GTAGAAAAAACGGCGTGTCCTAGTGGCAAAAAAGCACGTGAAAT TGATGAAAGTTTAATTTTTTATAAAAAATGGGAATTAGAAGCAT GTGTCGATGCAGCATTACTAGCTACACAAATGGATCGTGTTAAT GCTATTCATTACATATGAACAATTAGATGTTTTAAAGCATAA ATTAGACGAATTATATCCACAAGGTTATCCAGAATCAGTTATTC AACATTTAGGTTACTTATTTTTTAAAAATGAGTCCAGAAGACATA CGCAAATGGAATGTTACAAGTTTAGAAACATTAAAAGCGCTTTT AGAAGTTAACAAAGGTCATGAAATGAGTCCACAAGTTGCTACGT TAATTGATAGATTTCGTTAAAGGCCGTGGTCAATTAGATAAAGAT ACTTTAGATACATTAACAGCATTTTATCCTGGCTACTTATGCAG TTTATCACCAGAAGAATTAAGTTCGTTCCACCGAGTAGTATCT GGGCAGTTCGTCCGCAAGATTTAGATACATGCGACCCAGTCAA TTAGATGTTTTATATCCAAAAGCAAGATTAGCTTTCCAAAATAT GAACGGTAGTGAATATTTTCGTAAAAATTCAATCCTTTTTAGGTG GTGCACCAACTGAAGATCTAAAAGCATTAAGCCAACAAAATGTA AGTATGGATTTAGCTACGTTTATGAAATTACGTACAGATGCAGT TCTACCATTAACAGTTGCAGAAGTTCAAAAATTATTAGGTCCAC ACGTAGAAGGATTAAAAGCAGAAGAACGTCACCGTCCAGTTTCGC GATTGGATTTTACGTCAACGTCAAGATGATTAGATACATTAGG TTTAGGTTTACAAGGCTAAGAGCTC
Human mesothelin amino acid, deleted SS, deleted GPI anchor. (SEQ ID NO:75)	RTLAGETGQEAAPLDGVLTNPPNIISSLSRQLLGFP EVSGLSTERVRELAVALAQKNVKLSTEQLRCLAHRLSE PPEDLDALPLDLLFLNPDAFSGPQACTRFFSRITKAN VDLLPRGAPERQRLPAALACWGVRSLLSEADVRLG GLACDLPRFVAESAIEVLLPRLVSCPGLDQDQQAAR AALQGGGPPYPGPSTWSVSTMDALRGLLPVLGQPIIRS IPQGIIVAAWRQRSSRDPSWRQPERTILRPRFRREVEKT ACPSGKKAREIDESLI FYKKWELEACVDAALLATQMDR

	VNAIPFTYEQLDVLKHKLDELYPQGYPESVIOHLGYLF LKMSPEDIRKWNVTSLKALLEVNGHEMSPQVATL IDRFVKGRGQLDKDTLDTLTAFYPGYLCSLSPEELSSV PPSSIWAVRPQDLDTCDPRQLDVLYPKARLAFQNMNGS EYFVKIQSFLGGAPTEDLKALSQQNVSMDLATFMKLRT DAVLPLTVAEVQKLLGPHVEGLKAEERHRPVRDWILRQ RQDDLDTLGLGLQG
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[0453] The PCR amplicons of 1932 bps (full-length mesothelin) and 1637 bps (mesothelin Δ SP/ Δ GPI) were purified, digested with *Pst*I, purified, and ligated into the unique *Pst*I site of plasmid pPL2-hlyP-Np60 CodOp(1-77), treated by digestion with *Pst*I, and digestion with CIAP. The consistent amino terminus to carboxy terminus orientation of the p60 and Mesothelin domains was confirmed by restriction endonuclease mapping. These plasmids are known as pPL2-hlyP-Np60 CodOp(1-77)-mesothelin and pPL2-hlyP-Np60 CodOp(1-77)-mesothelin Δ SP/ Δ GPI, and were introduced into selected *L. monocytogenes* strains.

[0454] The sequence of the *Kpn*I-*Bam*HI sub-fragment of plasmid pPL2-hlyP-Np60 CodOp(1-77)-mesothelin containing the *hly* promoter linked functionally to the p60-human mesothelin protein chimera encoding gene has the sequence shown below.

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GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCAT
TAACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAG
CAAGCATATAATATTGCGTTTCATCTTTAGAAGCGAATTTGCGCCAATATTATAATT
ATCAAAAGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTAAAAAATGTA
GAAGGAGAGTGAAACCCATGAATATGAAAAAAGCTACGATTGCAGCTACAGCCG
GCATTGCCGTAACAGCTTTTGCAGCACCAACTATTGCCTCAGCCTCTACAGTTGTT
GTCGAAGCAGGAGACACATTATGGGGAATCGCACAAATCAAAGGTACAACGGTT
GATGCTATTAAAAAAGCGAATAATTTAACAACAGATAAAATCGTGCCAGGTCAA
AACTGCAGGCATTGCCAACTGCACGTCCATTACTAGGTAGTTGCGGTACACCAG
CACTAGGTTCTTTATTATTTTTGTTATTTTCTCTAGGTGGGTTCAACCAAGTCGT
ACATTAGCAGGTGAAACAGGTCAAGAAGCAGCACCCTTGACGGTGTATTAACG
AATCCACCAAATATATCAAGTTTAAGTCCACGTCAATTATTAGGTTTTCCATGTG
CAGAAGTTTCAGGTTTAAGTACAGAACGTGTCCGTGAGTTAGCAGTTGCATTAGC
ACAAAAAACGTTAAATTATCTACAGAACAGTTACGTTGTTTAGCCCATAGATTA
AGCGAACCACCAGAAGACTTAGATGCACTTCCTTTAGACCTTCTTTTATTCTTAA
ATCCAGATGCATTTTCAGGACCACAAGCATGTACACGTTTTTTTAGTCGAATTAC

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AAAAGCCAATGTTGATTTATTACCTCGTGGGGCTCCTGAAAGACAACGTTTATTA
CCTGCTGCATTAGCATGCTGGGGTGTTCGCGGTAGCTTATTAAGTGAAGCCGATG
TTCGTGCTTTAGGGGGTTTAGCATGTGATTTACCTGGTTCGTTTCGTTGCAGAATCA
GCAGAAGTGTTATTACCGAGATTAGTTTCATGCCCAGGACCTTTAGATCAAGATC
AACAAGAGGCAGCTAGAGCAGCTCTTCAAGGAGGAGGCCACCATATGGCCCAC
CAAGTACATGGAGTGTCTTCTACAATGGATGCGTTAAGAGGTTTATTACCGGTTTT
AGGACAACCAATTATTCGTAGTATTCCACAAGGCATTGTAGCAGCATGGCGTCAA
CGTAGTTCTCGTGATCCGTCTTGGCGACAACCAGAACGTACAATTCTACGTCCAA
GATTCGTAGAGAAGTAGAAAAAACGGCGTGTCTAGTGGCAAAAAAGCACGTG
AAATTGATGAAAGTTTAATTTTTTATAAAAAATGGGAATTAGAAGCATGTGTCTGA
TGCAGCATTACTAGCTACACAAATGGATCGTGTTAATGCTATTCCATTCACATAT
GAACAATTAGATGTTTTAAAGCATAAATTAGACGAATTATATCCACAAGGTTATC
CAGAATCAGTTATTCAACATTTAGGTTACTTATTTTTAAAAATGAGTCCAGAAGA
CATACGCAAATGGAATGTTACAAGTTTAGAAACATTAAAAGCGCTTTTAGAAGTT
AACAAAGGTCATGAAATGAGTCCACAAGTTGCTACGTTAATTGATAGATTCGTTA
AAGGCCGTGGTCAATTAGATAAAGATACTTTAGATACATTAAACAGCATTTTATCC
TGGCTACTTATGCAGTTTATCACCAGAAGAATTAAGTTCCGTTCCACCGAGTAGT
ATCTGGGCAGTTCGTCCGCAAGATTTAGATACATGCGACCCACGTCAATTAGATG
TTTTATATCCAAAAGCAAGATTAGCTTTCCAAAATATGAACGGTAGTGAATATTT
CGTAAAAATTCAATCCTTTTTTAGGTGGTGCACCAACTGAAGATCTAAAAGCATT
AGCCAACAAAATGTAAGTATGGATTTAGCTACGTTTATGAAATTACGTACAGATG
CAGTTCTACCATTAAACAGTTGCAGAAGTTCAAAAATTATTAGGTCCACACGTAGA
AGGATTAAGCAGAAAGAACGTCACCGTCCAGTTCGCGATTGGATTTTACGTCA
ACGTCAAGATGATTTAGATACATTAGGTTTAGGTTTACAAGGCGGTATTCCGAAT
GGATATTTAGTGTTAGATTTATCTGTTCAAGAAGCATTAAAGTGGTACACCGTGTT
TATTAGGTCCAGGTCCAGTTTTAACAGTGTTAGCATTATTATTAGCCAGTACATTA
GCTCTGCAGGTAAATAATGAGGTTGCTGCTGCTGAAAAACAGAGAAATCTGTT
AGCGCAACTTGGTTAAACGTCCGTAAGTGGCGCTGGTGTTGATAACAGTATTATTA
CGTCCATCAAAGGTGGAACAAAAGTAACTGTTGAAACAACCGAATCTAACGGCT
GGCACAAAATTACTTACAACGATGGAATAAAGTGGTTTCGTTAACGGTAAATACTT
AACTGACAAAGCAGTAAGCACTCCAGTTGCACCAACACAAGAAGTGAAAAAAG
AACTACTACTCAACAAGCTGCACCTGTTGCAGAAACAAAAACTGAAGTAAAC
AACTACACAAGCAACTACACCTGCGCCTAAAGTAGCAGAAACGAAAGAACTC
CAGTAATAGATCAAAATGCTACTACACACGCTGTCAAAAGCGGTGACACTATTTG

GGCTTTATCCGTAAAATACGGTGTTTCTGTTCAAGACATTATGTCATGGAATAAT
 TTATCTTCTTCTTCTATTTATGTAGGTCAAAAGCTTGCTATTAAACAAACTGCTAA
 CACAGCTACTCCAAAAGCAGAAGTGAAAACGGAAGCTCCAGCAGCTGAAAAAC
 AAGCAGCTCCAGTAGTTAAAGAAAATACTAACACAAATACTGCTACTACAGAGA
 AAAAAGAAACAGCAACGCAACAACAAACAGCACCTAAAGCACCAACAGAAGCT
 GCAAAACCAGCTCCTGCACCATCTACAAACACAAATGCTAATAAAACGAATACA
 AATACAAATACAAACAATACTAATAACACCATCTAAAAATACTAATACAAACCTCA
 AATACTAATAACGAATACAAACTCAAATACGAATGCTAATCAAGGTTCTTCCAACA
 ATAACAGCAATTCAAGTGCAAGTGCTATTATTGCTGAAGCTCAAAAACACCTTGG
 AAAAGCTTATTCATGGGGTGGTAACGGACCAACTACATTTGATTGCTCTGGTTAC
 ACTAAATATGTATTTGCTAAAGCGGGTATCTCCCTTCCACGTACATCTGGCGCAC
 AATATGCTAGCACTACAAGAATTTCTGAATCTCAAGCAAAACCTGGTGATTTAGT
 ATTCTTCGACTATGGTAGCGGAATTTCTCACATTGGTATTTATGTTGGTAATGGTC
 AAATGATTAACGCGCAAGACAATGGCGTTAAATACGATAACATCCACGGCTCTG
 GCTGGGGTAAATATCTAGTTGGCTTCGGTCGCGTATAATAAGGATCC (SEQ ID
 NO:136).

[0455] The sequence of the *KpnI*-*BamHI* sub-fragment of plasmid pPL2-hlyP-Np60
 CodOp(1-77)-mesothelin Δ SS/ Δ GPI containing the *hly* promoter linked functionally to the
 p60-human mesothelin Δ SS/ Δ GPI protein chimera encoding gene has the sequence shown
 below.

GGTACCTCCTTTGATTAGTATATTCCTATCTTAAAGTTACTTTTATGTGGAGGCAT
 TAACATTTGTTAATGACGTCAAAAGGATAGCAAGACTAGAATAAAGCTATAAAG
 CAAGCATATAATATTGCGTTTCATCTTTAGAAGCGAATTTGCGCCAATATTATAATT
 ATCAAAAGAGAGGGGTGGCAAACGGTATTTGGCATTATTAGGTTAAAAAATGTA
 GAAGGAGAGTGAAACCCATGAATATGAAAAAAGCTACGATTGCAGCTACAGCCG
 GCATTGCCGTAACAGCTTTTGCAGCACCAACTATTGCCTCAGCCTCTACAGTTGTT
 GTCGAAGCAGGAGACACATTATGGGGAATCGCACAATCAAAAGGTACAACGGTT
 GATGCTATTAAAAAAGCGAATAATTTAACAACAGATAAAATCGTGCCAGGTCAA
 AAACCTGCAGCGTACATTAGCAGGTGAAACAGGTCAAGAAGCAGCACCACTTGAC
 GGTGTATTAAACGAATCCACCAAATATATCAAGTTTAAGTCCACGTCAATTATTAG
 GTTTTCCATGTGCAGAAGTTTCAGGTTTAAGTACAGAACGTGTCCGTGAGTTAGC

AGTTGCATTAGCACAAAAAACGTTAAATTATCTACAGAACAGTTACGTTGTTTA
GCCCATAGATTAAGCGAACCACCAGAAGACTTAGATGCACTTCCTTTAGACCTTC
TTTTATTCTTAAATCCAGATGCATTTTCAGGACCACAAGCATGTACACGTTTTTTT
AGTCGAATTACAAAAGCCAATGTTGATTTATTACCTCGTGGGGCTCCTGAAAGAC
AACGTTTATTACCTGCTGCATTAGCATGCTGGGGTGTTTCGCGGTAGCTTATTAAG
TGAAGCCGATGTTTCGTGCTTTAGGGGGTTTAGCATGTGATTTACCTGGTCGTTTTCG
TTGCAGAATCAGCAGAAGTGTTATTACCGAGATTAGTTTCATGCCCAGGACCTTT
AGATCAAGATCAACAAGAGGCAGCTAGAGCAGCTCTTCAAGGAGGAGGCCACC
ATATGGCCCACCAAGTACATGGAGTGTTTCTACAATGGATGCGTTAAGAGGTTTA
TTACCGGTTTTAGGACAACCAATTATTCGTAGTATTCCACAAGGCATTGTAGCAG
CATGGCGTCAACGTAGTTCTCGTGATCCGTCTTGGCGACAACCAGAACGTACAAT
TCTACGTCCAAGATTTTCGTAGAGAAGTAGAAAAAACGGCGTGTCTAGTGGCAA
AAAAGCACGTGAAATTGATGAAAGTTTAATTTTTTATAAAAAATGGGAATTAGA
AGCATGTGTCGATGCAGCATTACTAGCTACACAAATGGATCGTGTTAATGCTATT
CCATTCACATATGAACAATTAGATGTTTTAAAGCATAAATTAGACGAATTATATC
CACAAGGTTATCCAGAATCAGTTATTCAACATTTAGGTTACTTATTTTTAAAAAT
GAGTCCAGAAGACATACGCAAATGGAATGTTACAAGTTTAGAAACATTAAAAGC
GCTTTTAGAAGTTAACAAAGGTCATGAAATGAGTCCACAAGTTGCTACGTTAATT
GATAGATTCGTTAAAGGCCGTGGTCAATTAGATAAAGATACTTTAGATACATTAA
CAGCATTTTATCCTGGCTACTTATGCAGTTTATCACCAGAAGAATTAAGTTCCGTT
CCACCGAGTAGTATCTGGGCAGTTCGTCCGCAAGATTTAGATACATGCGACCCAC
GTCAATTAGATGTTTTATATCCAAAAGCAAGATTAGCTTTCCAAAATATGAACGG
TAGTGAATATTTTCGTAAAAATTCAATCCTTTTTTAGGTGGTGCACCAACTGAAGAT
CTAAAAGCATTAAGCCAACAAAATGTAAGTATGGATTTAGCTACGTTTATGAAAT
TACGTACAGATGCAGTTCTACCATTAAACAGTTGCAGAAGTTCAAAAATTATTAGG
TCCACACGTAGAAGGATTAAAAGCAGAAGAACGTACCCGTCCAGTTCGCGATTG
GATTTTACGTCAACGTCAAGATGATTTAGATACATTAGGTTTAGGTTTACAAGGC
CTGCAGGTAAATAATGAGGTTGCTGCTGCTGAAAAAACAGAGAAATCTGTTAGC
GCAACTTGGTTAAACGTCCGTACTGGCGCTGGTGTTGATAACAGTATTATTACGT
CCATCAAAGGTGGAACAAAAGTAACTGTTGAAACAACCGAATCTAACGGCTGGC
ACAAAATTACTTACAACGATGGAAAAACTGGTTTCGTTAACGGTAAATACTTAAC
TGACAAAGCAGTAAGCACTCCAGTTGCACCAACACAAGAAGTGAAAAAGAAA
CTACTACTCAACAAGCTGCACCTGTTGCAGAAACAAAACTGAAGTAAACAAA
CTACACAAGCAACTACACCTGCGCCTAAAGTAGCAGAAACGAAAGAACTCCAG

TAATAGATCAAAATGCTACTACACACGCTGTCAAAAGCGGTGACACTATTTGGGC
 TTTATCCGTAAAATACGGTGTCTTCTGTTCAAGACATTATGTCATGGAATAATTTAT
 CTTCTTCTTCTATTTATGTAGGTCAAAAGCTTGCTATTAAACAAACTGCTAACACA
 GCTACTCCAAAAGCAGAAGTGAAAACGGAAGCTCCAGCAGCTGAAAAACAAGC
 AGCTCCAGTAGTTAAAGAAAATACTAACACAAATACTGCTACTACAGAGAAAAA
 AGAAACAGCAACGCAACAACAACAGCACCTAAAGCACCAACAGAAGCTGCAA
 AACCAGCTCCTGCACCATCTACAAACACAAATGCTAATAAAAACGAATACAAATA
 CAAATACAAACAATACTAATACACCATCTAAAAATACTAATACAAACTCAAATA
 CTAATACGAATACAAACTCAAATACGAATGCTAATCAAGGTTCTTCCAACAATAA
 CAGCAATTCAAGTGCAAGTGCTATTATTGCTGAAGCTCAAAAACACCTTGGA
 AGCTTATTCATGGGGTGGTAACGGACCAACTACATTTGATTGCTCTGGTTACACT
 AAATATGTATTTGCTAAAGCGGGTATCTCCCTTCCACGTACATCTGGCGCACAAT
 ATGCTAGCACTACAAGAATTTCTGAATCTCAAGCAAAACCTGGTGATTAGTATT
 CTTGACTATGGTAGCGGAATTTCTCACATTGGTATTTATGTTGGTAATGGTCAA
 ATGATTAACGCGCAAGACAATGGCGTTAAATACGATAACATCCACGGCTCTGGC
 TGGGGTAAATATCTAGTTGGCTTCGGTCGCGTATAATAAGGATCC. (SEQ ID
 NO:76)

EXAMPLE VII. ActA-N100-based fusion proteins; LLO-based fusion proteins (synthesis; vaccination; immunogenicity).

[0456] Table 11 discloses some of the bacterial strains that were prepared. The bacteria were used for vaccination into tumor-bearing mice. Where indicated, vaccination resulted in anti-tumor immune responses, reduction in tumor number and size, and increased survival.

Table 11. Recombinant <i>L. monocytogenes</i> bacteria of the present invention. "Delta" means deleted. The E30R mutation and the E30M mutation, where indicated, occur in the Bacillus Protective Antigen (BaPA) secretory sequence. The S28D mutation and S28R mutation, where indicated, occur in p60.					
Strain (trivial name)	Construct	Genetic background	Locus of integration	Promoter	Secretory sequence (SS)
--	Full length (FL) hMesothelin	Δ ActA Δ inlB	tRNA Arg	Hly	BaPA
hMeso1	hMeso [Δ SS Δ GPI]	Δ ActA Δ inlB	tRNA Arg	Hly	BaPA
hMeso2	HMeso [Δ SS Δ GPI]	Δ ActA Δ inlB prfA*	tRNA Arg	Hly	BaPA
hMeso3	hMeso [Δ SS Δ GPI]	Δ ActA Δ inlB	ActA	ActA	BaPA

Strain (trivial name)	Construct	Genetic background	Locus of integration	Promoter	Secretory sequence (SS)
hMeso4	HMeso [deltaSS deltaGPI]	Δ ActA Δ inlB	inlB	Hly	BaPA
hMeso5	p60-hMeso [deltaSS deltaGPI]	Δ ActA Δ inlB	tRNA Arg	Hly	p60
hMeso6	ActA-N100 hMeso [deltaSS deltaGPI]	Δ ActA Δ inlB	ActA	act	ActA
hMeso8	hMeso [deltaSS deltaGPI]- rasG12D	Δ ActA Δ inlB	tRNA Arg	hly	BaPA
hMeso10	ActA-N100 hMeso [deltaSS deltaGPI]-rasG12D	Δ ActA Δ inlB	ActA	ActA	ActA
hMeso11	HMeso [deltaSS deltaGPI]- rasG12D	Δ ActA Δ inlB	inlB	Hly	BaPA
hMeso12	hMeso [deltaSS deltaGPI]- rasG12D	Δ ActA Δ inlB	tRNA Arg	Hly	BaPA (E30R)
hMeso13	hMeso [deltaSS deltaGPI]- rasG12D	Δ ActA Δ inlB	tRNA Arg	hly	BaPA (E30M)
hMeso14	LLO62-hMeso [deltaSS deltaGPI]-rasG12D	Δ ActA Δ inlB	tRNAArg	hly	LLO(62)
hMeso15	LLOopt62 hMeso [deltaSS deltaGPI]-rasG12D	Δ ActA Δ inlB	tRNA Arg	Hly	LLO(opt62)
hMeso18	A30R ActA-N100-hMeso [deltaSS deltaGPI]-12ras (the ras has a G12D mutation)	Δ ActA Δ inlB	ActA	ActA	ActA (A30R)
hMeso19	S28D p60hMeso [deltaSS deltaGPI]	Δ ActA Δ inlB	tRNA Arg	hly	p60
hMeso20	S28R deltap60hMeso [deltaSS deltaGPI]	Δ ActA Δ inlB	tRNA Arg	hly	p60
hMeso22	LLO441-hMeso [deltaSS deltaGPI]-rasG12D	Δ ActA Δ inlB	tRNA Arg	hly	LLO
hMeso26	ActA-N100 hMeso [deltaSS deltaGPI]	Δ ActA Δ inlB	inlB	ActA	ActA
hMeso31	ActA-N100 (A30R in ActA-N100)-hMeso [deltaSS deltaGPI] diploid	Δ ActA Δ inlB	ActA and inlB	ActA and ActA	ActA and ActA
hMeso32	ActA-N100-hMeso [deltaSS deltaGPI] diploid	Δ ActA Δ inlB	inlB and tRNA ^{Arg}	ActA and ActA	ActA and ActA
hMeso33	ActA-N100 deltaSS (containing GPI)	Δ ActA Δ inlB	tRNA ^{Arg} integrated with pINT	ActA	ActA

Strain (trivial name)	Construct	Genetic background	Locus of integration	Promoter	Secretory sequence (SS)
hMeso37	ActA-N100 [deltaSS] (containing GPI)	Δ ActA Δ inlB	tRNA ^{Arg} integrated with pINT	ActA	ActA
hMeso37 differs from hMeso33 in that hMeso37 was treated with a plasmid encoding Cre recombinase to effect removal of loxP-flanked DNA. Cre recombinase was provided via the plasmid pCON2. pCON2 is temperature sensitive. Shifting temperature results in removal of loxP-flanked DNA and results in loss of pCON2 from the cell. pCON is described (see, e.g., Behari, <i>et al.</i> (1998) <i>J. Bacteriol.</i> 180:6316-6324; Milenbachs, <i>et al.</i> (2004) <i>Microbiology</i> 150:321-333).					
hMeso38	ActA-N100-hmeso [deltaSS] (not deleted in GPI). (hmeso33allele)	Δ ActA Δ inlB	inlB	ActA	ActA
hMeso40 (see Table 12)	hMeso26 with this additional integration: pINT-ActA-N100-dbl2ras3	Δ ActA Δ inlB	inlB and tRNA ^{Arg}	ActA and ActA	ActA and ActA
hMeso41 (see Table 12)	hmeso26 with this additional integration: pINT-ActA-N100-dbl-12ra s4	Δ ActA Δ inlB	inlB and tRNA ^{Arg}	ActA and ActA	ActA and ActA
hMeso42 (see Table 12)	hMeso26 with this additional integration: pINT-ActA-N100-dbl-12ra s5	Δ ActA Δ inlB	inlB and tRNA ^{Arg}	ActA and ActA	ActA and ActA
hMeso43 (see Table 12)	hMeso26 with this additional integration: pINT-ActA-N100- dbl-12ras6	Δ ActA Δ inlB	inlB and tRNA ^{Arg}	ActA and ActA	ActA and ActA
Where a polynucleotide is integrated at the ActA locus, the ActA gene is deleted during homologous recombination, unless otherwise specified. Where a polynucleotide is integrated at the ActA locus, and where the construct comprises a fusion protein that includes ActA-N100, and where the secretory sequence is listed as the ActA secretory sequence, the ActA secretory sequence comes from the ActA-N100 fusion protein partner (not from the genomic ActA gene, for the reason that the genomic ActA gene was deleted during homologous recombination), as in hMeso6, hMeso10, and hMeso18.					

Table 12. Sequences in expression cassettes of hMeso40, hMeso41, hMeso42, and hMeso43. "ActA-N100" indicates that the ActA-N100 sequence immediately precedes the indicated amino acids that follow.

Db112ras3 sequence of hMeso40	(ActA-N100) GSAKVLEEDDEEALPTARPLLGSCGTPALGSLFLFLFSLGWVQ PSRTLAGEGTGQEAEEDEEEADLVLAQVLMTEYKLVVVGADGVGKSALTIQLIQ ADLVLAQVLMTEYKLVVVGAVGVGKSALTIQLIQADLVLAQVLESIINFEKLAD LVAEQKLISEEDLV (SEQ ID NO:77)
Db112ras4 sequence of hMeso41	(ActA-N100) GSAKVLEEDDEETPALGSLFLFLFSLGWVQPEEEDDEEADLVLAQ VLMTEYKLVVVGADGVGKSALTIQLIQADLVLAQVLMTEYKLVVVGAVGVGKSA LTIQLIQADLVLAQVLESIINFEKLADLVAEQKLISEEDLV (SEQ ID NO:78)

Db112ras5 sequence of hMeso42	(ActAN100) GSAKVLMT Y KL V VVGADGVGKSALT I QLIQADLV L AKVLMTEY KL V VVGAVGVGKSALT I QLIQADLV L AKVLEE E EE E ALPTARPLL G SCGTPAL G SLLFLLFSLG V WVQPSRTL A GETGQEA A EE E EE E ADLV L AKVLESI I NFEKLADL VAEQKLISEEDLV (SEQ ID NO:79)
Db112ras6 sequence of hMeso43	(ActAN100) GSAKVLMT Y KL V VVGADGVGKSALT I QLIQADLV L AKVLMTEY KL V VVGAVGVGKSALT I QLIQADLV L AKVLEE E EE E ETPALGSLLFLLFSLG V WVQ PEE E EE E ADLV L AKVLESI I NFEKLADLV A EQKLISEEDLV (SEQ ID NO:80)
Identification of details within above sequences	
rasG12D (a.k.a. 12rasD)	MTEYKL V VVGADGVGKSALT I QLIQ (SEQ ID NO:81)
rasG12V (a.k.a. 12rasV)	MTEYKL V VVGAVGDGKSALT I QLIQ (SEQ ID NO:82)
Meso secretory sequence (MesoSS)	ALPTARPLL G SCGTPALGSLLFLLFSLG V WVQPSRTL A GETGQEA A (SEQ ID NO:83)
MesoA2 epitope occurring within MesoSS	TPALGSLLFLLFSLG V WVQ (SEQ ID NO:84)
Spacer	EEDEEE (SEQ ID NO:85)

[0457] The following *Listeria* Δ ActA Δ inlB constructs, suitable as control constructs, were found not to detectably express: (1) hMeso deltaSS deltaGPI ras (the ras had a G12D mutation). This construct had an ActA promoter, BaPA signal sequence, with an ActA locus of integration; (2) A30R ActA-N100 hMeso Δ SS Δ GPI ras (the ras had a G12D mutation). This construct had an ActA promoter, BaPA signal sequence, with an ActA locus of integration; and (3) A30L ActA-N100 hMeso Δ SS Δ GPI ras (the ras had a G12D mutation). This particular construct had an ActA promoter, ActA signal sequence, with an ActA locus of integration.

[0458] Promoters of the present invention can include one or more of the following operably linked with a nucleic acid encoding an antigen: hly, ActA, p60, pHyper, and so on. pHyper is disclosed (see, e.g., U.S. Pat. Appl. 2005/0147621 of Higgins, *et al.*). The present invention provides signal sequences, such as one or more of the signal sequence of LLO, ActA, BaPA, BsPhoD, p60, and so on. Fusion protein partners of the present invention can include one or more of LLO₁₋₆₂, LLO₁₋₄₄₁, ActA-N100, p60, PFO₁₋₃₉₀, BaPA₁₋₈₂, and the like.

[0459] Constructs containing an ActA-based fusion protein partner or an LLO-based fusion protein partner were synthesized as follows. When synthesis was complete, the construct was integrated into the genome of *L. monocytogenes*. While integration was mediated by vectors such as pKSV7, pPL2, and pINT, the present invention is not limited to any particular integration vector or mechanism of integration. Various polynucleotides were assembled in a modular fashion, that is, by ligating prefabricated nucleic acids together on the pKSV7 scaffold. These prefabricated nucleic acids were as follows:

[0460] The ActA promoter/ActA-N100/human Mesothelin Δ SS Δ GPI construct was assembled using the following components: A first polynucleotide consisting of a first nucleic acid encoding native listerial ActA promoter sequence (including the Shine Dalgarno site) connected directly to a second nucleic acid encoding ActA-N100 (the first polynucleotide had a 5'-*HindIII* site and a 3'-*BamHI* site.) A second polynucleotide consisted of human Mesothelin Δ SS Δ GPI (5'-*BamHI* site and 3'-*SacI* site). The pKSV7 received an insert consisting of the first polynucleotide connected directly to the second polynucleotide. In a variation of this construct, the second polynucleotide consisted of a first nucleic acid encoding human Mesothelin Δ ss Δ GPI connected directly to a second nucleic acid encoding 12ras (5'-*BamHI* site and 3'-*SacI* site). Human mesothelin is intended as a non-limiting example.

[0461] The hly promoter/LLO62/ human Mesothelin Δ SS Δ GPI/12ras construct was assembled using the following components. LLO62 means a nucleic acid encoding amino acids 1-62 of listeriolysin (LLO). A first polynucleotide was prepared that consisted of a first nucleic acid encoding native listerial hly promoter sequence (including the Shine Dalgarno site) connected directly to a second nucleic acid encoding LLO62 (the first polynucleotide had a 5'-*KpnI* site and a 3'-*BamHI* site). A second polynucleotide was prepared that consisted of a first nucleic acid encoding human Mesothelin Δ SS Δ GPI connected directly to a second nucleic acid encoding 12ras (the second polynucleotide had a 5'-*BamHI* site and a 3'-*SacI* site.) The pKSV7 received an insert consisting of the first polynucleotide connected directly to the second polynucleotide. A variation of this construct used LLO60 (codon optimized) in place of LLO62.

[0462] Figure 7 discloses a number of embodiments of the present invention, including LLO-based fusion proteins and actA-N100-based fusion proteins.

[0463] Figure 8 discloses expression of various constructs from cell cultures of engineered *Listeria*. In this context, expression means protein biosynthesis and secretion into the medium, where the indicated construct had been integrated into the listerial genome.

Expression was conducted in a medium containing yeast extract without glucose at a bacterial density corresponding to $OD_{600} = 0.8$. The term pPL2 indicates that the construct was inserted by way of site-specific recombination using the vector pPL2, pKSV7 means that the construct was inserted by homologous recombination using the vector pKSV7 (see Table 13).

[0464] The antibody for detecting mesothelin expression was a rabbit polyclonal antibody, produced by immunizing rabbit with three peptides from human mesothelin, where the antibody was purified by a single peptide that is completely conserved between mouse and human mesothelin (SEADVRLGGLAC (SEQ ID NO:86)).

Table 13. Legend for Figure 8.

	Construct	Promoters	Secretory sequences (SS) of construct:	Integration mediated by:
Lane P.	Parent <i>L. monocytogenes</i> Δ ActA Δ inlB.	N.A.	N.A.	N.A.
Lane 1.	pPL2 LLO BaPA Δ SS hMeso Δ SS Δ GPI-12-ras.	hly	LLO	pPL2
Lane 2.	pPL2 LLO BaPA E30R hMeso Δ SS Δ GPI-12-ras.	hly	LLO and BaPA	pPL2
Lane 3.	pPL2 LLO BaPA E30M hMeso Δ SS Δ GPI-12-ras.	hly	LLO and BaPA	pPL2
Lane 4.	pPL2 LLO _{natural} hmeso Δ SS Δ GPI-12-ras.	hly	LLO	pPL2
Lane 5.	pPL2 LLO _{opt} hmeso Δ SS Δ GPI-12-ras.	hly	LLO	pPL2
Lane 6.	pKSV7 ActA::ActA-N100 mMeso Δ SS Δ GPI.	ActA	ActA	pKSV7
Lane 7.	pKSV7 ActA:: ActA-N100 hMeso Δ SS Δ GPI-12-ras.	ActA	ActA	pKSV7
Lane 8.	pKSV7 ActA:: ActA N100 hMeso Δ SS Δ GPI.	ActA	ActA	pKSV7
Lane 9.	pKSV7 inlB::BaPA hMeso Δ SS Δ GPI-12-ras.	inlB	BaPA	pKSV7
Lane 10.	Molecular weight markers.	N.A.	N.A.	N.A.
The double colon of "ActA::ActA-N100" means that the locus of insertion was the ActA gene. LLO means listeriolysin. The hly gene encodes listeriolysin.				

[0465] The results from the gel (Figure 8) show proteins in the supernatant (secreted proteins).

[0466] Lane P, a control experiment using the parental *Listeria*, does not show any obvious stained band.

[0467] Lanes 1-4 show little or no bands.

[0468] Lane 5 shows some secretion of LLO_{opt} hmeso Δ SS Δ GPI-12-ras, where integration was by pPL2-mediated integration in the listerial tRNA^{Arg} gene.

[0469] Lane 6, which represents an attempt to secrete mouse mesothelin, does not show any obvious stained band.

[0470] Lane 7 shows marked secretion of the ActA-N100 hMeso Δ SS Δ GPI-12-ras, where integration was mediated by pKSV7 at the ActA site of the listerial genome.

[0471] Lane 8 shows even greater secretion, where the construct was ActA N100 hMeso Δ SS Δ GPI, and where integration was mediated by pKSV7 at the ActA site of the listerial genome (Figure 7).

[0472] Lane 9 shows little or no band.

[0473] Figure 9 demonstrates protein secretion from *L. monocytogenes* Δ ActA Δ inlB, where the *Listeria* expressed various fusion proteins comprising human mesothelin Δ SS Δ GPI. All mesothelin constructs were expressed from *L. monocytogenes* by nucleic acids codon optimized for *L. monocytogenes*. Various constructs were prepared for the secretion study (see Table 14). In these experiments also, the antibody for detecting mesothelin expression was a rabbit polyclonal antibody, produced by immunizing rabbit with three peptides from human mesothelin, where the antibody was purified by a single peptide that is completely conserved between mouse and human mesothelin (SEADVRLGGLAC (SEQ ID NO:86)).

Table 14. Legend for Figure 9. Western blot analysis for secretion of human mesothelin (hMeso).

Lane	Construct	Promoters	Secretory sequences (SS) of construct:	Integration mediated by:
P.	Parent <i>L. monocytogenes</i> Δ ActA Δ inlB (no mesothelin).	N.A.	N.A.	N.A.
1.	<i>L. monocytogenes</i> Δ ActA Δ inlB LLO441 _{opt} human mesothelin Δ SS Δ GPI-12-ras	ActA	LLO	pPL2.
2.	<i>L. monocytogenes</i> Δ ActA Δ inlB ActA::BaPA ActA-N100(A30R)-human mesothelin Δ SS Δ GPI (clone 2.25).	ActA	BaPA	pKSV7 at ActA locus.
3.	<i>L. monocytogenes</i> Δ ActA Δ inlB ActA::BaPA ActA-N100 (A30R)-human mesothelin Δ SS Δ GPI (clone 2.69).	ActA	BaPA	pKSV7 at ActA locus.
4.	<i>L. monocytogenes</i> Δ ActA Δ inlB ActA::BaPA ActA-N100 (A30R)-human mesothelin Δ SS Δ GPI-12-ras (clone 1.1)	ActA	BaPA.	pKSV7 at ActA locus.
5.	<i>L. monocytogenes</i> Δ ActA Δ inlB	ActA	ActA	pKSV7 at

	ActA::ActA-N100 (A30R)-human mesothelin Δ SS Δ GPI (clone 1.46). A30R indicates mutation in the ActA upon which ActA-N100 is based.			ActA locus.
6.	<i>L. monocytogenes</i> Δ ActA Δ inlB ActA::ActA-N100 (A30R)-human mesothelin Δ SS Δ GPI (clone 2.14). A30R indicates mutation in the ActA upon which ActA-N100 is based.	ActA	ActA	pKSV7 at ActA locus.
7.	<i>L. monocytogenes</i> Δ ActA Δ inlB inlB::ActAN100-human mesothelin Δ SS Δ GPI (clone BH77). ActA-N100 is based on wild type ActA.	inlB	ActA	pKSV7 at inlB locus.
8.	<i>L. monocytogenes</i> Δ ActA Δ inlB inlB::ActAN100-human mesothelin Δ SS Δ GPI (clone BH78). ActA-N100 is based on wild type ActA.	inlB	ActA	pKSV7 at inlB locus.
9.	<i>L. monocytogenes</i> Δ ActA Δ inlB inlB::ActA-N100(A30R)-human mesothelin Δ SS Δ GPI (clone BH85). A30R indicates mutation the ActA upon which ActA-N100 is based.	inlB	ActA	pKSV7 at inlB locus.
10.	<i>L. monocytogenes</i> Δ ActA Δ inlB inlB::ActA-N100(A30R)-human mesothelin Δ SS Δ GPI (clone BH85). A30R indicates mutation the ActA upon which ActA-N100 is based.	inlB	ActA	pKSV7 at ActA locus.
11.	<i>L. monocytogenes</i> Δ ActA Δ inlB ActA-N100 Ndegcon-human mesothelin (clone A11-2).	ActA	ActA.	pKSV7 at ActA locus.
12.	<i>L. monocytogenes</i> Δ ActA Δ inlB ActA-N100 Ndegcon-human mesothelin (clone A11-2).	ActA	ActA.	pKSV7 at ActA locus.
13.	<i>L. monocytogenes</i> Δ ActA Δ inlB ActA-N100 Ndegcon human mesothelin Δ SS Δ GPI-12-ras (clone 1-3).	ActA	ActA.	pKSV7 at ActA locus.
14.	Molecular weight markers.	N.A.	N.A.	N.A.
N.A. means not applicable. The double colon found in "inlB::ActAN100" indicates the locus of the construct, i.e., at the inlB gene. "Ndegcon" refers to constructs that include consensus sequences modeled after the sequences set forth by Suzuki and Varshavsky (1999) EMBO J. 18:6017-6026.				

[0474] The construct used for Lane 1 used LLO441 as the source of secretory sequence, where the nucleic acid for LLO441 had been codon optimized for expression in *L. monocytogenes*, and where the heterologous antigen was human mesothelin Δ SS Δ GPI (Lane 1). This construct produced the highest level of secretion in this particular experiment (Lane 1). The high molecular weight material shown in the western blot represents LLO₄₄₁ fused to mesothelin, where the lower molecular weight material likely represents degradation products.

[0475] The constructs used for Lanes 2 and 4 were based on ActA-N100, but with the ActA's signal sequence deleted and replaced with the the signal sequence of BaPA. Expression from these constructs was relatively low (Lanes 2 and 4) (Figure 9).

[0476] All of the remaining constructs contained full-length ActA-N100 as the source of secretory sequence, but where ActA-N100 had an A30R mutation (Lane 5); where ActA-N100 had no mutation (Lane 7); where ActA-N100 had an A30R mutation (Lane 9); and where ActA-N100 had four mutations (designated "Ndegcon") (Lane 11). The four mutations in ActA-N100 designated by "Ndegcon" were Arg-29, Lys-32, Lys-37, and Lys-44. The Ndegcon was situated (or inserted) in between ActA-N100 and the mesothelin. Secreted protein was collected by precipitation with trichloroacetic acid from mid-exponential cultures grown in yeast extract without glucose.

[0477] Figure 10 shows immune stimulation, as determined after a single vaccination with the indicated *L. monocytogenes* Δ ActA Δ inlB construct, where spleens were harvested seven days after vaccination and used as the source of splenocytes. Mesothelin-specific immune responses were found after vaccination with each of the four constructs: (1) hly promoter was operably linked with BaPA signal sequence and hMeso (integrated at tRNA^{Arg} locus); (2) hly promoter was operably linked with BaPA signal sequence and hMeso (integrated at inlB locus); (3) ActA promoter was operably linked with ActA-N100 and hMeso (integrated at ActA); and (4) hly promoter was operably linked with p60 and hMeso (integrated at tRNA^{Arg} locus).

[0478] The results indicate a role of the ActA promoter in stimulating immune response; a role of the ActA-N100 fusion partner in enhancing immune response; as well as a role of integration at ActA locus in increasing immune response; and demonstrate enhanced ability to stimulate immune response where the ActA promoter is operably linked with ActA-N100 fusion protein partner and integration is at ActA locus (Figure 10).

[0479] Further details of the above study are described as follows. Mice were injected with *Listeria*, followed by a period of time (7 days) to allow the *Listeria* to be taken up and processed by antigen presenting cells (APCs). After uptake of the *Listeria*, the APC presented *Listeria*-encoded antigens to T cells, resulting in the activation and clonal expansion of the T cells. Spleens were removed, and the splenocytes (including T cells and APCs) were isolated. To the isolated splenocytes was added either buffer or a pool of human mesothelin peptides (0.002 mg/ml final concentration of pool). After adding the peptides, the dendritic cells (DCs) in the splenocyte preparation were allowed to present peptide to any activated T cells. Successful presentation resulted in the T cell's secretion of

interferon-gamma, as reflected by signals in spot forming assays (spot forming cells; SFC) (Figure 10).

[0480] The mesothelin peptide pool (also known as 15 x 11 pool) consisted of 153 different peptides, all of them 15 mers, spanning the entire sequence of human mesothelin, where succeeding peptides overlapped by eleven amino acids. The results demonstrated that interferon-gamma (IFGgamma) expression was greater where the peptide pool had been added to the splenocytes, than where no peptide pool was used. Figures 10-12 compare immune response where mice were vaccinated with 1×10^7 CFU or 3×10^7 CFU (Figure 10); 1×10^6 CFU or 1×10^7 CFU (Figure 11); or 1×10^6 CFU or 1×10^7 CFU of *L. monocytogenes* (Figure 12). In most cases disclosed here, immune response was greater where mice were injected with greater numbers of bacteria.

[0481] Figures 11 and 12 disclose similar studies using spot forming cell assays.

[0482] The raw data (photographs of spot forming cell assays) from Figure 11 are graphed in Figure 12. Figure 12 discloses the number of cells that produce an IFNgamma signal (spot forming cell; SPC) per number of splenocytes. The data disclose comparable mesothelin-specific immune responses, where the construct was with hly promoter operably linked with BaPA signal sequence and hMeso (inlB locus), or where the construct was with ActA promoter operably linked with ActA signal sequence and ActA-N100 and hMeso (ActA locus).

[0483] Figures 13-14 disclose tumor metastasis data. The study measured metastasis of CT-26 human mesothelin expressing cells to the lungs. At $t = 0$ days, CT-26 tumor cells were injected i.v. (2×10^5 cells). At $t = 3$ days, mice were administered the indicated *Listeria* vaccine. At $t = 18$ days, lungs were harvested. " 2×10^5 cells" means 2×10^5 cells.

[0484] Tumor cell-inoculated mice were treated as follows: (1) Salt water only (HBSS); (2) *L. monocytogenes* Δ ActA Δ inlB encoding no heterologous antigen (negative control); (3) *L. monocytogenes* Δ ActA Δ inlB encoding the AH1-A5 peptide derived from the gp70 tumor antigen (an antigen different from mesothelin -- positive control); and (4)-(7) *Listeria* Δ ActA Δ inlB encoding various mesothelin constructs. The AH1-A5 peptide is derived from the gp70 tumor antigen. AH1-A5 is used as a positive control in the present experiments (see, e.g., Brockstedt, *et al.* (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; Slansky, *et al.* (2000) Immunity 13:529-538).

[0485] Figure 14 reveals equivalent effects of the four mesothelin-expressing *Listeria* constructs in eliminating tumor metastasis.

Table 15. Groups of mice challenged with CT26 tumor cells and treated with *Listeria* vaccines.

Group	<i>Listeria</i> vaccine	Site of integraton.
1	Hanks Buffered Salt Solution only (HBSS) (no <i>Listeria</i>) (negative control).	no <i>Listeria</i>
2	<i>L. monocytogenes</i> Δ ActA Δ inlB (parental strain) (negative control).	none
3	<i>L. monocytogenes</i> Δ ActA Δ inlB-OVA-AH1-A5. The AH1-A5 epitope was inserted in-frame within OVA by using a unique <i>Ava</i> II site (expressed from <i>hly</i> promoter as part of pPL2 vector) (positive control).	tRNA ^{Arg} locus
4	<i>L. monocytogenes</i> Δ ActA Δ inlB <i>prfA</i> * (E77K)-BaPa signal sequence-human Mesothelin Δ SS Δ GPI (see, e.g., Mueller and Freitag (2005) Infect. Immun. 73:1917-1926).	ActA locus
5	<i>L. monocytogenes</i> Δ ActA Δ inlB-BaPa signal sequence-human mesothelin Δ SS Δ GPI (expressed from ActA promoter).	ActA locus
6	<i>L. monocytogenes</i> Δ ActA Δ inlB-BaPa signal sequence-human mesothelin Δ SS Δ GPI (expressed from <i>hly</i> promoter).	inlB locus
7	<i>L. monocytogenes</i> Δ ActA Δ inlB-ActA signal sequence-ActA-N100-human mesothelin Δ SS Δ GPI (expressed from ActA promoter).	ActA locus

[0486] Figure 15 demonstrates that various mesothelin-expressing *Listeria* are effective in reducing lung tumors, where three different doses of each mesothelin-expressing *Listeria* were tested. hMeso6 is more effective than, for example, hMeso2 or hMeso4, in stopping lung metastasis (Figure 15).

[0487] Figure 16 discloses survival to tumors with various listerial vaccines. With negative control treatments (HBSS; parental *Listeria*), none of the mice survived beyond 22 days. The positive control *Listeria* expressed an antigen derived from gp70. The antigen (AH1-A5) was derived from the immunodominant antigen from CT26 cells (Slansky, *et al.* (2000) Immunity 13:529-538). Mice treated with the positive control vaccine survived up to or beyond 60 days (Figure 16).

[0488] Figure 17 discloses gels, with western blot analysis, for detecting secreted mesothelin (top blot) and total expressed mesothelin (lower blot). *L. monocytogenes* Δ ActA Δ inlB engineered to contain a polynucleotide encoding the indicated secretory sequences and antigens were cultured, and the total or secreted mesothelin was measured. The secretory sequences were BaPA or Bs phoD, as indicated. The antigens were full length (FL) human mesothelin or human mesothelin deleted in its secretory sequence and GPI anchor (hMeso Δ SS Δ GPI), as indicated. The results indicate that total expression was somewhat greater with Bs phoD (lanes 4-5; lower gel) than with BaPA (lanes 2-3; lower gel). The

results also demonstrate that, at least with the Bs phoD containing constructs, secretion was greater with hMeso (Δ SS Δ GPI) (lanes 4-5; top gel) than with full length hMeso (lanes 8-9; top gel).

[0489] Figure 18 compares the mesothelin-specific immune response to vaccination with hMeso1, hMeso2, hMeso3, and hMeso4. Side-by-side comparison of hMeso1 and hMeso2 reveals that a *Listeria* construct comprising a nucleic acid encoding for constitutively active PrfA (prfA*) increases immune response, as compared to a *Listeria* construct not comprising that nucleic acid. Side-by-side comparisons of hMeso1 and hMeso4 reveals that increased immune response is found with genomic integration at the *inlB* locus (hMeso4), as compared to immune response where genomic integration is at the tRNA^{Arg} locus (hMeso1).

Comparison of immune response to hMeso3 and hMeso4 suggests that immune response can be enhanced by using *hly* promoter, as compared to immune response with ActA promoter. Elispot analysis was used to assess immune response. Splenocytes (plus or minus stimulation of splenocytes with a pool of mesothelin peptides) for elispot assays, where the elispot assays measured IFN γ expression.

[0490] The gels of Figure 18 disclose western blots sensitive to total expression of mesothelin or to secretion of mesothelin. hMeso2 produced the highest levels of secretion, indicating the usefulness of the following combination for increasing secretion: (1) prfA* nucleic acid; (2) Integration at tRNA^{Arg} locus; (3) The *hly* promoter; and (4) BaPa secretory sequence. Again, the usefulness of the prfA* nucleic acid is demonstrated.

[0491] Figure 19 compares immune response to hMeso12 and hMeso1. Mesothelin-specific immune response is depicted by the raw data (elispot assays) and by histograms showing the number of spot forming splenocytes per 2×10^5 splenocytes. The results indicate that the ras sequence present in the fusion protein of hMeso Δ SS Δ GPI (hMeso12) results in lower immune response (elispot assays) and lower expression (western blots), as compared to results where the fusion protein did not comprise ras (hMeso1) (Figure 19).

[0492] Mice were vaccinated with the two strains (hMeso12 or hMeso1), and splenocytes were removed and used for elispot assays, where assay mixtures were pulsed with the standard hMeso pool of peptides. As disclosed above, hMeso1 (the BaPA secretory sequence is wild type) stimulated a greater mesothelin-specific immune response than hMeso12 (the BaPA secretory sequence is E30R).

[0493] Figure 20 compares immune response to hMeso1, hMeso5, hMeso19, and hMeso20. The results demonstrate that the greatest mesothelin-specific immune response was to hMeso1, where there was also some detectable mesothelin-specific response to hMeso5. The

results demonstrate that BaPA secretory sequence results in greater immune response, as compared to p60 secretory sequence, or to derivatives of p60 secretory sequence. The gel demonstrates that the p60 secretory sequence supports secretion of mesothelin. See lanes labeled hMeso5 or hMeso20 (Figure 20).

[0494] Figure 21 compares immune responses to hMeso11, hMeso6, hMeso10, and hMeso18. Mesothelin-specific immune responses occurred with each of these vaccines, where the highest responses were provoked by hMeso10 and hMeso18. In comparing hMeso6 and hMeso10, it can be seen that the ras (hMeso10) can enhance mesothelin-specific immune response. Here, both *Listeria* strains ActA secretory sequence was used, ActA promoter was used, and ActA locus of integration was used. The high degree of immune response to hMeso18 can be due to the use of the ActA (A30R) secretory sequence. The present invention provides a *Listeria* containing a polynucleotide comprising a first nucleic acid encoding ActA (A30R), operably linked with and in frame with a second nucleic acid encoding a heterologous antigen, e.g., an antigen derived from a tumor, such as mesothelin antigen, or an antigen derived from an infectious agent. The gel reveals that the hMeso18 *Listeria* strain secreted relatively low amounts of mesothelin, as compared with secretion by hMeso10 and hMeso6 (Figure 21).

[0495] Figure 22 compares immune responses to hMeso1, hMeso14, hMeso15, and hMeso22. Mesothelin-specific immune responses to hMeso1, hMeso14, and hMeso22 were comparable, while that to hMeso15 was greater. The secretory sequences (SS) of each these four vaccine strains are different. The secretory sequences (SS) of hMeso1 is BaPA; hMeso14 (LLO62); hMeso15 (LLO opt62); hMeso22 (LLO441).

[0496] Figure 23 reveals immune response in healthy human volunteers, to listeriolysin (LLO) and to mesothelin. Immune response to epitopes of LLO and to mesothelin was found in all three subjects tested.

[0497] Figure 24 illustrates expression of human mesothelin by hMeso6 or hMeso5, in BHI broth and in J774 macrophages, where expression was assessed by gel separation and detection by the western blot method. The results demonstrate relatively low expression by hMeso6 in broth (and high expression by hMeso5 in broth), and relatively high expression by hMeso6 inside mammalian cells (and low expression by hMeso5 inside mammalian cells). The graph demonstrates relatively high immune response (meso-specific response; elispot assays) after vaccination with hMeso6, and low immune response after vaccination with hMeso5 (Figure 24).

[0498] Figure 25 discloses mesothelin-specific immune response, where mice had been vaccinated with *Listeria* containing a polynucleotide comprising a first nucleic acid encoding p60, BaPA, LLO441, ActA-N100, as indicated, and a second nucleic acid encoding hMeso. Integration was at the tRNA^{Arg} locus, ActA locus, or inlB locus, of the listerial genome, as indicated.

[0499] Figure 26 illustrates *in vivo* expression of mesothelin from J774 macrophages, as detected by western blotting using an anti-mesothelin antibody. Similar *in vivo* expression occurred when the J744 macrophages were infected with hMeso6 or with hMeso26.

[0500] Figure 26, as well as Figure 27, shows show that mesothelin-specific mounted after vaccination with various engineered *Listeria* were greater with the hMeso26 strain than with the other tested strains.

[0501] Figures 28 (photographs of lungs), 29 (histograms of lung data), and 30 (mouse survival) reveal the successful treatment of lung tumors by administering hMeso6 and hMeso26. Mice were treated with a negative control (HBSS); positive control (*Listeria* expressing AH1-A5); or the indicated numbers of hMeso6 or hMeso26. The tumors were induced by an injection with CT26 cells. The results demonstrate that both hMeso6 and hMeso26 were effective in reducing tumor metastasis, where hMeso26 was more effective than hMeso6 (Figure 30).

[0502] Figure 31 compares expression, and immune responses to vaccination, with various *Listeria* strains engineered to contain integrated expression cassettes at different points in the listerial genome. The control bacterium (*L. monocytogenes* Δ ActA Δ inlB) contained no expression cassette, while hMeso26 contained only one integrated expression cassette. The strains hMeso40, hMeso41, hMeso42, and hMeso43, each contained two different expression cassettes (integrated at two different points in the genome), where expression from these *Listeria* strains and immune response to these *Listeria* strains are shown (Figure 31).

[0503] Figure 32 shows *in vivo* expression of mesothelin, that is, *in vivo* within J744 macrophages, after infection with (1) hMeso6; (2) hMeso26; or (3) *L. monocytogenes* Δ ActA Δ inlB (three identical constructs) bearing an expression cassette encoding full length human mesothelin, and integrated at tRNA^{Arg} locus. The three identical constructs, or siblings, are labeled 1-1, 7-1, and 8-1.

[0504] Figure 33 discloses *in vivo* expression of mesothelin by hMeso6, hMeso26, and hMeso38 within J774 murine macrophages (gels with western blots). The control bacterium was *L. monocytogenes* Δ ActA Δ inlB. Also shown are mesothelin-specific immune responses (elispot assays). The results demonstrate comparable expression of mesothelin where

hMeso6, hMeso26, and hMeso38 are located in macrophages, and comparable immune response to hMeso26 and hMeso38.

[0505] Figure 34 discloses mesothelin-specific immune response generated seven days after a single injection of hMeso26 or hMeso38, at the indicated doses. The dose response curves reveal a marked increase in going from one million bacteria to ten million bacteria. The dose response curves found with the two strains are similar to each other (Figure 34). The present invention provides hMeso26; hMeso38; a vaccine comprising hMeso26 and/or hMeso38; a method of administering hMeso26 and/or hMeso38 to a mammalian subject; a method of stimulating mesothelin-specific immune response against a cancer or tumor comprising administering hMeso26 and/or hMeso38; a method of increasing survival to a cancer or tumor comprising administering hMeso26 and/or hMeso28, and so on (Figure 34).

[0506] Figures 35A and 35B continue the narrative on hMeso26 and hMeso38, and shows photographs of fixed lungs. Tumor cells were injected at $t = 0$ days. *Listeria* vaccines were injected (i.v.) at $T = 3$ days. Lungs were harvested at $t = 19$ days, where the histogram quantitates the metastasis results represented by the lung photographs (Figure 35A,B). With titration of mice with the indicated numbers of bacteria, the results show similar responses for both listerial strains, hMeso26 and hMeso38.

[0507] Figure 36 also continues the narrative of *Listeria* strains hMeso26 and hMeso38. The results demonstrate that both strains result in similar increases in survival to inoculated CT26 tumor cells.

[0508] Figure 37 dissects mesothelin-specific immune response to *Listeria* strains hMeso26 and hMeso38 into $CD4^+$ T cell response and $CD8^+$ T cell response. Immune response was monitored by intracellular staining assays (ICS). Both strains of *Listeria* were tested with Balb/c mice, while only the hMeso26 *Listeria* strain was tested with CD-1 mice. The results demonstrate that the proportion of immune response that is $CD4^+$ T cell response, or $CD8^+$ T cell response, can differ in different strains of mice.

[0509] Figure 38 demonstrates that hMeso38 increases survival to tumors, and dissects the contribution to survival by cells that are $CD4^+$, $CD8^+$, and NK cells. Mice were treated with antibodies that depleted one of $CD4^+$ cells, $CD8^+$ cells, or NK cells. Treating with the anti- $CD8$ antibodies resulted in only slight impairment of hMeso38-mediated increased survival. Treating with anti-NK cell antibodies resulted in moderate impairments of hMeso38-mediated increased survival. Treating with anti- $CD4$ antibodies resulted in a large impairment in hMeso38-mediated increased survival (Figure 38). Antibody-mediated depletion of the mouse's cells were effected by administering antibodies on $t = \text{minus } 8$ days,

minus 4 days, and on minus 1 days. At $t = 0$ days, mice were injected (i.v.) with tumor cells. At $t = 3$ days, mice were injected with *Listeria* vaccine (i.v.). Weekly antibody boosts were given to provoke depletion of the mouse's cells. Figure 39 shows a similar experiment, but where only antibody was administered, where only hMeso38 was administered, or where both hMeso38 and the indicated antibody were administered.

[0510] The above-disclosed data are not intended to limit the present invention to embodiments comprising *L. monocytogenes* Δ ActA Δ inlB containing a nucleic acid encoding human mesothelin. The present invention provides other attenuated listerial vaccine platforms, e.g., KBMA *L. monocytogenes*, *L. monocytogenes* Δ inlB; *L. monocytogenes* Δ ActA; *L. monocytogenes* Δ hly; KBMA *L. monocytogenes* Δ inlB; KBMA *L. monocytogenes* Δ ActA; KBMA *L. monocytogenes* Δ ActA Δ inlB; KBMA *L. monocytogenes* Δ hly. Moreover, what is also provided are constructs encoding antigens other than, or in addition to, human mesothelin.

EXAMPLE VIII. Nucleic acids encoding phage integrases, phage attachment sites (attPP'), and bacterial attachment sites (attBB').

[0511] Site-specific integration of a first nucleic acid into a polynucleotide can be mediated by a phage integrase, an attPP' site residing in the first nucleic acid, and a corresponding or compatible attBB' site residing in the polynucleotide. The present invention provides a number of nucleic acids, encoding phage integrases, attPP' sites, and attBB' sites, useful for mediating integration of a first nucleic acid into a polynucleotide, where the polynucleotide can be a plasmid or bacterial genome, to provide some non-limiting examples.

[0512] Figure 40, Figure 41, Figure 42, Figure 43, and Figure 44, disclose the amino acid sequences of some of the phage integrases of the present invention. What is encompassed is polynucleotides encoding these phage integrases, nucleic acids that hybridize under stringent conditions to these polynucleotides where the nucleic acids encode functional phage integrases. Also encompassed are other polynucleotides that are bracketed by a pair of PCR primers, where the pair of PCR primers corresponds exactly to two positions of a polynucleotide encoding a phage integrase of the present invention.

[0513] Provided are nucleic acids encoding the following phage integrases, the phage integrase polypeptides, nucleic acids encoding relevant phage attachment sites (attPP') and nucleic acids encoding corresponding bacterial attachment sites (attBB'). The present invention encompasses the following integrases: (1) *L. innocua* 0071 integrase; (2) *L.*

innocua 1231 integrase; (3) *L. innocua* 1765 integrase; (4) *L. innocua* 2610 integrase; and (5) *L. monocytogenes* f6854_2703 integrase.

[0514] Identification of a nucleic acids encoding integrases, attPP' sites, and attBB' sites, was according to the following multi-step procedure. Candidate nucleic acid sequences were initially acquired, and homologies can be identified, using, e.g., the protein or nucleotide BLAST feature on the world wide web at ncbi.nlm.nih.gov, and using the completed microbial genomes feature on the world wide web at tigr.org.

[0515] Step 1. Novel phage integrase sequences were identified as follows. Nucleic acids of a known phage integrase were used to search for a similar sequence in a listerial genome, where the listerial genome harbors a prophage. The known phage integrases sequences used at this step of the search were those encoding PSA integrase and U153 integrase.

[0516] Step 2. Once a nucleic acid encoding a new phage integrase is identified, review the DNA 3-prime to the nucleic acid encoding the integrase for the appearance of an attachment site. The attachment site typically takes the form of a hybrid of the phage attachment site and the bacterial attachment site (attPB'). The attachment site takes the form of this hybrid because the phage has integrated itself into the listerial genome.

[0517] Step 3. Regions of the listerial genome containing a putative attPB' site were compared with the corresponding region of another listerial strain or listerial species, where this other listerial strain or species is not expected to contain an integrated phage. The crossover point (crossover point in between phage sequence and bacterial sequence in attPB') takes the form of a discontinuity. The crossover point can occur in an open reading frame or in an intergenic region.

[0518] Step 4. The sequence of nucleotides residing immediately downstream from (immediately 3-prime end of) the integrase gene, and upstream to the crossover point, is identified as phage-derived sequence, and constitutes "a first half" of the phage attachment site.

[0519] Step 5. The "second half" of the phage attachment site can be identified by reviewing the nucleic acid sequences residing upstream to (5-prime to) the integrase gene, comparing with the corresponding regions of a listerial strain or species expected not to contain any integrated phage (no integrated phage in the genomic region of interest), and identifying a region of discontinuity. The combination of the first half of the phage attachment site and the second half of the phage attachment site is attPP'.

[0520] Step 6. Phage attachment sites and bacterial attachment sites typically contain a region of identity, for example, of between three to 10, 20, 30, or more nucleotides. A region

of identity can help in finding the general location of the phage attachment site and bacterial attachment site.

[0521] Step 7. Where the listerial species of interest is a species other than *L. monocytogenes*, e.g., *L. innocua*, the identified bacterial attachment site in the *L. innocua* genome can be used as a computer-probe to search the *L. monocytogenes* genome for homologous sequences. The result of this search of the *L. monocytogenes* genome where the result of the probe will be the bacterial attachment site (attBB').

[0522] Step 8. Where the region of identity is relatively long, e.g., 40-50 nucleotides, this region of identity can constitute the entire phage attachment site (attPP') and entire bacterial attachment site (attBB').

[0523] Most site-specific integrases are of the tyrosine recombinase family or serine recombinase family. About 100 phage-encoded integrase genes have been identified. These genes, encoded by the phage genome, can be found in the phage genome and/or also with a bacterial genome after integration of the phage into the bacterial genome.

[0524] The serine recombinases have a catalytic domain at the N-terminus, which includes a number of invariant residues, including Arg-8, Ser-10, and Arg-68. The N-terminal catalytic domain is followed by a region of about 220 amino acids, which contains at least ten conserved residues (including three cysteines). This region is followed by about 125 amino acids on non-conserved residues, by a 30-amino acid region rich in Leu, Ile, Val, and/or Met, and finally a C-terminal tail of 4-200 amino acids in length (see, e.g., Smith and Thorpe (2002) Mol. Microbiol. 44:299-307; Nunes-Darby, *et al.* (1998) Nucleic Acids Res. 26:391-406; Esposito and Scocca (1997) Nucleic Acids Res. 25:3605-3614).

[0525] Phage integrases of the tyrosine recombinase family can be identified by a conserved R-H-R-Y motif. The R-H-R-Y motif is a hallmark for the integrase family of recombinases. The histidine (H) can be substituted by arginine, lysine, asparagine, or tyrosine. In phage lambda integrase, for example, the amino acids of the R-H-R-Y motif occur at amino acids R212, H308, R311, and Y342 (see, e.g., GenBank Acc. No. P03700) (Nunes-Duby, *et al.*, *supra*). Phage integrases are further identified by Box I (see, e.g., A202-G225 of phage lambda integrase), Box II (see, e.g., T306-D344 of phage lambda integrase), and by certain motifs occurring before or between Box I and Box II. Box II can include the consensus sequence LLGH, where the glycine (G) can be replaced by A, S, or T (Nunes-Duby, *et al.*, *supra* (SEQ ID NO: 137)). In addition to the Box I motif and Box II motif, three "patches" of conserved sequences occur in prokaryotic integrases, such as phage integrases. Patch I is upstream of Box I, and has the consensus sequence LT-EEV--LL (SEQ ID NO:88). In phage

lambda integrase, Patch I has the sequence LTAD EYLKIY (SEQ ID NO:87) (amino acids 180-189 of GenBank Acc. No. P03700). Patch II is lysine (K235 of phage lambda integrase) flanked on both sides by serine, threonine, glycine, or methionine. In phage lambda integrase, Patch II occurs as SKT, while in Cre recombinase Patch II occurs as TKT, and in XerD recombinase it occurs as GKG. Patch III, which occurs between Boxes I and II, is [D,E]-[F,Y,W,V,L,I,A]₃₋₆[S,T] (SEQ ID NO:89, 138, 139, 140). In phage lambda integrase, Patch III occurs at amino acids 269-274 (Nunes-Duby, *et al.*, *supra*). In using a candidate phage integrase sequence as a query sequence, for comparison with established phage integrase sequences, it might be useful to introduce a gap or extension to bring Box I and Box II into alignment.

[0526] The conserved R-H-R-Y motif (Table 16) resides in the phage integrases of the present invention. The positions were determined by manual inspection. Esposito and Scocca provide additional conserved sequences within Box I (a.k.a. Box A) and Box II (a.k.a. Box B) (Esposito and Scocca (1997) *Nucleic Acids Res.* 25:3605-3614). Esposito and Scocca disclose that that Arginine (in Box I (Box A) of the R-H-R-Y motif) resides in the following context: TGLRXTEL (SEQ ID NO:91), and that the histidine and the second arginine (in Box II (Box B) of the R-H-R-Y motif) reside in the following context:

HXLRHAXATXLXXXG (SEQ ID NO:90). The histidine (H) and second arginine (R) of the R-H-R-Y motif is bolded and underlined. Sequences corresponding to these two contexts can readily be found, by manual inspection, in Boxes I and II of *L. innocua* 0071. Esposito and Scocca place the Tyrosine (Y) of the R-H-R-Y motif in a motif identified as Box C, where the Box C of Esposito and Scocca is: VXXXLGHXXXXXTXXYXH (SEQ ID NO:92). The Y of the of R-H-R-Y motif is bolded and underlined. Inspection of the *L. innocua* 0071 integrase sequence demonstrates that the Box C consensus sequence resides in *L. innocua* 0071 integrase of the present invention.

[0527] Inspection reveals that Esposito and Scocca's Box B and Box C exists in *L. innocua* 1765 integrase of the present invention. Furthermore, inspection demonstrates that Esposito and Scocca's Box A resides in *L. innocua* 2601 integrase of the present invention. In addition, inspection of the *L. monocytogenes* f6854_2703 integrase sequence shows the occurrence of Box A, B, and C. Taken together, the consensus sequences of Nunes-Duby, *et al.*, *supra*, and of Esposito and Scocca, *supra*, confirm the identified sequences as phage integrases. Inspection of PSA phage integrase sequence reveals motifs similar to Esposito and Scocca's Boxes A, B, and C.

[0528] *L. innocua* 1231 integrase of the present can be identified as a serine recombinase. Yang and Steitz disclosed a number of invariant motifs, and conservatively substituted motifs, of the serine recombinase family (Yang and Steitz (1995) Cell 82:193-207). The YxRVSTxxQ (SEQ ID NO:93) motif of Yang and Steitz occurs in *L. innocua* 1231 integrase. Also, the VLVxxLDRLxR (SEQ ID NO:141) motif of Yang and Steitz can be found in *L. innocua* 1231 integrase. Furthermore, Yang and Steitz's VAQAERxxxxERxxxG (SEQ ID NO:94) motif is found in *L. innocua* 1231 integrase of the present invention.

Table 16. Conserved R-H-R-Y motifs in phage integrases.

	Arginine (R)	Histidine (H)	Arginine (R)	Tyrosine (Y)
<i>L. innocua</i> 0071 integrase.	382	595	598	631
<i>L. innocua</i> 1765 integrase.	241	334	337	369
<i>L. innocua</i> 2601 integrase (90.9% identical to PSA integrase).	199	309	312	344
<i>L. monocytogenes</i> f6854_2703 integrase.	204	328	331	364
Lambda phage. GenBank Acc. No. P03700.	212	308	311	342
PSA phage. GenBank Acc. No. CAC85582.	199	309	312	344

<i>L. innocua</i> 0071 integrase. Coding sequence plus Shine Dalgarno and terminator. See, e.g., GenBank Acc. No. AL596163.1 (Segment 1/12). (SEQ ID NO:95)	AggagggccttatttATGGTAAAAAAGTAAAAGGTAGGCGTTATGAGGGTTCTATT GAACAACGTAGCAAAAAATTCATGGCGTATGCGCGTGAAGTATGAGGCTATGACTACAA AGGTACGCCGATTTCGAGCTGACAGAACGACGCGAACAAAAATGAGAGGGAGCGAG AAAGAGAGTTAAGAAATTTTCATCACAGAATTAGAGCAAAATGGATATACAGCTCCT GCAAGAATGACATTTAAAGCATTGTGTTGAGAATGAGTATATGCCGAAACATGCACA AAATAACCTAGAAGTTAAAACCTGGACAGAATACTACAAATCTATAGTAGCAAGAG CTTACCCAGCCTTTGGCGGGCGTTCAAATGGATAAAATAACTACACTTCATATAGTT AACTTAGTCGCAAAATTACAAAAGCCCCGGCGCAAGATTAGATGTTAAACCTACAGA TTCAGACGAAAAGAAAAATAAGCCGCTTCGCGCGGATCTATCAGAAATATTTATT TTGCGATAAATTCAGTATTTGAACTGCGGTTGAGTGGAAGTAATCCCAATTAAC CCCGCAGAGGGTGTAAGGCTTCCAAAAACAATAAGAGACCGCTACTATTTTATAC TCCTGCTGAAATTGAATTGTTAAATGCAGCTCTAGTGAAAGAGCCACTTAGATTGC AAGTAATGATTTATATAGCGCTGATTTTCAAGTTGTAGAGAAGCTGAATTAGCAGCA TTAGAAGTAAACACGTGAACCTTAATAGAAGATGAGCTAACATTGCAACAAACGCT AGTTGCAAAAGCAGGAGAAGGTTTACTTCTTAAAGAATCAACTAAGAATGATGTAG CTGGGATAGTTTCTATACCCGCTTGGTTAACTAATTTAATAGAAACATATATAAGC AATGAAGTTTTAGACCTAAAACCTGAAGGGAATGGGCCAATCACAATTTTTTATT CGCCGACATGGAAGGCAAACCGATTAGGCCTGATTTCGATTTATCAGCGTTGGAAC GATTTTTAGAAAGACACAACCTGCGCGTGATTTCGTTTTTCATGATTTGCGTCACACA TCTGCTACACTTTTATTGAACAAAGGTAGAGATATAAAAAATTATCCAAGAGCGGCT TAGACATAAATCTAGTGTGACCACTTCAAACATTTATGCACATGTTTTGAAAGATA CGCACAAAGATGCAGCTAGCGATTTTGAGAACCCTTTTTTAAGctttctgccccacc tctgccccacttaataaaaaaaggcaatttttaaActAaaatttcacaaacaaaaaa ccgcttaaacgctttgttttaggcgg
Coding sequence only of integrase. <i>L. innocua</i> 0071. (SEQ ID NO:96)	ATGGTAAAAAAGTAAAAGGTAGGCGTTATGAGGGTTCTATTGAACAACGTAGCAA AAATTCATGGCGTATGCGCGTGAAGTATGAGGCTATGACTACAAAGGTACGCCGATTC GAGCTGACAGAACGACGCGAACAAAAATGAGAGGGAGCGAGAAAGAGAGTTAAGA AATTTTCATCACAGAATTAGAGCAAAATGGATATACAGCTCCTGCAAGAATGACATT TAAAGCATTGTGTTGAGAATGAGTATATGCCGAAACATGCACAAAATAACCTAGAAG TTAAAACCTGGACAGAATACTACAAATCTATAGTAGCAAGAGCTTACCCAGCCTTT GGCGGCGTTCAAATGGATAAAATAACTACACTTCATATAGTTAACTTAGTCGCAAA ATTACAAAAGCCCCGGCGCAAGATTAGATGTTAAACCTACAGATTTCAGACGAAAAGA AAAATAAGCCGCTTTCGCGCGGATCTATCAGAAATATTTATTTTTCGCGATAAATTC GTATTTGAAACTGCGGTTGAGTGGAAGTAATCCCAATTAACCCCGCAGAGGGTGT AAGGCTTCCAAAAACAATAAGAGACCGCCTACTATTTTACTCCTGCTGAAATTG AATTGTTAAATGCAGCTCTAGTGAAAGAGCCACTTAGATTGCAAGTAATGATTTAT ATAGCGCTGATTTTCAGGTTGTAGAGAAGCTGAATTAGCAGCATTAGAAGTAAACA CGTGAACCTTAATAGAAGATGAGCTAACATTTCGAACAAACGCTAGTTGCAAAAGCAG GAGAAGGTTTACTTCTTAAAGAATCAACTAAGAATGATGTAGCTGGGATAGTTTCT ATACCCGCTTGGTTAACTAATTTAATAGAAACATATATAAGCAATGAAGTTTTAGA CCTAAAAACTGAAGGGAATGGGCCAATCACAATTTTTTATTCGCCGACATGGAAG GCAAACCGATTAGGCCTGATTTCGATTTATCAGCGTTGGAACGATTTTTTAGAAAGA CACAACCTGCGGCTGATTTCGTTTTTCATGATTTGCGTCACACATCTGCTACACTTTT ATTGAACAAAGGTAGAGATATAAAAAATTATCCAAGAGCGGCTTAGACATAAATCTA GTGTGACCACTTCAAACATTTATGCACATGTTTTGAAAGATACGCACAAAGATGCA GCTAGCGATTTTGAGAACCCTTTTTTAA
<i>L. innocua</i> 0071	MVKKVKGRRYEGSIEQSRKNSWRMRVTVGYDYKGTPIRADRTTRTKNERERERELR NFITELEQNGYTAPARMTFKAFVENEYMPKHAQNNLEVKTWTEYYKSIVARAYPAF GGVQMDKITTLHIVNLVAKLQKPGARLDVKPTDSDEKKNKPLSPRSIRNIYFAINS

integrase amino acid sequence. (SEQ ID NO:97)	VFETAVEWKVIPINPAEGVRLPKTTKRPPTIYTPAEIELLNAAALVKEPLRLQVMIY IALISGCREAELAALEVKHVNLIIEDELTFEQLVAKAGEGLLLKESTKNDVAGIVS IPAWLTNLIETYISNEVLDLKTEGKWANHKLFLFADMEGKPIRPDSIYQRWKRFLER HNLPVIRFHDRLRHTSATLLLNKGRDIKIIQERLRHKSSVTTSNIYAHVLKDKTHKDA ASDFENPF
<i>L. innocua</i> 0071. Bacterial attachment site (between <i>L. monocytogenes</i> f2365_0095 & <i>L. monocytogenes</i> f2365_0096, in the tRNA-lys gene (attachment site underlined). (SEQ ID NO:98)	taccgaaaaatatagccgcagcgagtggtgctgcggctgtgttttatcgctgaattat ggtataatatatttttgtcgggaatacgacaacgggttggttagctcagttggttagagc agctgactcttaatcagcgggtcgggggttcgaaaccctcacaaccataaaaaaca aacgccagtgactgttaaagtcggttggtgtttgtcggttttacgggcaaaatggt aataatttcaataataagctgatttctttttgattatttatcgattacatagaaaa taagtggaatttcaaagtatctaataatttActAcatgatatacaaaaggagttgt ttca
<i>L. innocua</i> 0071 phage attachment site. (Common sequence between phage and chromosome (attP and attB)). (SEQ ID NO:99)	ACTCTTAATCAGCGGGTCGGGGGTTCGAAACCCTCACAACCCATA

<i>L. innocua</i> 1231 integrase nucleic acid sequence. <i>L. innocua</i> Clip11262 complete genome GenBank Acc. No. AL596168.1 (segment 6/12 nucleotides 29,995 to 28,563). (SEQ ID NO:100)	TggaggtgagaaagttcATGACTGTAGGGATTTATATAAGGGTTTCC ACTGAAGAACAAGTGAAGGAAGGCTTTTCTATATCAGCACAGAAAGA GAAGTTAAAGCATATTGCACAGCGCAAGGATGGGAAGATTTCAAGT TTTACGTCGATGAAGGTAAATCAGCAAAAGATATGCACCGCCCTCTT CTACAAGAAATGATTTACATATATAAAAAAAGGACTTATAGACACAGT CCTAGTATATAAATTGGATCGTCTTACTAGGTCCGTTGTAGATTTGC ATAATTTATTAAGTATATTTGATGAATTTAACTGTGCATTTAAAAGC GCTACTGAAGTCTACGATACTTCTTCCGCTATGGGCAGATTTTTTAT TACAATAATAAGTTCAGTTGCTCAATTTGAAAGAGAGAATACCTCTG AACGAGTTAGCTTTGGGATGGCTGAGAAAGTGCGTCAAGGAGAATAT ATTCCTCTCGCTCCCTTCGGTTATACTAAGGGGACTGACGGAAAAC AATAGTAAATAAAATAGAAAAAGAAATATTTTACAAGTAGTTGAAA TGTTTTCAACCGGTTATTCTTTACGACAAACTTGTGAATATTTAACA AATATTGGTTTTGAAAAACAAGGCGTTCAAAATGATGTGTGGAAGTATC TACATTAATTTGGATGTTAAAAAATCCTGCTGTCTACGGAGCGATAA AATGGAATAATGAAATATATGAAAATACACATGAGCCTCTAATCGAT AAGGCAACATTTAATAAAGTAGCCAAAATACTATCAATAAGAAGTAA ATCAACAACAAGCCGTCGTGGACACGTTTCATCACATTTTTTAAAAATA GATTAATTTGTCCAGCTTGTGGAAAAAGATTATCTGGATTAAGAACA AAATATATAAATAAAAAATAAGGAAACTTTTTATAACAATAACTATCG TTGTGCTACCTGCAAGAACATAGACGTCCAGCAGTACAGATAAGCG AGCAAAAAATAGAGAAAGCATTATTTGATTATATTTCAAACATATA CTCAATAAAGCAAATATCTCTTCTAAAAAATTAGATAATAATTTGAG AAAACAAGAAATGATTCAAAAAGAAATATTTCACTTCAAAGAAAAC GTGAAAAGTTTCAGAAAGCATGGGCTGCTGACCTTATGAATGATGAT GAATTTTCTAAATTAATGATTGATACAAAAATGGAGATTGATGCTGC AGAAGATAGAAAAAAGAAATATGACGTATCATTATTTGTATCTCCTG AAGATATTGCTAAAAAGAAATAACATTCTTCGTGAACATAAAAAATAAT TGGACTTCATTATCTCCTACTGAAAAACAGATTTTATAAGTATGTT TATTGAAGGAATTGAATATGTAAAAGATGATGAAAATAAAGCGGTTA TAACGAAAATAAGTTTTTTTATAA
<i>L. innocua</i> 1231 integrase amino acid sequence. (SEQ ID NO:101)	MTVGIYIRVSTEEQVKEGFSISAQKEKLKAYCTAQGWEDFKFYVDEGKSA KDMHRPLLQEMISHIKKGLIDTVLVYKLDRLTRSVVDLHNLISIFDEFNC AFKSATEVYDTSSAMGRFFITIISSVAQFERENTSERVSFGMAEKVRQGE YIPLAPFGYTKGTDGKLIVNKIEKEIFLQVVEMVSTGYSLRQTCEYLTNI GLKTRRSNDVWKVSTLIWMLKNPAVYGAIKWNNIYENTHEPLIDKATFN KVAKILSIRSKSTTSRRGHVHHIFKNRLICPACGKRLSGLRTRYINKNKE TFYNNNYRCATCKEHRRAVQISEQKIEKAFIDYISNYTLNKANISSKKL DNNLRKQEMIQKEIISLQRKREKFQKAWAADLMNDDEFKLMIDTKMEID AAEDRKKEYDVSLFVSPEDIKRNNILRELKINWTSLSPTKTDFFISMF EGIEYVKDDENKAVITKISFL

<i>L. innocua</i> 1231 phage attachment site attPP'. This site resides in <i>L. monocytogenes</i> strain 4bF2365 (complement to 2495122 to 2495193), and is essentially the same as a sequence found in <i>L. monocytogenes</i> strain EGD (nt 145171 to 145423 of GenBank Acc. No. AL591983.1 segment 11/12). (SEQ ID NO:102)	Taaataattgtcagtcagtcaatcaaaagaattatattataggtttttgtcaaata Tggtgatgtgtacttataacccatttttcttgcaataaaaagcttggttatt ccccgttcta
<i>L. innocua</i> 1231 attachment site attBB' within <i>L. monocytogenes</i> 1263: (SEQ ID NO:103)	Ttcataaaagaatttcaaatcgcacattaaaatttcacttagaataa Cagcatttttgtgtgatagtctaacagttcctttttcaatgttactg Taacctgatgtgtacctatagcccatccgtcgcgcaatgaaagcttg Ggtgattcctcgctgcaatcgtaattctcgaatttttgttgatttaa ttcttctgggtgtctactgttttcac

<i>L. innocua</i> 1765 integrase. See also <i>L. innocua</i> Clip11262 complete genome, segment 7/12 (nucleotide 210,321 to 211,089). (SEQ ID NO:104)	AggatgaaagagaATGGCAAAGAACAAATGGCAACCCACTAAA CATTTAGGAATTTATGAATACATGACTAAAAAAGGAAAGCGTT ATGGGATACGAGTTCGTTATAAGCAAGGTAATGATTATCCTGA AATAAATAAATCTGGTTTTGAGACAATTGCAGCTGCAAAAGTTT ATAAAAAACAACATTGAAAATTTGAAAGCTAATAAAAAAGAATAT GTTTTTACAAATGAAAAATTAACATTAAATACTTGGTTTTGCTTC TTACATGGAAATGTTTAAAAAGAAAAACAAAAGTAAAGACACAA TAGCGAATAAATATAGTATTTATAATAATCACTTAGAAATCCCT TTTGGTAATTACTATTTAACTGATATAAGTTTAGATATTTACGA AGACTTTTTGCGCGAAAAAATTAAAAATGGATACGCAAACAAC CAGTCAAAGCGATGCATAAATTAATGAAAAGCATTTTAAACGCT GCTGTTAGATATGAGAACTAGAAAAAACAGACTTCAATTTGCT TGAAATAGAGCAATTAGAAGAAAATGAAGTTATTGAGCTTAAGG TATTAGAAACAGATGAGTTTAAATGTATTTATATCAGCTTGTA GCATTTTTTACTAAATATGATTTTACAATGATTTATCTTGCA TTGGGGGATGCGTCGCGGTGAAGTTATGGGGGTAAACTTAAAA ATCTTACTTTTGATGATGCTAAACAACAAGTACGTATTACACTA GATTCCACTCGAACCCTTCGTACTCCCGAGGGAAAAGGTACGAA AACACCAGCTGGTAGAAGAATATTACTAATAGACGGCGAAGGTT ATCGACTACTTAAATATTCGGTAGAAAAAGCGGTTAGCATTGCT AAAGACCATGGATCTGTTTTGCACCAGGATGATTTTATTTT AAACCCAACCTCTAATCGTCCTTGGGCGGTACGCGTATGAATG ATTTACTACGAAAATTAGAAAAAGAATACGACATAAAAGTTTAC CCTCATCTATTACGCCATAACTTTAATACTCAGGCATTATTGGC TGGAGCTAATAGCAATGATTTACGAAAATTTATTGGCCACAAAA ACAGTAGCATGACTGATCATTATTACATGCGACAGACGAGGGA CGAGAAAAATTAATGAATACGATGAAAGACAGATTGTCAGGAAT CTAG
<i>L. innocua</i> 1765 integrase amino acid sequence. (SEQ ID NO:105)	MAKNKWQPTKHLGIYEYMTKKGKRYGIRVRYKQGN DYPEINKSGFETIAA AKVYKNNIENLKANKKEYVFTNEKLT LNTWFASYMEMFKKKNKSKDTIAN KYSIYNNHLEIPFGNYLTDISLDIYEDFLREKIKNGYANNSVKAMHKLM KSILNAAVRYEKLKLNRLQFAEIEQLEENEVIELKVLETDEFNVFISACR AFFTKYDFTMIYLAVWGMRRGEVMGVKLKNLTFDDAKQQVRITLDSTRTL RTPEGKGT KTPAGRRILLIDGEGYRLKYSVEKAVSI AKDHGSVLHQDDF IFRNPTSNRPWAVTRMNDLLRKLEKEYDIKVYPHLLRHNFN TQALLAGAN SNDLRKFIGHKNSSMTDHYSHATDEGREKLMNTMKDRLSGI

<i>L. innocua</i> 1765 Phage attachment site. (SEQ ID NO:106)	Aaaattgtgggataaaaaattaaatataaaaaatatcccacaaa Aaatcccacaatagtttgatattgtatgatattcaaagaaa Tcaaaaaataaaaaaccccggtatttcctaagaaaatacgggg ttttgatatcatataaaatcaattaaaaattgac
<i>L. innocua</i> 1765. bacterial attachment site. This sequence resides in <i>L. monocytogenes</i> EGDe (complete genome) GenBank Acc. No. AL591824 at nt 1,705,630 to nt 1,706,203. Similar sequences occur in <i>L. mono-cytogenes</i> strain 4bF2365 (nt 216008 to 216262 of section 6) and in <i>L. innocua</i> Clip11262 nt 77369 to 77270. (SEQ ID NO:107)	Tcttgttgccctcctttttgtaatcaatagttgcaatgcaa Gagtatcataaaaaagcgatgtataaccaaaaatgtaatg aaatgtccgattcttctgtcgtgaacgActAgaaaaatggagc ttatttagagatattccttacacaacgtgagtatcattaag ttttttggtcataagataatactcattatgagttActAtt cacatttttaaacattcctgttttctattttatcacaaaaaat acatatcaatccaagatatgcggtattttcacttatgaata ttccttattttatttaattattttatcagttttattttattac taggtgaataatatagataattatttcacctacgacagac gagacacgagaaaaattaatgaatacagatgaaagacagat tgtcaggaatctagaaaaattgtgggataaaaaattaaatat aaaaatatcccacaaaaaatcccacaataatttgatattg tatgatattcaaagaaatcaaaaaaatcaaaaccccgca tttcctaagaaaatacggggttttgatatcatataaaatc gatttaaaatggac
<i>L. innocua</i> 2610. Integrase gene from <i>L. innocua</i>. The present invention also provides the nucleic acid and polypeptide of <i>L. innocua</i> Clip11262 complete genome segment 11/12 GenBank Acc. No. AL596173.1 (nucleotides 14,676 to 15,804). (SEQ ID NO:108)	ATGAAAATAAAAAAATGAAAAATGGTAAATATACTGTTTCGTTTGCGTAT TAAAGTTGATGGAGAGTGGAAAGAAAAACGTTTGACAGATACAAGTGAAA CAAATTTGATGTACAAAGCATCAAATTTATTAACAAGTTGAACATGAT AGTAATTCATAAAAGAATGGAATTTCAAAGAATTCTATTTCGCTATTTAT GAAAACTTTCAAAGAAAAATAAAGTAGTCAATCAACAATTAACCTGTATG ACTTAGCTTATAATCAGTTCGTTAATTATTTTCGACGAAAAAATAAGTTA AATTCATTGACGCTGTTCAATATCAGCAATTTATTAATCATTTAGCATT AGATTACGCTGTCTGCTACTATAGATACACAGACACCGCAAAATTAGAGCGA TTTTCAATAAAGCCGTCCTATTTAGGTTACATGAAAAAAACCCCTGCTCTG GGCGCTCACATAAGCGGTCATGATATAGCAAAAACAAAAGCGCAATATTT AGAAACAGATAAAGTACATCTATTATTAGAAGAGCTTGCAAAACTTCATT CTATATCAAGAGCAGTTATTTTTTTTAGCAGTTCAAACAGGAATGCGATTT GAAGAAATTATTGCACTGACAAAAAAGATATTAATTTTACTAAACGTTT TATATCAGTGAATAAGGCATGGGATTATAAATACACTAACACGTTTACGG ACACTAAAACAAAAAGTCACGAGTAATCTATATTGATAATTCAACTGTT CAATATTTACAGTCTTACCTTGCTTGGCATGCTGATTATATGAAAGAGCA TGCAATTGAAAATCCGGTGATGTTGTTATTCATTACTTATCACAATAAAC CTGTTGACAACGCTTCATGTAACAAAGCACTGAAGAAAATATGTACTACA ATTAATTCTGAAACAGTAACATTACACAAGCTTCGACACACGCACACAGG TCTATGTGTAGAGGCTGGTATGGATATTATTTATGTAGCTGACAGGCTTG GTCATGATGATATTAATACAACATTAATAATATTATAGTCATCTGAGTTCT AATTTACGACAACAAAATCAATCTAAAGTAGATGCTTTTTTTCACACTAAA AACAGATGAAAATACCACAAAATTTGCCACAAATGCCACAAAAACAACGG AA
<i>L. innocua</i> 2610 integrase, amino acid sequence (90.9%)	MKIKMKNGKYTVRLRIKVDGEWKEKRLTDTSETNLMYKASKLLKQVEHD SNSLKEWNFEKYSLEFMKTFKENKSSQSTINLYDLAYNQFVNYFDEKIKL NSIDAVQYQQFINHLALDYAVATIDTRHRKIRAI FNKAVHLGYMKKNPAL

identical to PSA integrase). (SEQ ID NO:109)	GAHISGHDIAKTKAQYLETDKVHLLLEELAKLHSISRAVIFLAVQTGMRF EEIIALTKKDINFTRKRSISVNKAWDYKYTNFTDTTKKSRVIYIDNSTV QYLQSYLAWHADYMKEHAIENPVMLLFITYHNKPVDNASCNKALKKICTT INSETVTLHKLRLHTHTGLCVEAGMDIIYVADRLGHDDINTTLKYYSHLSS NLRQQNQSKVDAFFTLKTDENTTKFATNATKTTE
<i>L. innocua</i> 2610. This sequence is an attBB' site from <i>L. innocua</i> . Attachment site (tRNA-Arg5 gene plus surrounding sequences, integrates <i>Listeria innocua</i> strain). Core attachment site in bold (atgccctcggaggga). (SEQ ID NO:110)	Taaaacgggtattgcaagggtataaaaaaatctctaaaacattcgtttattc CtttaatatcaaggatttccaacgtttttagagatttctttacatcActAc Tta <u>atgccctcggaggga</u> atcgaacccccatttttaagaaccggaatctta Cgtgctatccggttgaccacgagggctttatgtacaaagaaaatgtttac Cgtacgaataataattatagcgaaattcgtagtgtttttacaagctttatt Ttgaatgaagaagccagcgcctcctgagatttgctggcttcaatagtta
Core attachment site (in bold). (SEQ ID NO:111)	atgccctcggaggga
This sequence is an attBB' site from <i>L. monocytogenes</i> f2365. Attachment site of non-integrated strain (<i>L. monocytogenes</i> F2365; attachment site in tRNA-Arg5 gene underlined. Core att site is in BOLD (atgccctcggaggga). (SEQ ID NO:112)	Taaaatgaaaaaacatcttacaacatggcttttgccagatgtgggatgt Ttttttagt <u>atgccctcggaggga</u> atcgaacccccatttttaagaaccgg Aatcttacgtgctatccggttgaccacgagggctatatgtaggccagaa Atgcttaccgtacgaataataattatagcgaaattcgtagtgttttaca Agttttattttaaatgaagaagccagcgcctccaaagatttgctggctc aagtatta

<i>L. monocytogenes</i> f6854_2703 integrase - 2680803: 2681963 (Most of this sequence is available at tigr.org). (SEQ ID NO:113)	ATGGCTAGCTATGTAAATTTAGGAAATAATAAATATGAGCTAAGAGTTT CAAAGGGATATGATGCACGTGGAAAACAAATACGCAAAACAAAAACGT CACAGTTAAAACAGTAAAAGCGTTAAAAC TAGAACTTTCTAATTTTGAA GCTTATGTCTATTCAAGCGATTACACAGAAATAAAAGATATGCGATTTA TTGACTTTGTGGAAAAATGGCGCTTAAATTACGCAAAAAGAGAACTAAA AGGTAATACTATTGATAAGTATAACCTCTTTCTCGAAAACTGGATTATA CCTTATTTTGGAGAGGAAGAAAATAAGTAAAATTACAACATATGCAGTTGC TCGACTACTTTTCATGAAGTTCAAAAAAAGGAGTTGGTCCAAGCGCTTT AGAGGGACATCATCGAGTTATAAGAAGTTTATTTAAATATGCTACCTTG TGGGGAATTACTGAAACAGACGTATCTTTATCAGTGAAAAAACCTACCT ATAAAGTGCCAGAAAAAATATTTATAATAGACGAGAAATAGAAGTGT AATAGATCGCATTAAAGATATTACAAAAATATCAACAAGTAATGATTAAA TTAGCGCTATACTGCGGTCTTAGACGTGGCGAAGTTATCGGTTTAAACAA CTAAAGATATGAATTACAATAAAAAATACAATTAACGTTTATAGAGCGGT TATAAAGAGTGCTAGCGAAGGTATAAACTAGATGAACTAAAAATAAG CGAAAAAGAATTGTCCCGCTCCCGCTGGACTGATGCAAGAAATTAAAG AACTTGCAAAAGAAAAGCAAAAAACAAAGATAAATTAGGTTTGTGTG GAAAGGAACAAAAGATTTAGATGGGAAAAGTGTGTATTAATTTTCAGT CATGACGACGGCACCCCTTTACCCCGCTTCTGTCACTAGAATGTTTA ATCGATTTT TAGAGAAAGAAGAAAAATAACGATCTTACTAAAATATCATT TCATGATTTGCGTCATTCTGCTGCAAGCTTCCTTCTCGAACAAGGTATT AATGTAAAAGTCATTCAAAACATTTTAGGACATT CAGACATTAAAGTTA CATTAATACGTATGCACATATCACTGAAGATGGTTACTCAGAAGCAGC AAAACTTTTGATAATTTCTATAAATCTAGTAAA
<i>L. monocytogenes</i> f6854_2703 integrase 2,680,803: 2,681,963. (SEQ ID NO:114)	MASYVNLGNNKYELRVSKGYDARGKQIRKTKNVTVKTVKALKLELSNFEA YVYSSDYTEIKDMRFIDFEKWRNLNYAKRELKGNTIDKYNLFLENWIIIPY FERKKISKITTMQLLDYFHEVQKKGVGPSALEGHHRVIRSLFKYATLWGI TETDVSLSVKKPTYKVPEKNIYNRREIEVLIDRIKILQKYQQVMIKLALY CGLRRGEVIGLTTKDMNYNKNTINVYRAVIKSASEGIKLDETKNKRKRIV PAPAGLMQEIKELAKEKQKNKDKLGLLWKGTDLGKTVVLIFSHDDGTP FTPASVTRMFNRFLEKEENNDLTKISFHDLRHSAASFLEQGINVKVIQN ILGHSDIKVTLNTYAHITEDGYSEAAKTFDNFYKSSK

<i>L. monocytogenes</i> f6854_2703. Phage attachment site. (SEQ ID NO:115)	TaagggtgtcgaataaggtgttttgcctatTTTTtaggcaaataaAaaaagc Ttcgcataattagcgaacacctacagcaccaacgttttatattaagcca Cttgtcggatttgaaccgacgaccccttccttaccatggaagtgtctta Ccaactgagctaaagcggcagcaaagcctttcaaataaaaaaatggctc Cacaggcaggactcgaacctgacgacgatcggttaacagccgattgtctc Taccaactgagctactgtggaataataaattgcccggcagcgacactact CtcgcagggggaagcccccaActAccattggcgagagaagcttaActA CcggtgttcgggatgggaacgggtgtgaccttctcgccataActAccaga CaatattgagttgttgaaagattgctctctcaaaaActAgagaagaaagt Gttcagttaggttaacttcgtttcattttttgggttaagtccctcgatcgat Tagtatttgcgcgtccatgtatcgctacacttccactccaaacctatc Tacctgatcatctttcagggatcttactttccgaagaaatgggaaatct Catcttgagggggggttcacgcttagatgctttcagcgtttatccctgc Cacacatagctaccagcgatgctcctggcggaacaactggtacaccag CgggtgtgtccatcccggtcctctcgtActAaggacagctcctctcaaat Ttctgcgcccgcgacggatagggaaccgaactgtctcacgacgttctga Acccagctcgcggtgcgctttaatggggaacagcccaacccttggggac CgActAca
Phage attachment site (attPP'). Phi6854.3 attachment site is within the tRNA- Thr-4 gene Phage attachment site highlighted in bold and underlined, and is annotated as a phage attachment site in the F2365 genome (Nelson, <i>et al.</i> (2004) Nucleic Acids Res. 332:2386-2395). (SEQ ID NO:116)	AaaaacacccccaccggttctgtattataccatagtagtataatcGatttatActAc CtAttcaagatatccataataaatatcattattCtttaacaatAaaaaagcct cgcAtActAgcgaacatAcaaattatccatatattat <u>ttaagccacttgcggatttgaaccgacgaccccttccttaccatggaag</u> tgcctaccaactgagCtaaagcggcagcaaagcctttcaaataaaaaaatgg ctccacaggcaggactCgaacctgcgaCcGatcggtaacagccgattgct ctaccaactgagctactgtGgaataataaattgcccggcagcgacactactctc cagggggaagcccccaActAccattggcgagagaagcttaa
Phage (attPP') Phi6854.3 attachment site (same as above) is within the tRNA- Thr-4 gene, where the tRNA-Thr-4 gene is shown outlined in a	AaaaacacccccaccggttctgtattataccatagtagtataatcGatttat ActAcctAttCaagatatccataataaatatcattattCtttaacaatA aaaaaagcctcgcAtActAgcgaacatAcaaattatccatatattattta <u>agccacttgcggatttgaaccgacgaccccttccttaccatggaagtgcctaccaact</u> gagCtaaagcggcagcaaagcctttcaaataaaaaaatggctccacaggcaggact CgaacctgcgaCcGatcggtaacagccgattgctctaccaactgagctactgt Ggaataataaattgcccggcagcgacactactctcgcagggggaagcccccaact Accattggcgagagaagcttaa

box. (SEQ ID NO:117)	
Bacterial (attBB') Phi6854.3 attachment site is within the tRNA- Thr-4 gene Phage attachment site highlighted in bold and underlined, and is annotated as a phage attachment site in the F2365 genome (Nelson, <i>et al., supra</i>). tRNA- Thr-4 gene is <u>outlined</u> . (SEQ ID NO:118)	Aaaaacacccccaccggttctgtattatacccatagtataatcgatttat ActAcctattcaagatatccataataaatatcattattctttaacaata aaaaaagcctcgcatActAgcgaaacatacaaatatccatatattat <u>ttaagccacttgcggattgaaccgacgaccccttccttaccatggaagtgcctaccaact</u> gagctaaagcggcagcaaaagccttcaataaaaaaatggctccacaggcaggactcgaacct gcgaccgatcgggtaacagccgattgctctaccaactgagctactgtggaataataaattgcc cggcagcgacctactctcgcagggggaagcccccaActAccattggcgcagagaagcttaa
Nucleic acid sequences can be found on the world wide web at tigr.org and, clicking: (1) Comprehensive microbial resources; (2) Searches; (3) CMR BLAST; and (4) inputting a listerial integrase sequence as a query sequence. If an accession number is known, a sequence can be found on the world wide web at tigr.org, by clicking: (1) Comprehensive microbial resource; (2) Genomes; (3) <i>Listeria monocytogenes</i> 1/2a F6854; (4) Searches; (5) Locus; (6) typing "LMO6854_2703" in the box; and (7) clicking at TIGR sequences on the sidebar.	

[0529] A phage attachment site (attPP') or bacterial attachment site (attBB') of the present invention can be implanted into a polynucleotide by way of site-specific recombination, homologous recombination, by use of restriction sites, by methods of synthetic organic chemistry, or by other methods. In particular, where homologous recombination is used, an attBB' site can be implanted into a virulence gene, where integration results in a simple

insertion or, alternatively, in insertion with deletion of a corresponding region of the virulence gene.

[0530] Thus, the present invention provides methods for implanting a phage attachment site (attPP') into a plasmid. Provided are methods for implanting a bacterial attachment site (attBB') into a plasmid, as well as downstream methods where the plasmid can later be used to transfer the attBB' into a bacterial genome. In one aspect, the plasmid contains a first nucleic acid encoding an attPP' site and a second nucleic acid encoding a heterologous antigen. In this case, the invention contemplates methods for incorporating the second nucleic acid into an attBB' site residing in a target polynucleotide, where the target polynucleotide can be a bacterial genome.

[0531] The target polynucleotide of site-specific recombination, homologous recombination, or engineering by using restriction sites, is not to be limited to virulence genes, but also encompasses without limitation any polynucleotide, plasmid, episome, extrachromosomal element, bacterial genome, listerial genome, genome of *Bacillus anthracis*, or genome of *Francisella tularensis*.

[0532] The present invention encompasses a nucleic acid encoding a phage integrase, an attPP' site, or an attBB' site, where the nucleic acid can hybridize under stringent condition to one of the nucleic acids claimed as part of the present invention, that is, to one of the nucleic acids encoding a phage integrase, attPP' site, or attBB site, and where the hybridizing polynucleotide can encode a functional phage integrase, attPP' site, or attBB' site.

[0533] Also encompassed is a nucleic acid derived from a polymerase chain reaction (PCR), where the pair of PCR primers matches exactly and brackets a functional region of one of the nucleic acids of the present invention, disclosed herein, encoding a phage integrase, attPP' site, or attBB site. The PCR reaction can be carried out in silico. The present invention encompasses a nucleic acid derived from the PCR reaction, where the nucleic acid encodes a functional phage integrase, attPP' site, or attBB' site. The PCR primers can be designed to bracket the entire nucleic acid encoding the phage integrase, attPP' site, or attBB' site, disclosed herein, or they can be designed to bracket a shorter, functionally active, part of the nucleic acid.

EXAMPLE IX. Reagents and methods used for Examples X and XI

[0534] The heterologous antigen in recombinant *L. monocytogenes* (Lm) strains can be alternatively constructed to contain the nucleotide sequence encoding the eight amino SIINF EKL (SEQ ID NO:142) peptide (also known as SL8 and ovalbumin₂₅₇₋₂₆₄), positioned in-frame at the carboxyl terminus of an ActAN100-PSCA fusion protein. Compositions such

as the SL8 epitope serve as a surrogate to demonstrate the ability of recombinant *L. monocytogenes* vaccine strains to be taken up by antigen presenting cells and subsequently program presentation of antigens via the MHC class I pathway, using an *in vitro* antigen presentation assay.

[0535] This presentation assay can be performed using the cloned C57BL/6-derived dendritic cell line DC2.4 together with the B3Z T cell hybridoma cell line. The DC2.4 line was generated from bone marrow-derived DC that were subsequently transformed with the oncogenes *myc* and *raf*. DC2.4 cells have been shown previously to present exogenously added antigen by both MHC class I and class II pathways. Presentation of peptide by DC2.4 cells on class I molecules following phagocytosis of recombinant *L. monocytogenes* was measured after incubation with B3Z cells. B3Z is a lacZ-inducible CD8⁺ T-cell hybridoma specific for the SL8 epitope presented on the murine K^b class I molecule. Class I-restricted presentation of SL8 to B3Z cells results in the induction of beta-galactosidase synthesis by B3Z. The amount of beta-galactosidase produced can be measured by the hydrolysis of the chromogenic substrate CPRG and is an indication of the amount of SL8 / K^b complexes presented on the surface of antigen presenting cells. Thus, the *in vitro* antigen presentation assay is a direct measure of the ability of recombinant *L. monocytogenes* strains utilizing any predetermined ActAN100-heterologous antigen fusion protein to express and secrete the antigen fusion protein within infected host cells, leading to appropriate processing and presentation on MHC molecules and stimulation of antigen-specific T lymphocytes (Dubensky, *et al.*, Pat. Appl. Publ. No. US 2004/0228877).

[0536] Alternatively, the ability of recombinant *L. monocytogenes* strains utilizing any selected ActAN100-heterologous antigen composition to express and secrete the antigen fusion protein within infected host cells can be measured by Western Blot analysis of recombinant *L. monocytogenes*-infected host cell lysates, using an antibody that is specific for the ActAN100 N-terminal region fusion partner. The anti-ActA antibody used in this Example was a rabbit polyclonal antibody raised against a synthetic peptide (ATDSEDSSLNTDEWEEEEK (SEQ ID NO:143)) corresponding to the mature N-terminal 18 amino acids of ActA (Mourrain, *et al.* (1997) Proc. Natl. Acad. Sci. USA 94:10034-10039).

[0537] The following lysis buffer was used to extract *L. monocytogenes*-encoded proteins that had been expressed and secreted within a mammalian host cell, e.g., into the cytosol of the mammalian host cell. Lysis buffer was prepared in a total volume of 50 ml, and contained 2.5 ml 1 M Tris HCl, pH 6.8; 10 ml 10% sodium dodecylsulfate; 5 ml glycerol; 0.1

g bromophenol blue; and 5 ml 1 M dithiothreitol. Extraction was with heating at 100° for 5 min.

[0538] Immune response in mice vaccinated with recombinant *L. monocytogenes* strains was assessed as follows. Elispot assays were used to assess immune responses that had been generated in mice inoculated with *L. monocytogenes* vaccine constructs. In short, Balb/c mice were inoculated with the indicated *L. monocytogenes* strain (5×10^6 cfu). After one week, spleens were harvested, and the isolated splenocytes (2×10^5 splenocytes) were incubated overnight with or without the indicated peptides. The overnight incubations were conducted in wells coated with immobilized anti-interferon- γ antibody. The immobilized antibody served to capture any secreted IFN- γ , thus permitting subsequent measurement of secreted IFN- γ , and assessment of the immune response to the vaccine. To view the overall picture, immune responses generated in the mouse were visualized in the *in vitro* splenocyte IFN- γ expression assays. In Figure 50, the peptide pool spanned the amino acid sequence of human PSCA. In Figure 52, the added peptides were either the PSCA pool, the mesothelin pool, or a single peptide corresponding to an epitope of the listerial protein, p60. The p60 peptide was p60₂₁₇₋₂₂₅ (KYGVSVDI (SEQ ID NO:144)).

[0539] In Elispot assays, the PSCA peptide pool was a pool of 28 peptides, where the 28 peptides spanned the length of human PSCA. Each peptide was a 15-mer, where adjacent peptides overlapped each other by 11 amino acids. The concentration of each peptide was 1 microgram/ml.

[0540] The Mesothelin peptide pool was a pool of 153 different peptides spanning the entire sequence of human Mesothelin, where succeeding peptides overlapped by 11 amino acids. The concentration of each peptide was 1 microgram/ml. Each peptide was a 15-mer.

EXAMPLE X. Construction and Immunogenicity of recombinant *L. monocytogenes* strains encoding Prostate Stem Cell Antigen (PSCA)

[0541] The construction and immunogenicity of recombinant *L. monocytogenes* containing an expression cassette encoding an ActAN100-Prostate Stem Cell Antigen (PSCA) based fusion protein is yet a further non-limiting example of the utility of the compositions and methods set forth in this invention. The Examples disclose expression and secretion of heterologous antigens from recombinant *Listeria* within infected mammalian host cells, leading to a cellular immune response specific for the antigen.

[0542] Prostate stem cell antigen (PSCA) is a cell surface antigen associated with human cancer. A number of PSCA sequences and PSCA-derived sequences are shown below. What is disclosed is the amino acid sequence of human PSCA; the nucleic acid sequence of codon optimized PSCA (codon optimized for expression in *Listeria monocytogenes*), where the PSCA was deleted in its signal sequence and the GPI-anchor sequence; the amino acid sequence encoded by this codon optimized nucleic acid sequence; and the amino acid sequence of an ActAN100-human PSCA fusion protein, modified to contain a standard immunogenic peptide (SIINFELK (SEQ ID NO:142)). GPI-anchor sequence refers to a sequence of amino acids occurring at the C-terminus of PSCA for post-translational modification by glycosylphosphatidylinositol (GPI).

Human PSCA amino acid sequence (SS & GPI in *italics*):

*MKAVLLALLMAGLALQPGTALLCYSCKAQVS*NEDCLQVENCTQLGEQCWTARIRAVG
LLTVISKGCSLNCVDDSDQDYVVGKKNITCCD~~TDLCNASGAHALQ~~*PAAAILALLPALGLL*
LWGPGQL- (SEQ ID NO:145)

Codon-optimized PSCA, BamHI-SpeI delta SS, delta GPI (amino acids 18-101):

ggatccGGTACAGCACTTTTATGTTATTCCTGTAAAGCACAAAGTATCAAATGAAGAC
TGTTTACAAGTAGAAAATTGTACACAATTGGGAGAACAATGTTGGACTGCGAGA
ATTCGAGCCGTAGGTTTATTAAGTAAAGGATGTTTCGTTAAACTGTG
TAGATGACTCACAAGATTATTACGTTGGCAAAAAAATATTACATGCTGTGACAC
TGATTTATGCAATGCAAGTGGCGCTCACGCTCTTCAAactagt (SEQ ID NO:146)

Translation of codon-optimized PSCA BamHI-SpeI delta SS, delta GPI (GS and TS are BamHI and SpeI restriction sites, respectively):

GSGTALLCYSCKAQVSNECLQVENCTQLGEQCWTARIRAVGLLTVISKGCSLNCVD
DSQDYVVGKKNITCCD~~TDLCNASGAHAL~~QTS (SEQ ID NO:147)

ActAN100-hPSCA 18-101-SIINFELK (predicted sequence based on nucleic acid sequence, the first amino acid is Valine). (PSCA sequence is underlined):

VGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEKTEEQPSEVNTG
PRYETAREVSSRDIEELEKSNKVKNTNKADLIAMLKAKAEKGGSGGTALLCYSCKAQV
SNEDCLOVENCTQLGEQCWTARIRAVGLLTVISKGCSLNCVDDSDQDYVVGKKNITC
CDTDLCNASGAHALQTSQLADLVAKVLQLESIIINFELKADLVA (SEQ ID NO:148)

ActAN100-hPSCA 18-101-SIINFEKL, (the first amino acid is methionine) (Actual sequence expressed in *Listeria monocytogenes* when first amino acid is the amino-terminal amino acid (PSCA sequence is underlined):

MGLNRFMRAMMVVFITANCITINPDIIFAATDSEDSSLNTDEWEEEEKTEEQPSEVNTG
 PRYETAREVSSRDIEELEKSNKVKNTNKADLIAMLKAKAEKGGSGTALLCYSCKAQV
SNEDCLOVENCTQLGEOCWTARIRAVGLLTVISKGCSLNCVDDSDQYYVGKKNITC
CDTDLCNASGAHALQTSQLADLVLAQVLQLESIINFEKLADLVA (SEQ ID NO:149)

[0543] Figure 47A-B discloses the schematic configuration of the vector, pINT-ActAN100-BamHI-SpeI-MfeI-SIINFEKL plasmid DNA. This vector contains an attPP' site, which mediates site-specific integration into a nucleic acid that contains a corresponding attBB' site. The vector also contains an actA promoter, actA-N100, a cloning site containing a number of restriction sites, and a SIINFEKL (SEQ ID NO:142) sequence. The vector also encodes antibiotic resistance genes (ErmC and CAT), which are flanked by loxP sites, where the loxP sites facilitate Cre-recombinase catalyzed elimination of the antibiotic resistance genes. Elimination of these genes is useful, for example, after the vector has been integrated into a bacterial genome, and after isolation of the bacterium with the successful integration.

[0544] Figure 48 discloses the schematic configuration of PSCA molecular constructs with a Kyte-Doolittle overlay. The diagrams include the following domains, namely, actA signal sequence (actA SS); actA-N100; PSCA signal sequence; PSCA; the PSCA GPI anchor region, and SIINFEKL ((SEQ ID NO:142) abbreviated as "SL8"). As indicated in the Figure, "delta half SS, delta half GPI" refers to the PSCA SS with half of this sequence deleted, and the PSCA GPI anchor with half of this sequence deleted. Amino acids 1-9 of the signal sequence were deleted, while amino acids 112-123 of the GPI anchor had been deleted.

[0545] The following concerns the terminology in Figure 48. The diagram separately identifies ActA SS and ActA-N100. ActA-N100 includes, within it, an ActA signal sequence. However, the amino acid sequence of ActA signal sequence was not duplicated in the constructs, and the separate identification of ActA SS and ActA-N100 is only for convenience. In this diagram, the notation PSCA means full length PSCA, but without the PSCA SS and without the PSCA GPI anchor. Thus, where the diagram shows that a construct contains both "SS" and "PSCA," there exists only one PSCA signal sequence in the construct.

[0546] Figure 49A discloses response of a reporter T cell line (B3Z cells) to peptides presented by dendritic cells. The bar graph on the left demonstrates that heterologous ActA-N100-PSCA fusion proteins encoded by recombinant *L. monocytogenes*, were expressed and secreted within infected host dendritic cells, and subsequently processed and presented on MHC molecules by the dendritic cells. Various recombinant *L. monocytogenes* were used to infect DC2.4 cells, as indicated in the Figure. With infection, the recombinant *L. monocytogenes* expressed and secreted the polypeptides within the DC2.4 cells. The DC2.4 cells presented peptides, where detection of presented peptides was by way of a reporter T cell hybridoma line (B3Z T cell hybridoma). CRS-100 is a *L. monocytogenes* strain with deletions in both the actA and inlB genes (*Lm* Δ actA Δ inlB). The strain CRS-100 is not engineered to express any heterologous antigen, though it can be modified to contain further mutations or to express heterologous antigens.

Table 17. *Listeria monocytogenes* strains expressing ActA-N100-PSCA fusion proteins

Strain	Construct	Background
BH137	pPL2_ActAN100-AH1A5-OVA	CRS-100
CRS-100		
BH419	p221_ActAN100_PSCA- β _SL8	CRS-100
BH470	p221_ActAN100_PSCA-1-100_SL8	CRS-100
BH472	p221_ActAN100_PSCA-10-110_SL8	CRS-100
BH474	p221_ActAN100_PSCA-16-122_SL8	CRS-100
BH476	p221_ActAN100_PSCA-16-100_SL8	CRS-100

[0547] Figure 49A discloses results from the B3Z T cell assays. Various *L. monocytogenes* strains (2×10^7 cfu) were incubated for one hour with 100,000 DC2.4 cells at 37°, to allow infection of the DC2.4 cells. After the one-hour incubation, 100,000 B3Z cells were added, together with gentamicin at a final concentration of 0.1 mg/ml. The final volume was 0.2 ml/well. The mixture was incubated overnight, followed by collection of all cells by centrifugation, lysis of cells, and assay of the beta-galactosidase expressed by the reporter B3Z T cell line. The results were a function of a number of steps, for example, bacterial expression and secretion, and the DC2.4 cell's presentation of the SL8 peptide by way of MHC Class I. Similar results were found with all of the indicated recombinant *L. monocytogenes* that encoded the SL8 epitope.

[0548] The Western blot (Figure 49B) demonstrates that recombinant *L. monocytogenes* infected J774 macrophage cells express and secrete the encoded heterologous ActA-N100-PSCA fusion proteins. The Western blot discloses results from recombinant *L. monocytogenes* infections of J774 cells. J774 macrophages were infected with recombinant

L. monocytogenes, where the figure identifies each of the recombinant *L. monocytogenes* strains. J774 cells (1×10^6 cells) were infected with *L. monocytogenes* (50×10^6 cfu), at a multiplicity of infection of 50. The mixture was incubated for 30 min, washed, then supplemented with gentamicin and incubated for 5-7 hours at 37°. After this incubation, the J774 cells were lysed, using lysis buffer, in order to release the expressed and secreted ActA-N100-PSCA fusion protein, while leaving the bacteria intact.

[0549] The Western blot demonstrates that the delta SS, delta GPI construct was expressed and secreted most efficiently (lane 6). The SS and GPI anchor domains contain regions of hydrophobicity that may complicate secretion. The invention provides antigen constructs deleted in SS and GPI domains.

Table 18. Legend for Figure 49B. Western blot analysis for expression and secretion of ActA-N100-PSCA fusion proteins

lane	strain	antigen
1	hMeso26	ActAN100-hMeso Δ SS, Δ GPI
2	BH419	ActAN100-PSCA Δ SL8
3	BH470	ActAN100-PSCA 1-100_SL8
4	BH472	ActAN100-PSCA 10-110_SL8
5	BH474	ActAN100-PSCA 16-122_SL8
6	BH476	ActAN100-PSCA 16-100_SL8
7	CRS-100	-

[0550] The expected molecular weight of the polypeptide expressed from *L. monocytogenes* strain BH419 (actA-N100-full length PSCA-SL8) is 24 kDa. The expected molecular weight for the polypeptide expressed from *L. monocytogenes* BH476 (actA-N100-PSCA 16-100-SL8) is 20.3 kDa.

[0551] *L. monocytogenes* strain BH476 produced the greatest levels of expression and secretion as demonstrated by the gel, where the gel revealed dense staining of polypeptides in a molecular weight range in the vicinity of the 19 kDa molecular weight marker.

[0552] Figure 50 discloses vaccine-induced PSCA-specific T cell immune responses in mice vaccinated with various recombinant *L. monocytogenes* strains expressing ActA-N100-PSCA fusion proteins.

[0553] Balb/c mice were inoculated with the indicated recombinant *L. monocytogenes* strain. After one week, spleens were harvested and the isolated splenocytes were incubated overnight with or without the indicated peptides, in Elispot assays. Incubations were in the presence or absence of the PSCA peptide pool. The results demonstrate that recombinant *L. monocytogenes* encoding full length PSCA induced the lowest immune response, while Lm-PSCA- Δ SS- Δ GPI (Lm-PSCA-16-100) induced the highest immune response (Figure 50). These results demonstrate that the extent of expression and secretion of the ActA-N100-

PSCA fusion protein by recombinant *L. monocytogenes* within infected host cells is directly correlated with its immunogenicity in vaccinated animals. This correlation can be observed by comparing the Western blots with the Elispot results.

EXAMPLE XI. Construction and immunogenicity of recombinant bivalent *L. monocytogenes* strains encoding ActA-N100-Prostate Stem Cell Antigen (PSCA) and ActA-N100-Mesothelin.

[0554] It is well known in the art that selected tumors express several antigens that are related to the tumor, and that inducing cellular immune responses to more than one of these tumor associated antigens can enhance the potency of a selected cancer vaccine. The invention provides compositions and methods for recombinant *L. monocytogenes* vaccine strains that express and secrete more than one heterologous antigen, where vaccination leads to the induction of cellular immunity in vaccinated animals to each of the expressed heterologous antigens. Example XI discloses, as a non-limiting example, the utility of compositions and methods of recombinant bivalent *L. monocytogenes* strains encoding two distinct ActAN100-heterologous antigen fusion proteins.

[0555] Figures 51A and 51B disclose a recombinant bivalent *L. monocytogenes* vaccine strain expressing two different human cancer antigens, Mesothelin and PSCA, each antigen taking the form of an ActA-N100 fusion protein. Utility was demonstrated by the induced T cell immunity, where T cell immunity was induced against each expressed and secreted antigen in animals vaccinated with the bivalent vaccine.

[0556] The schematic shown in Figure 51A shows a first fusion protein of actA-N100 and Mesothelin Δ SS Δ GPI, and a second fusion protein of actA-N100 and PSCA Δ SS Δ GPI. The diagram identifies the *L. monocytogenes* internalin B gene (*inlB* gene) as the site of integration of a first nucleic acid encoding the first fusion protein, and the *L. monocytogenes* tRNA^{Arg} locus as the site of integration of a second nucleic acid encoding the second fusion protein.

[0557] The Western blot (Figure 51B) demonstrates expression and secretion of both fusion proteins, i.e., the actA-N100-mesothelin Δ SS Δ GPI and actA-N100-PSCA Δ SS Δ GPI, by recombinant bivalent *L. monocytogenes* vaccine strains in infected J774 macrophage cells. The recombinant bivalent *L. monocytogenes* vaccine strain (Lm strain BH648) expressed and secreted both the PSCA and Mesothelin ActA-N100 fusion proteins in the infected J774 macrophages, as demonstrated by the Western blot (lane 3). Expression of ActAN100-PSCA was similar to that found with the recombinant monovalent *L. monocytogenes* strain encoding

only actA-N100-PSCA Δ SS Δ GPI (lane 2), while expression of actAN100-Mesothelin was similar to that found with the recombinant monovalent *L. monocytogenes* strain encoding only actA-N100-mesothelin Δ SS Δ GPI (lane 4).

[0558] Figure 52 discloses immunization of mice with recombinant monovalent *L. monocytogenes* encoding only ActAN100-PSCA (BH476) or with recombinant bivalent *L. monocytogenes* encoding both PSCA and Mesothelin ActA-N100 fusion proteins (BH648). The PSCA and Mesothelin sequences in these constructs are delta SS and delta GPI.

[0559] Balb/c mice were immunized with the indicated recombinant *L. monocytogenes* (5×10^6 cfu) vaccine strain. After one week, spleens were harvested, and the isolated splenocytes were incubated overnight with or without the indicated peptides, in Elispot assays.

[0560] Elispot assays disclose the results of immunizing with one of two different *L. monocytogenes* vaccine strains. Balb/c mice were immunized with recombinant monovalent *L. monocytogenes* strain BH476, which encoded only one human cancer antigen (PSCA), or with recombinant bivalent *L. monocytogenes* strain BH648, which encoded two human cancer antigens (PSCA and Mesothelin). The results demonstrate that the recombinant monovalent *L. monocytogenes* strain stimulated an immune response against PSCA, while the recombinant bivalent *L. monocytogenes* strain stimulated immune response against both PSCA and Mesothelin. The results demonstrate that the magnitude of PSCA-specific cellular immunity elicited in mice immunized with either recombinant monovalent or bivalent *L. monocytogenes* strains expressing ActAN100-PSCA fusion protein were the same, thus illustrating the utility of recombinant *L. monocytogenes* vaccine strains containing two or more antigen expression cassettes encoding ActA-N100-heterologous antigen fusion proteins, as applied to any desired selected heterologous antigen.

[0561] Many modifications and variations of this invention, as will be apparent to one of ordinary skill in the art, can be made to adapt to a particular situation, material, composition of matter, process, process step or steps, to preserve the objective, spirit, and scope of the invention. All such modifications are intended to be within the scope of the claims appended hereto without departing from the spirit and scope of the invention. The specific embodiments described herein are offered by way of example only, and the invention is to be limited by the terms of the appended claims, along with the full scope of the equivalents to which such claims are entitled; and the invention is not to be limited by the specific embodiments that have been presented herein by way of example.

CLAIMS

What is claimed is:

1. A polynucleotide comprising:
 - (a) a promoter; and
 - (b) a nucleic acid operably linked to the promoter, wherein the nucleic acid encodes a fusion protein comprising:
 - (i) a modified ActA comprising more than the first 59 amino acids of ActA, and less than the first 380 amino acids of ActA; and
 - (ii) a heterologous antigen.
2. The polynucleotide of claim 1, wherein the promoter is an *actA* promoter.
3. The polynucleotide of claim 1, wherein the modified ActA comprises less than the first 265 amino acids of ActA.
4. The polynucleotide of claim 1, wherein the modified ActA comprises at least the first 85 amino acids of ActA and less than the first 125 amino acids of ActA.
5. The polynucleotide of claim 4, wherein the modified ActA consists of amino acids 1-100 of ActA.
6. The polynucleotide of claim 5, wherein the promoter is an *actA* promoter.
7. The polynucleotide of claim 1, wherein the heterologous antigen is non-Listerial.
8. The polynucleotide of claim 1, wherein the heterologous antigen is from, or is derived from, a cancer cell, tumor, or infectious agent.
9. A plasmid comprising the polynucleotide of claim 1.
10. A *Listeria* bacterium comprising the polynucleotide of claim 1.
11. The *Listeria* bacterium of claim 10, which is *Listeria monocytogenes*.

12. The *Listeria* bacterium of claim 11 which is attenuated for cell-to-cell spread and/or entry into nonphagocytic cells.
13. The *Listeria* bacterium of claim 12 which is an *actA* deletion mutant or an *actA* insertion mutant.
14. The *Listeria* bacterium of claim 13, wherein the promoter is an *actA* promoter.
15. The *Listeria* bacterium of claim 10, wherein the *Listeria* bacterium comprises the polynucleotide in its genome.
16. The *Listeria* bacterium of claim 15, wherein the polynucleotide has been integrated into a virulence gene in the genome, wherein the integration of the polynucleotide:
 - (a) disrupts expression of the virulence gene; or
 - (b) disrupts a coding sequence of the virulence gene.
17. The *Listeria* bacterium of claim 16, wherein the virulence gene is *actA* or *inlB*.
18. The *Listeria* bacterium of claim 15, wherein the nucleic acid encoding the fusion protein has been integrated into a virulence gene in the genome, wherein the integration of the nucleic acid:
 - (a) disrupts expression of the virulence gene; or
 - (b) disrupts a coding sequence of the virulence gene.
19. A vaccine comprising the *Listeria* bacterium of claim 10.
20. A method for stimulating an immune response to the heterologous antigen in a mammal comprising administering an effective amount of the *Listeria* bacterium of claim 10, or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal.
21. A *Listeria* bacterium comprising a genome, wherein the genome comprises a polynucleotide comprising a nucleic acid encoding a heterologous antigen, wherein the

nucleic acid has been integrated into a virulence gene in the genome, wherein integration of the polynucleotide

- (a) disrupts expression of the virulence gene; or
- (b) disrupts a coding sequence of the virulence gene.

22. The *Listeria* bacterium of claim 21, wherein all or part of the virulence gene has been deleted.

23. The *Listeria* bacterium of claim 21, wherein none of the virulence gene has been deleted.

24. The *Listeria* bacterium of claim 21, wherein the virulence gene is actA or inlB.

25. The *Listeria* bacterium of claim 21, which is *Listeria monocytogenes*.

26. The *Listeria* bacterium of claim 21, wherein the heterologous antigen is from, or is derived from, a cancer cell, tumor, or infectious agent.

27. The *Listeria* bacterium of claim 21, further comprising:
a second nucleic acid encoding a second heterologous antigen that has been integrated into a second virulence gene.

28. The *Listeria* bacterium of claim 21, wherein the nucleic acid encodes a fusion protein comprising a modified ActA and the heterologous antigen.

29. A vaccine comprising the *Listeria* bacterium of claim 20.

30. A method for stimulating an immune response to the heterologous antigen in a mammal, comprising administering an effective amount of the *Listeria* bacterium of claim 21, or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal.

31. A method of producing a *Listeria* bacterium for use in a vaccine, comprising:

integrating a polynucleotide into a virulence gene in the genome of the *Listeria* bacterium, wherein the polynucleotide comprises a nucleic acid encoding a heterologous antigen and wherein the integration of the polynucleotide

- (a) disrupts expression of the virulence gene; or
- (b) disrupts a coding sequence of the virulence gene.

32. The method of claim 31, wherein the polynucleotide is integrated into the virulence gene by homologous recombination.

33. The method of claim 31, wherein all or part of the virulence gene is deleted during integration of the polynucleotide into the virulence gene.

34. The method of claim 31, wherein the virulence gene is *actA* or *inlB*.

35. A *Listeria* bacterium produced by the method of claim 31.

36. A polynucleotide comprising a first nucleic acid encoding actA-N100, operably linked and in frame with, a second nucleic acid encoding a heterologous antigen.

37. A *Listeria* bacterium comprising the polynucleotide of claim 36.

38. The *Listeria* bacterium of claim 37, wherein the polynucleotide is genomic.

39. The *Listeria* bacterium of claim 37, wherein the polynucleotide is integrated into *actA* or *inlB*.

40. The *Listeria* bacterium of claim 37, wherein the polynucleotide is plasmid-based.

41. The *Listeria* bacterium of claim 37 which is *Listeria monocytogenes*.

42. The *Listeria* bacterium of claim 37, wherein the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent.

43. The *Listeria* bacterium of claim 37, wherein the heterologous antigen is immunologically cross-reactive with, or shares at least one epitope with, the cancer, tumor, or infectious agent.
44. The *Listeria* bacterium of claim 37, wherein the polynucleotide is operably linked to an *actA* promoter.
45. A vaccine comprising the *Listeria* bacterium of claim 37.
46. A method for stimulating immune response to an antigen from, or derived from, a cancer or infectious agent, comprising administering the *Listeria* bacterium of claim 37 to a mammal having the cancer or infectious agent, and wherein the heterologous antigen shares at least one epitope with or is immunologically cross-reactive with the antigen from, or derived from, the cancer or infectious agent.
47. The method of claim 46, wherein the cancer comprises a tumor or pre-cancerous cell.
48. The method of claim 46, wherein the infectious agent comprises a virus, pathogenic bacterium, or parasitic organism.
49. A polynucleotide comprising a first nucleic acid encoding a modified actA, wherein the modified actA comprises:
- a. amino acids 1-59 of actA; and
 - b. an inactivating mutation in, deletion of, or truncation prior to, at least one domain for actA-mediated regulation of the host cell cytoskeleton,
- wherein the first nucleic acid is operably linked and in frame with a second nucleic acid encoding a heterologous antigen.
50. The polynucleotide of claim 49, wherein the domain is the cofilin homology region (KKRR) (SEQ ID NO:23).
51. The polynucleotide of claim 49, wherein the domain is the phospholipid core binding domain (KVFFKKIKDAGKWVRDKI) (SEQ ID NO:20).

52. The polynucleotide of claim 49, wherein the at least one domain comprises all four proline-rich domains (FPPPP, FPPPP, FPPPP, FPPIP) (SEQ ID NO:21, SEQ ID NO:22) of ActA.
53. A *Listeria* bacterium containing the polynucleotide of claim 49.
54. The *Listeria* bacterium of claim 53, wherein the polynucleotide is genomic.
55. The *Listeria* bacterium of claim 53, wherein the polynucleotide is not genomic.
56. The *Listeria* bacterium of claim 53, wherein the polynucleotide is operably linked with one or more of:
- a. *actA* promoter; or
 - b. a bacterial promoter that is not *actA* promoter.
57. The *Listeria* bacterium of claim 53 that is *Listeria monocytogenes*.
58. A vaccine comprising the *Listeria* bacterium of claim 57.
59. A method for stimulating immune response to an antigen from, or derived from, a cancer or infectious agent, comprising administering the *Listeria* bacterium of claim 53 to a mammal having the cancer or infectious agent, and wherein the heterologous antigen shares at least one epitope with or is immunologically cross-reactive with the antigen from, or derived from, the cancer or infectious agent.
60. The method of claim 59, wherein the cancer comprises a tumor or pre-cancerous cell.
61. The method of claim 59, wherein the infectious agent comprises a virus, pathogenic bacterium, or parasitic organism.
62. The method of claim 59, wherein the heterologous antigen is, or is derived from, a cancer cell, tumor, or infectious agent.

63. A plasmid comprising a first nucleic acid encoding a phage integrase, a second nucleic acid encoding a phage attachment site (attPP' site), and a third nucleic acid encoding a heterologous antigen or regulatory nucleic acid, wherein:

- a. each of the nucleic acids is derivable from *L. innocua* 0071;
- b. each of the nucleic acids is derivable from *L. innocua* 1765;
- c. each of the nucleic acids is derivable from *L. innocua* 2601;
- d. each of the nucleic acids is derivable from *L. monocytogenes* f6854_2703; or
- e. the first nucleic acid encodes a phiC31 integrase,

wherein the plasmid is useful for mediating site-specific integration of the nucleic acid encoding the heterologous antigen at a bacterial attachment site (attBB' site) in a bacterial genome that is compatible with the attPP' site of the plasmid.

64. A method of modifying a bacterial genome, comprising transfecting the bacterium with the plasmid of claim 63, and allowing integrase-catalyzed integration of the third nucleic acid into the bacterial genome under conditions suitable for integration.

65. A plasmid comprising:

- a. a first nucleic acid encoding a first region of homology to a bacterial genome,
- b. a second nucleic acid encoding a second region of homology to the bacterial genome, and

c. a third nucleic acid comprising a bacterial attachment site (attBB'), wherein the third nucleic acid is flanked by the first and second nucleic acids, wherein the first nucleic acid and second nucleic acid are operably linked with each other and able to mediate homologous integration of the third nucleic acid into the bacterial genome.

66. A bacterium modified by integration of the plasmid of claim 65.

67. A bacterium wherein the genome of the bacterium comprises a polynucleotide containing two operably linked heterologous recombinase binding sites flanking a first nucleic acid, wherein the two sites are:

- a. two lox sites; or
- b. two Frt sites,

and wherein the nucleic acid flanked by the two lox sites is excisable by Cre recombinase, and wherein the nucleic acid flanked by the two Frt sites is excisable by FLP recombinase.

68. A method of excising the first nucleic acid of claim 67 from the bacterial genome, comprising contacting the genome with Cre recombinase or FLP recombinase, and allowing the recombinase to catalyze excision of the first nucleic acid, under conditions allowing or facilitating excision:

- a. wherein the first nucleic acid is flanked by lox sites and the recombinase is Cre recombinase; or
- b. wherein the first nucleic acid is flanked by Frt sites and the recombinase is FLP recombinase.

69. The *Listeria* bacterium of claim 18, wherein the virulence gene is a prfA-dependent gene.

70. The *Listeria* bacterium of claim 18, wherein the virulence gene is not a prfA-dependent gene.

71. The plasmid of claim 63, further comprising a first promoter operably linked with the first nucleic acid, and a second promoter operably linked with the third nucleic acid.

72. The *Listeria* bacterium of claim 66, wherein the integration is in a region of the genome that is necessary for mediating growth or spread.

73. The *Listeria* bacterium of claim 66, wherein the integration is in a region of the genome that is not necessary for mediating growth or spread.

74. The *Listeria* bacterium of claim 67, wherein each lox site is a loxP site.

75. A polynucleotide comprising:

- (a) a promoter; and
- (b) a nucleic acid sequence operably linked to the promoter, wherein the nucleic acid encodes a fusion protein comprising:
 - (i) a modified ActA comprising more than the first 59 amino acids of ActA, and less than the first 380 amino acids of ActA; and
 - (ii) mesothelin, or an antigen derived from mesothelin.

76. The polynucleotide of claim 75, wherein the mesothelin is human mesothelin.
77. The polynucleotide of claim 75, wherein the promoter is *actA* promoter.
78. The polynucleotide of claim 75, wherein the modified ActA comprises at least the first 85 amino acids of ActA and less than the first 125 amino acids of ActA.
79. The polynucleotide of claim 75, wherein the modified ActA consists of amino acids 1-100 of ActA.
80. The polynucleotide of claim 79, wherein the promoter is the *actA* promoter.
81. A plasmid comprising the polynucleotide of claim 75.
82. A *Listeria* bacterium comprising the polynucleotide of claim 75.
83. The *Listeria* bacterium of claim 82, which is *Listeria monocytogenes*.
84. The *Listeria* bacterium of claim 82 which is an *actA* deletion mutant or an *actA* insertion mutant.
85. The *Listeria* bacterium of claim 82, wherein the *Listeria* bacterium comprises the polynucleotide in its genome.
86. The *Listeria* bacterium of claim 85, wherein the polynucleotide has been integrated into a virulence gene in the genome, wherein the integration of the polynucleotide:
 - (a) disrupts expression of the virulence gene; or
 - (b) disrupts a coding sequence of the virulence gene.
87. The *Listeria* bacterium of claim 86, wherein the virulence gene is *actA* or *inlB*.

88. The *Listeria* bacterium of claim 85, wherein the nucleic acid encoding the fusion protein has been integrated into a virulence gene in the genome, wherein the integration of the nucleic acid:

- (a) disrupts expression of the virulence gene; or
- (b) disrupts a coding sequence of the virulence gene.

89. A vaccine comprising the *Listeria* bacterium of claim 82.

90. A method for stimulating an immune response to mesothelin, or to an antigen derived from mesothelin, in a mammal comprising administering an effective amount of the *Listeria* bacterium of claim 82, or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal.

91. A *Listeria* bacterium comprising a genome, wherein the genome comprises a polynucleotide comprising a nucleic acid encoding mesothelin, or an antigen derived from mesothelin, wherein the polynucleotide has been integrated into a virulence gene in the genome, wherein the integration of the polynucleotide:

- (a) disrupts expression of the virulence gene; or
- (b) disrupts a coding sequence of the virulence gene.

92. The *Listeria* bacterium of claim 91, wherein all or part of the virulence gene has been deleted.

93. The *Listeria* bacterium of claim 91, wherein none of the virulence gene has been deleted.

94. The *Listeria* bacterium of claim 91, wherein the virulence gene is *actA* or *inlB*.

95. The *Listeria* bacterium of claim 91, which is *Listeria monocytogenes*.

96. The *Listeria* bacterium of claim 91, wherein the mesothelin is human mesothelin.

97. A vaccine comprising the *Listeria* bacterium of claim 91.

98. A method for stimulating an immune response to mesothelin, or to an antigen derived from mesothelin, in a mammal, comprising administering an effective amount of the *Listeria* bacterium of claim 91, or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal.

99. A method of producing a *Listeria* bacterium for use in a vaccine, comprising integrating a polynucleotide into a virulence gene in the genome of the *Listeria* bacterium, wherein the polynucleotide comprises a nucleic acid encoding mesothelin, or an antigen derived from mesothelin, and wherein the integration of the polynucleotide:

- (a) disrupts expression of the virulence gene; or
- (b) disrupts a coding sequence of the virulence gene.

100. A *Listeria* bacterium produced by the method of claim 99.

101. A polynucleotide comprising a first nucleic acid encoding actA-N100, operably linked and in frame with, a second nucleic acid encoding mesothelin, or an antigen derived from mesothelin.

102. The polynucleotide of claim 101, wherein the mesothelin is human mesothelin.

103. A *Listeria* bacterium comprising the polynucleotide of claim 101.

104. The *Listeria* bacterium of claim 103, wherein the polynucleotide is genomic.

105. The *Listeria* bacterium of claim 104, wherein the polynucleotide is integrated into the *actA* gene or *inlB* gene.

106. The *Listeria* bacterium of claim 103, wherein the polynucleotide is plasmid-based.

107. The *Listeria* bacterium of claim 103 that is *Listeria monocytogenes*.

108. The *Listeria* bacterium of claim 103, wherein the polynucleotide is operably linked to an *actA* promoter.

109. A vaccine comprising the *Listeria* bacterium of claim 103.
110. A method for stimulating immune response to mesothelin, or an antigen derived from mesothelin, in a mammal comprising administering the *Listeria* bacterium of claim 103 to the mammal.
111. A polynucleotide comprising a first nucleic acid encoding a modified ActA, where the modified ActA comprises:
- a. amino acids 1-59 of ActA; and
 - b. an inactivating mutation in, deletion of, or truncation prior to, at least one domain for actA-mediated regulation of the host cell cytoskeleton,
- wherein the first nucleic acid is operably linked and in frame with a second nucleic acid encoding mesothelin, or an antigen derived from mesothelin.
112. A *Listeria* bacterium containing the polynucleotide of claim 111.
113. The *Listeria* bacterium of claim 111, wherein the polynucleotide is operably linked with one or more of:
- a. *actA* promoter; or
 - b. a bacterial promoter that is not *actA* promoter.
114. The *Listeria* bacterium of claim 111 that is *Listeria monocytogenes*.
115. A vaccine comprising the *Listeria* bacterium of claim 111.
116. A method for stimulating immune response to mesothelin, or an antigen derived from mesothelin, in a mammal comprising administering the *Listeria* bacterium of claim 111 to the mammal.
117. The *Listeria* bacterium of claim 88, wherein the virulence gene is a prfA-dependent gene.
118. The *Listeria* bacterium of claim 88, wherein the virulence gene is not a prfA-dependent gene.

119. The polynucleotide or bacterium of claim 75, 91, or 111, wherein the antigen derived from mesothelin is mesothelin deleted of its signal peptide region, its GPI anchor region, or both its signal peptide and GPI anchor region.

120. The polynucleotide of any one of claims 1-8, 36, and 149-152, or the bacterium of any one of claims 21-28, wherein the heterologous antigen is PSCA or an antigen derived from PSCA.

121. The polynucleotide or bacterium of claim 120, wherein the antigen derived from PSCA is PSCA deleted of:

- (a) part or all its signal peptide region;
- (b) its GPI anchor region; or
- (c) its GPI anchor region and part or all of its signal peptide region.

122. The polynucleotide or bacterium of claim 120, wherein the PSCA is human PSCA.

123. A *Listeria* bacterium comprising a genome, wherein the genome comprises:

- (a) a first polynucleotide comprising a first nucleic acid encoding a first heterologous antigen, wherein the first polynucleotide has been integrated into a virulence gene in the genome and wherein the *Listeria* bacterium is attenuated by:

- (i) disruption of expression of the virulence gene; or
- (ii) disruption of a coding sequence of the virulence gene; and

- (b) a second polynucleotide comprising a second nucleic acid encoding a second heterologous antigen, wherein the second polynucleotide has also been integrated into the genome.

124. The *Listeria* bacterium of claim 123, wherein the virulence gene is *actA* or *inlB*.

125. The *Listeria* bacterium of claim 123, which is *Listeria monocytogenes*.

126. The *Listeria* bacterium of claim 123, wherein the first and second heterologous antigens are from, or are derived from, a cancer cell, tumor, or infectious agent.

127. The *Listeria* bacterium of claim 126, wherein the first and/or second heterologous antigen is mesothelin, an antigen derived from mesothelin, PSCA, or an antigen derived from PSCA.

128. The *Listeria* bacterium of claim 123, wherein the first and/or second nucleic acid encodes a fusion protein comprising a modified ActA and the first or second heterologous antigen.

129. The *Listeria* bacterium of claim 128, wherein the modified ActA is ActA-N100.

130. The *Listeria* bacterium of claim 123, wherein the first and/or second nucleic acid encodes a fusion protein comprising a signal sequence and the first or second heterologous antigen, wherein the signal sequence is an ActA signal sequence, a p60 signal sequence, an LLO signal sequence, a BaPa signal sequence, a Llusp45 signal sequence, or a PhoD signal sequence.

131. A vaccine comprising the *Listeria* bacterium of claim 123.

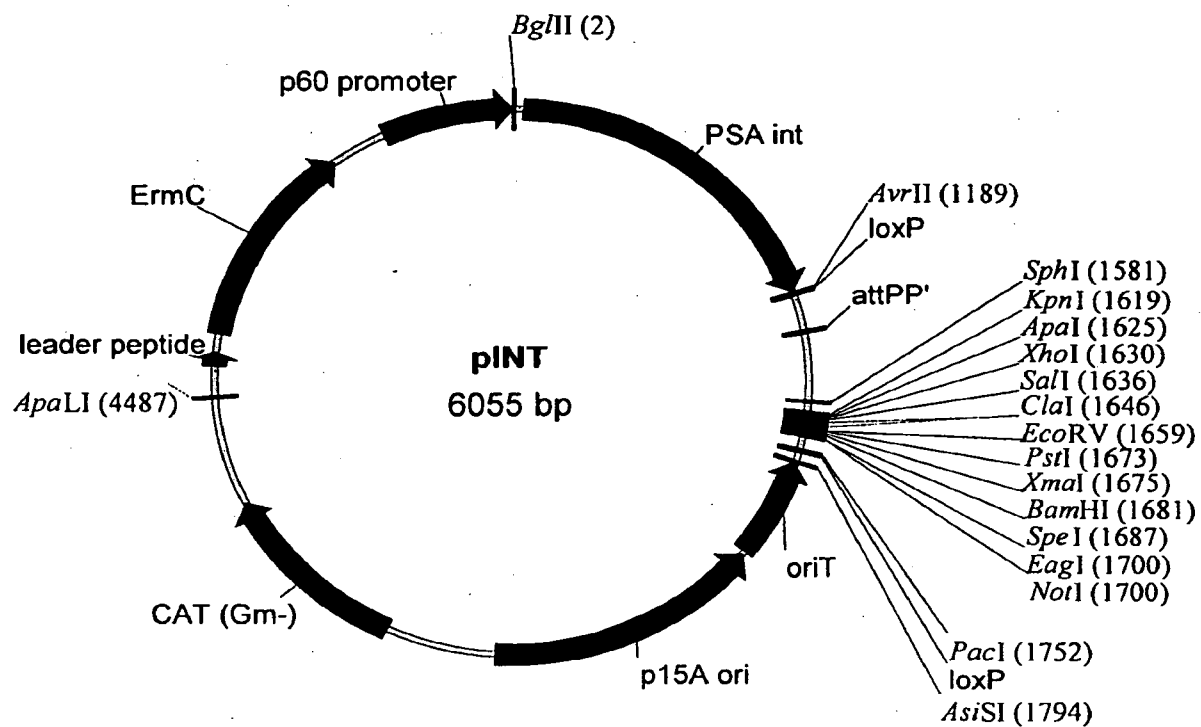
132. A method for stimulating an immune response to the heterologous antigen in a mammal, comprising administering an effective amount of the *Listeria* bacterium of claim 123, or an effective amount of a composition comprising the *Listeria* bacterium, to the mammal.

133. A method of producing a *Listeria* bacterium for use in a vaccine, comprising:
integrating a polynucleotide into a virulence gene in the genome of the *Listeria* bacterium, wherein the polynucleotide comprises a nucleic acid encoding a heterologous antigen and wherein the *Listeria* bacterium is or has been attenuated by mutation of the virulence gene.

134. A *Listeria* bacterium produced by the method of claim 133.

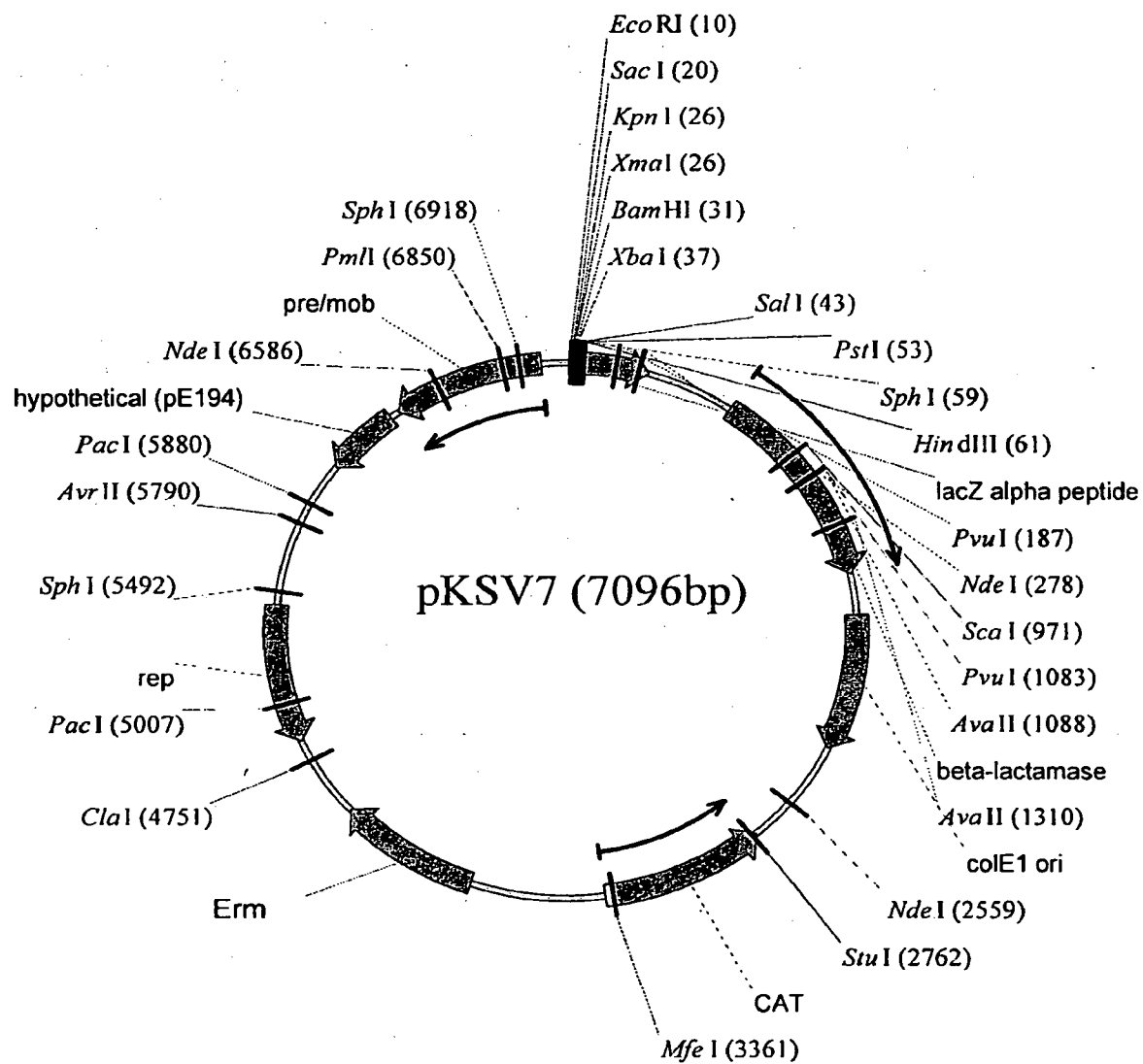
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Fig. 1



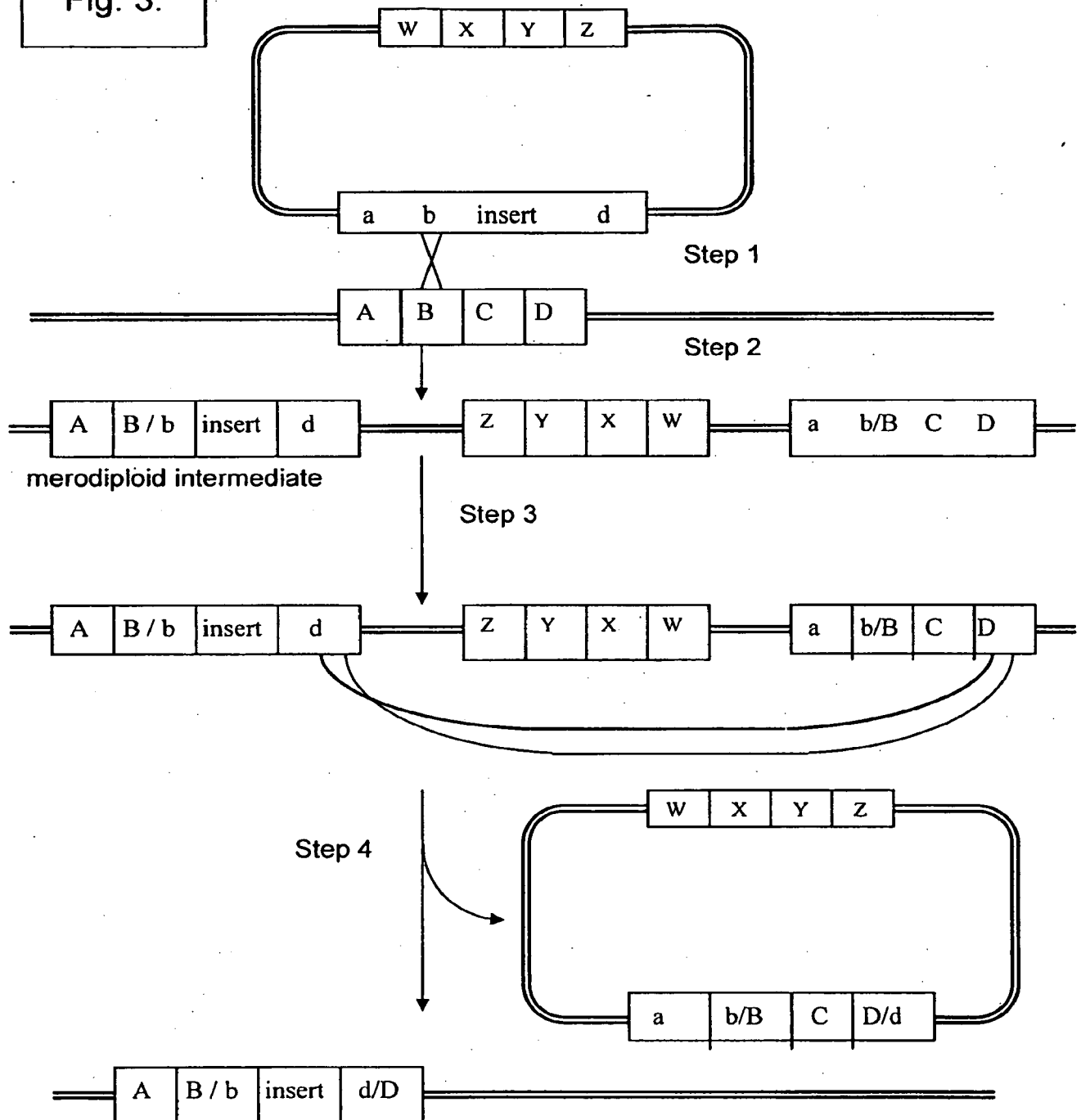
2/58

Fig. 2.



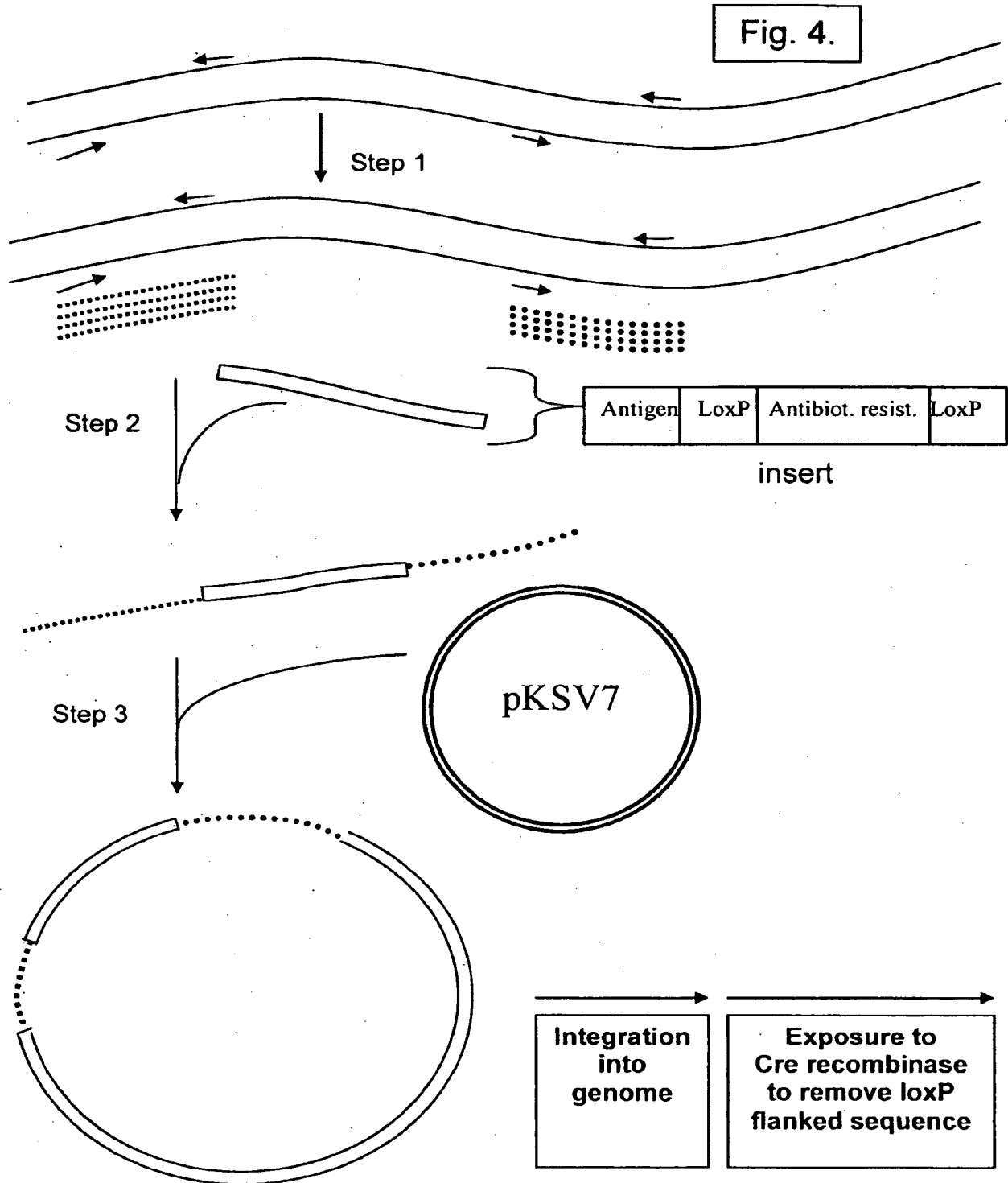
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Fig. 3.



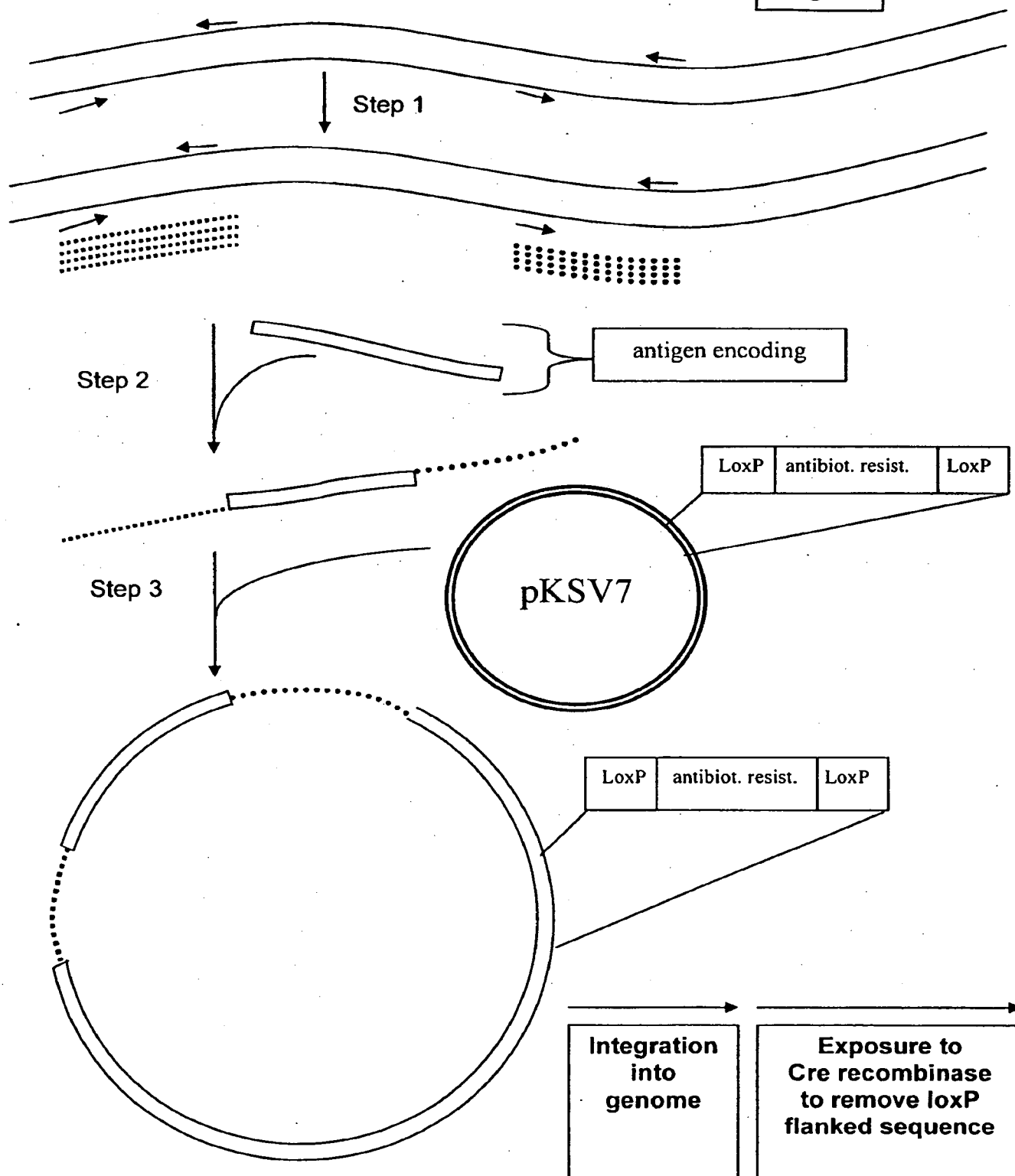
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Fig. 4.



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Fig. 5



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Fig. 6.

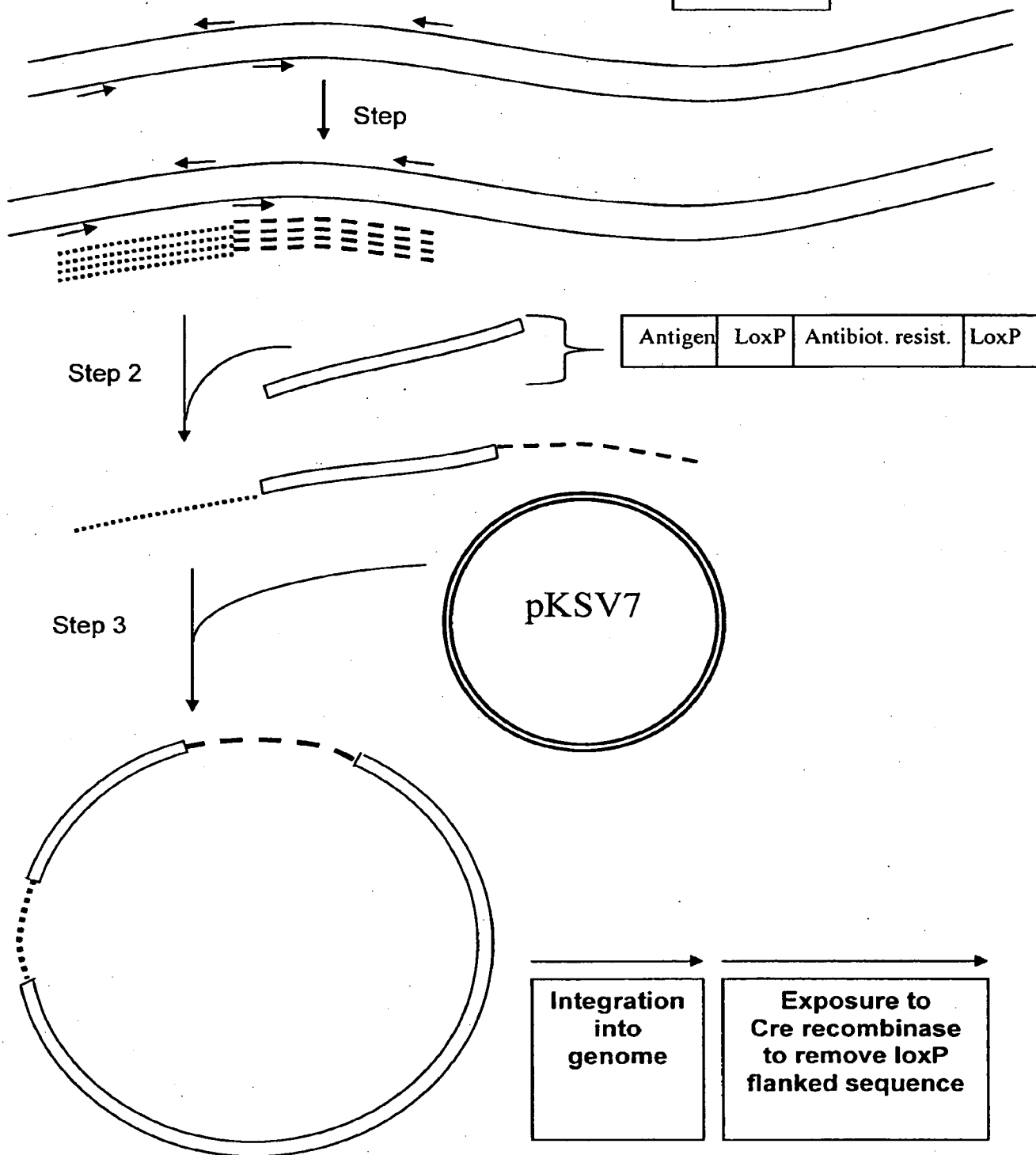


Fig. 7.

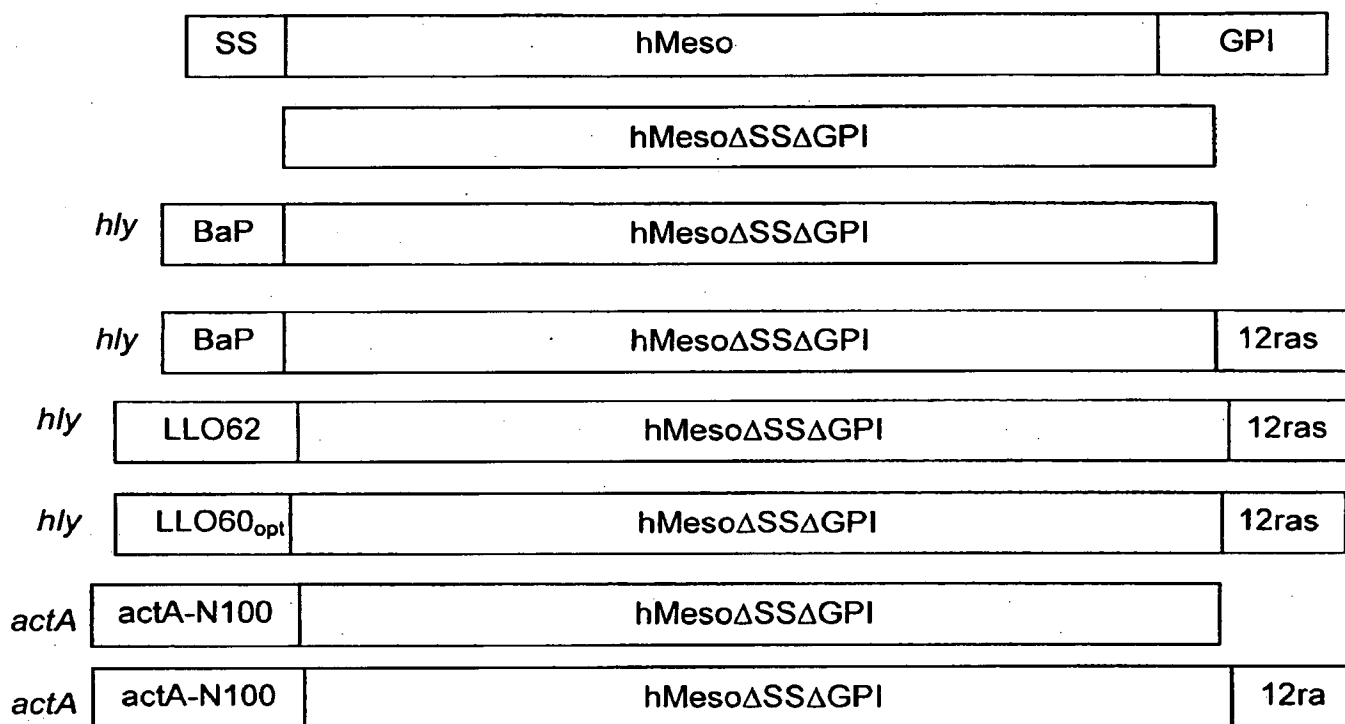
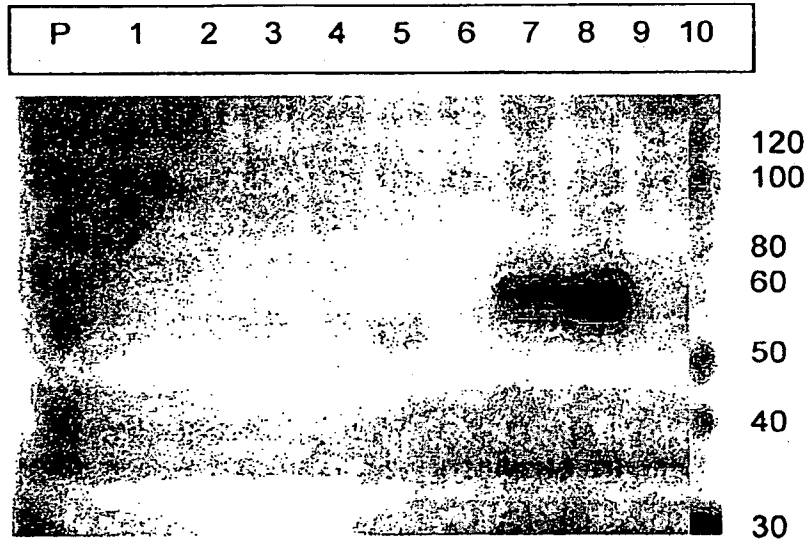


Fig. 8.



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FIG. 9

P 1 2 3 4 5 6 7 8 9 10 11 12 13 M



FIG. 10

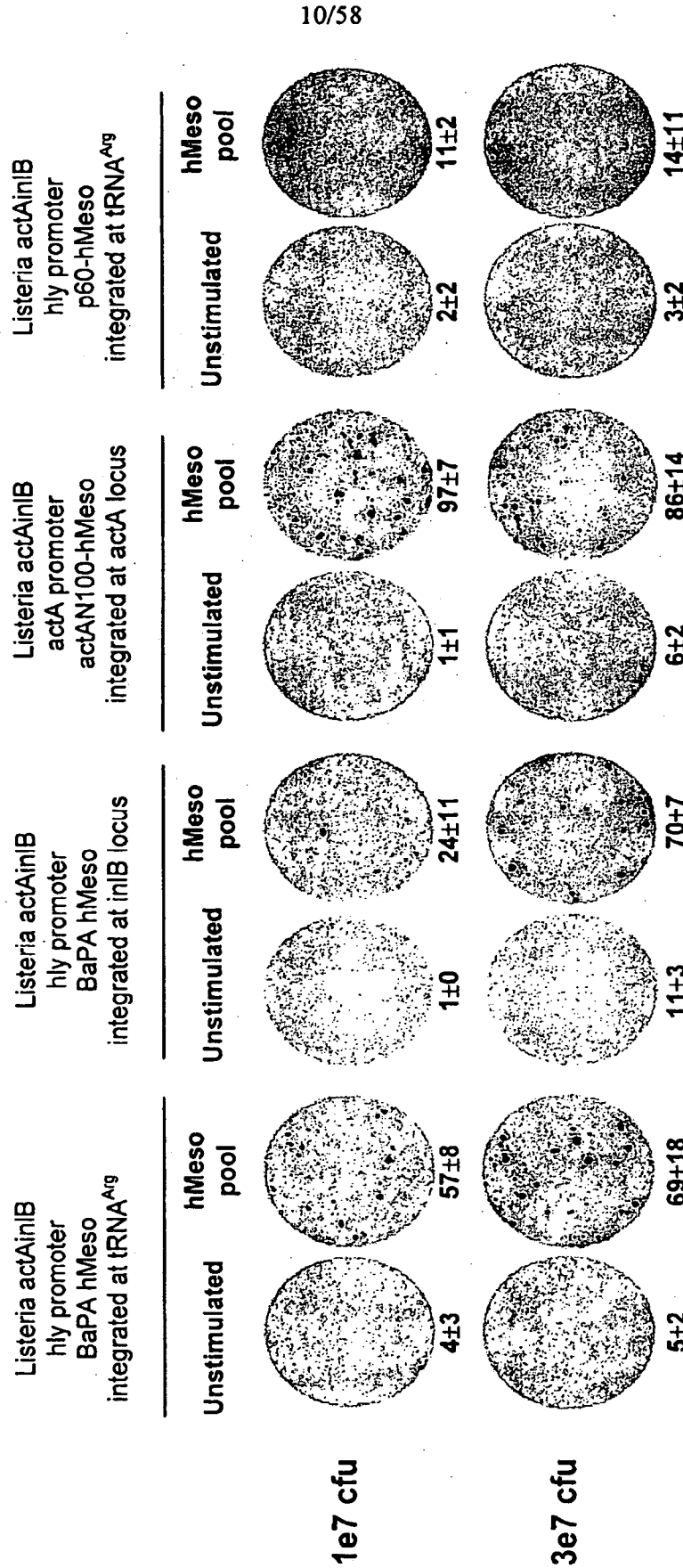
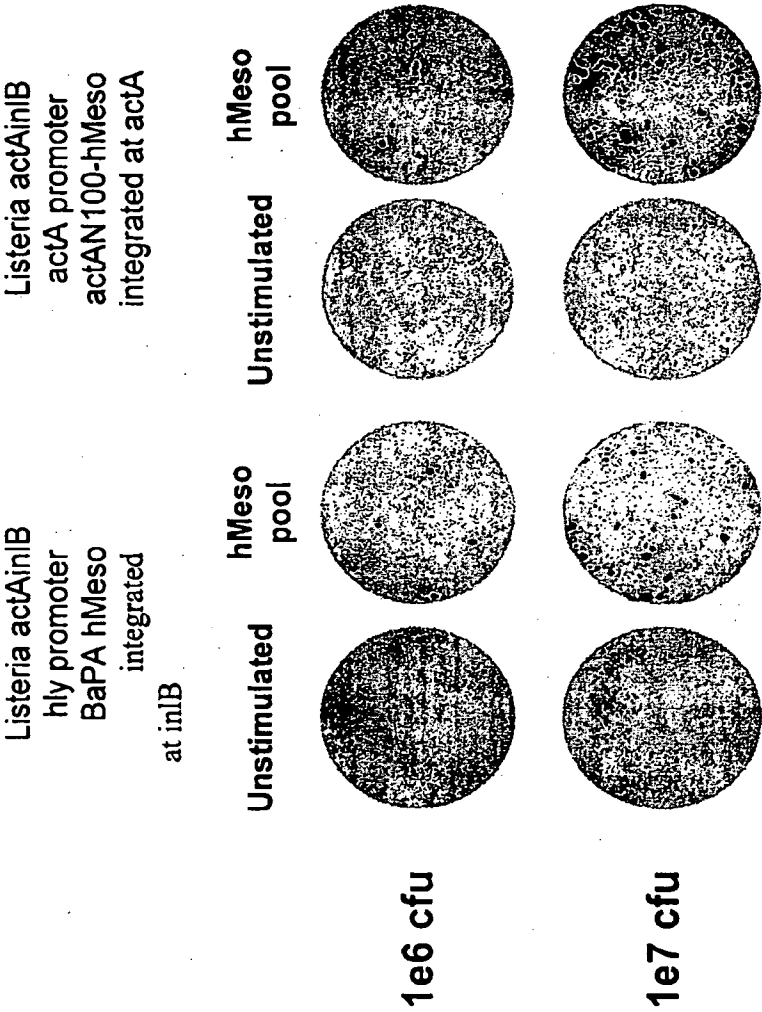
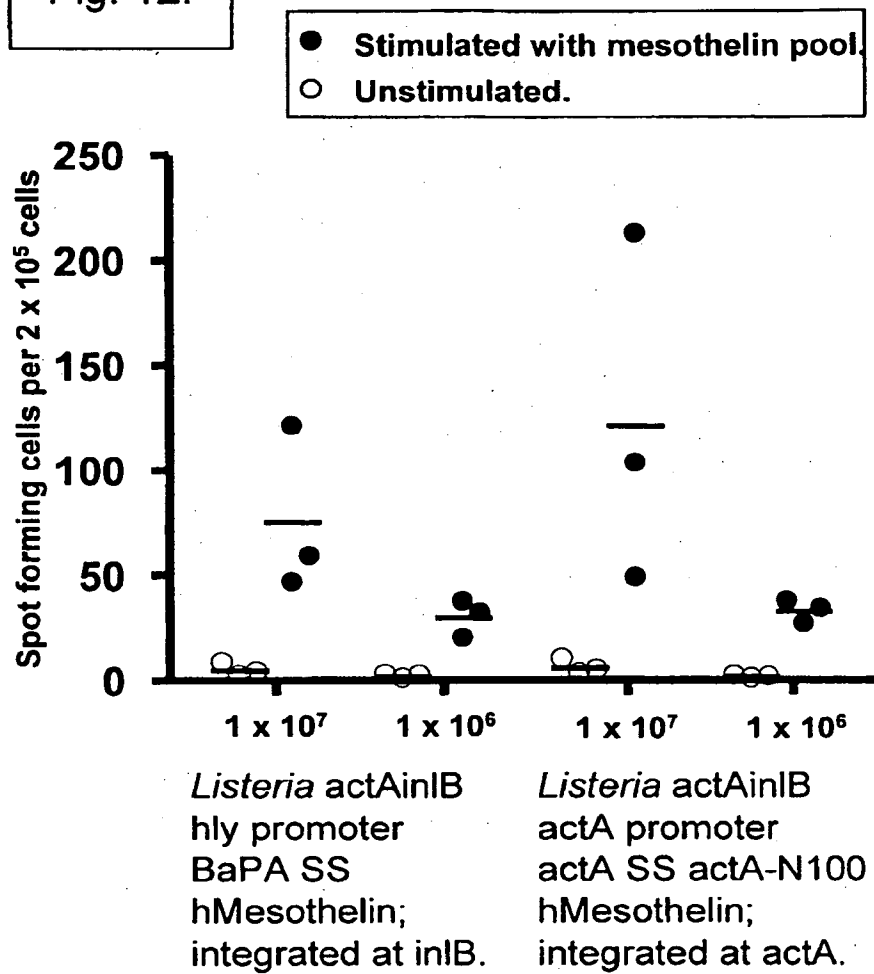


Fig. 11



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Fig. 12.



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Fig. 13

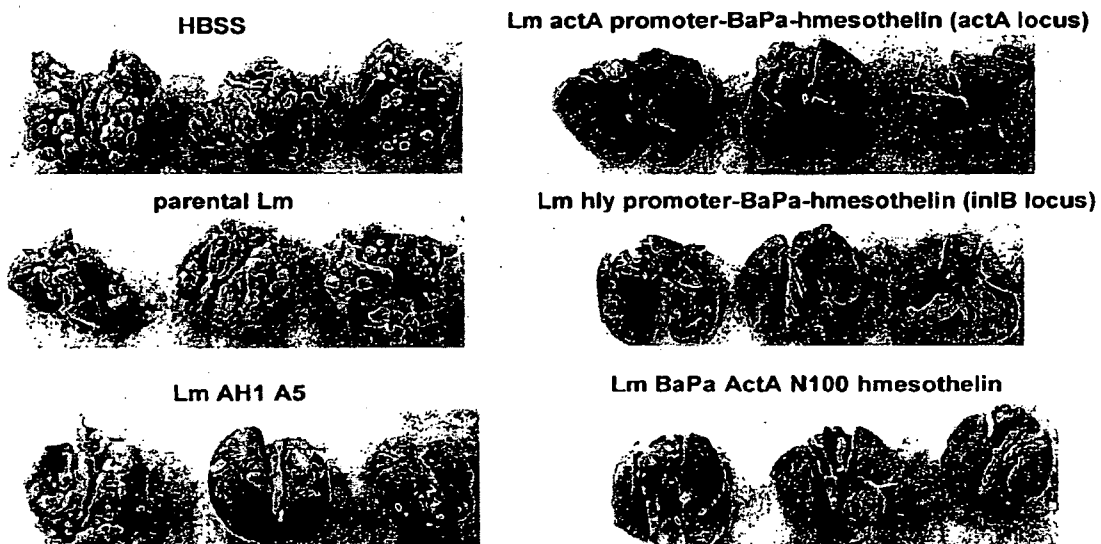
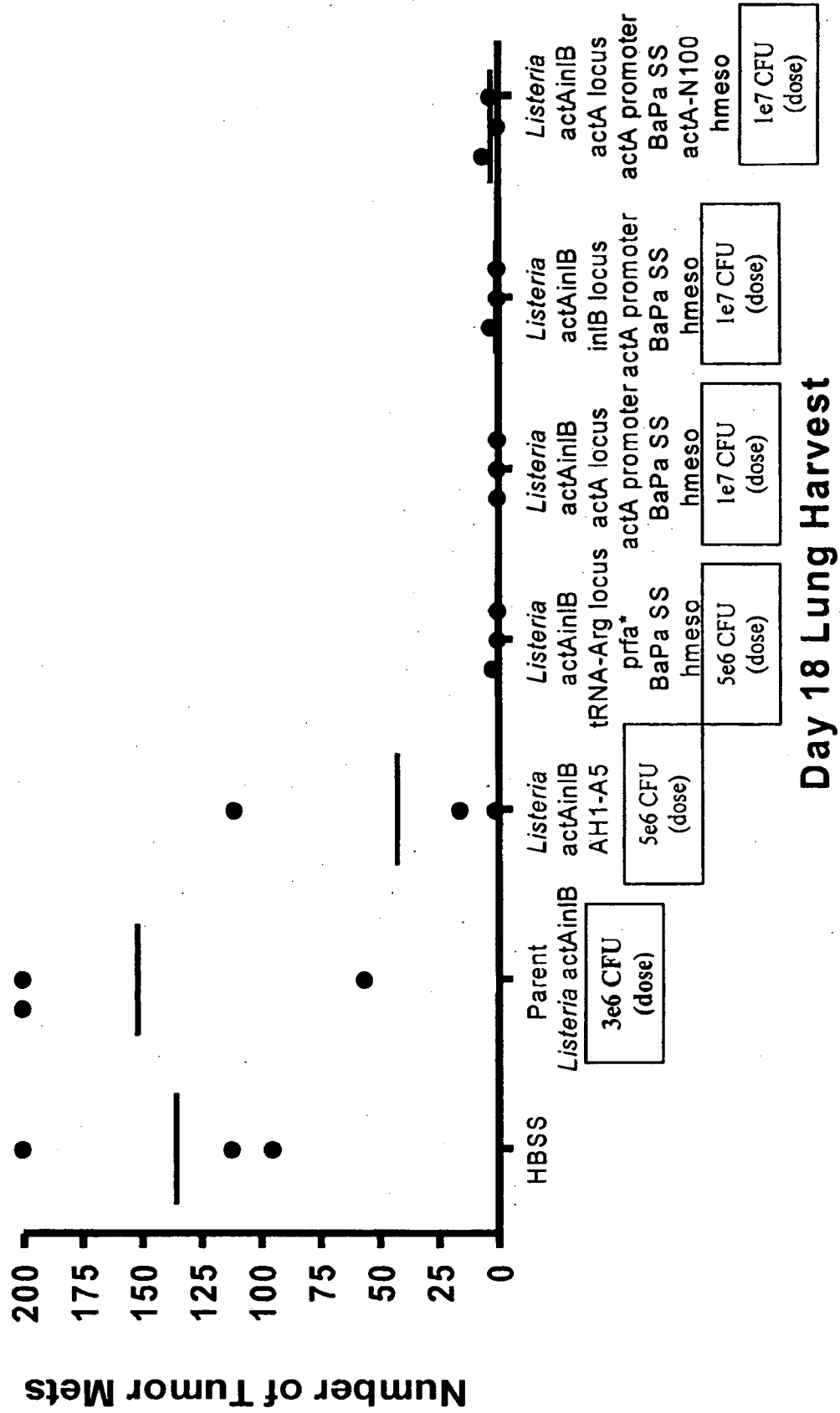


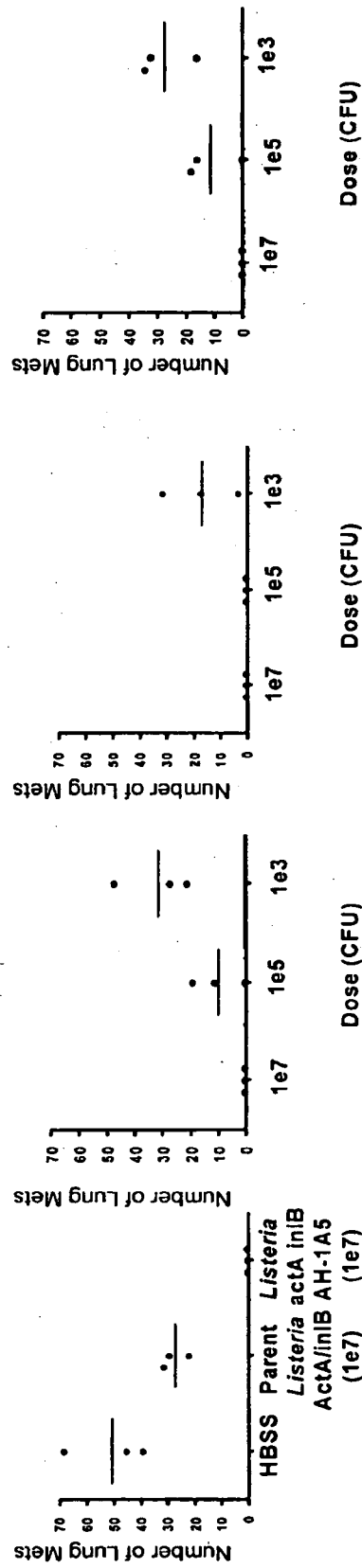
Fig. 14.



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Fig. 15A-G. Harvest splenocytes on day 23 post tumor implant.

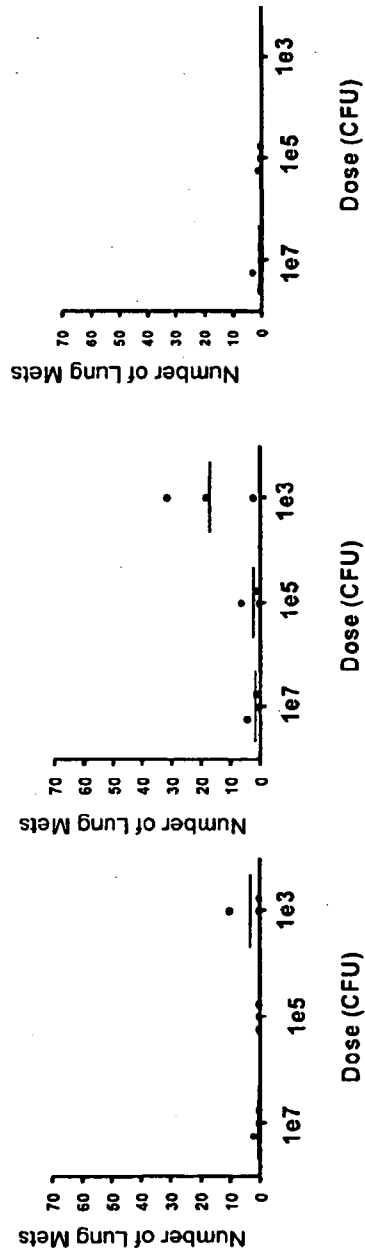
A. Controls:
(1) HBSS only; (2) Parent
L. monocytogenes
 Δ actA/inlB only; (3) *Listeria*
expressing AH1-A5 antigen.



E. hMeso6.

F. hMeso7.

G. hMeso8.



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Fig. 16

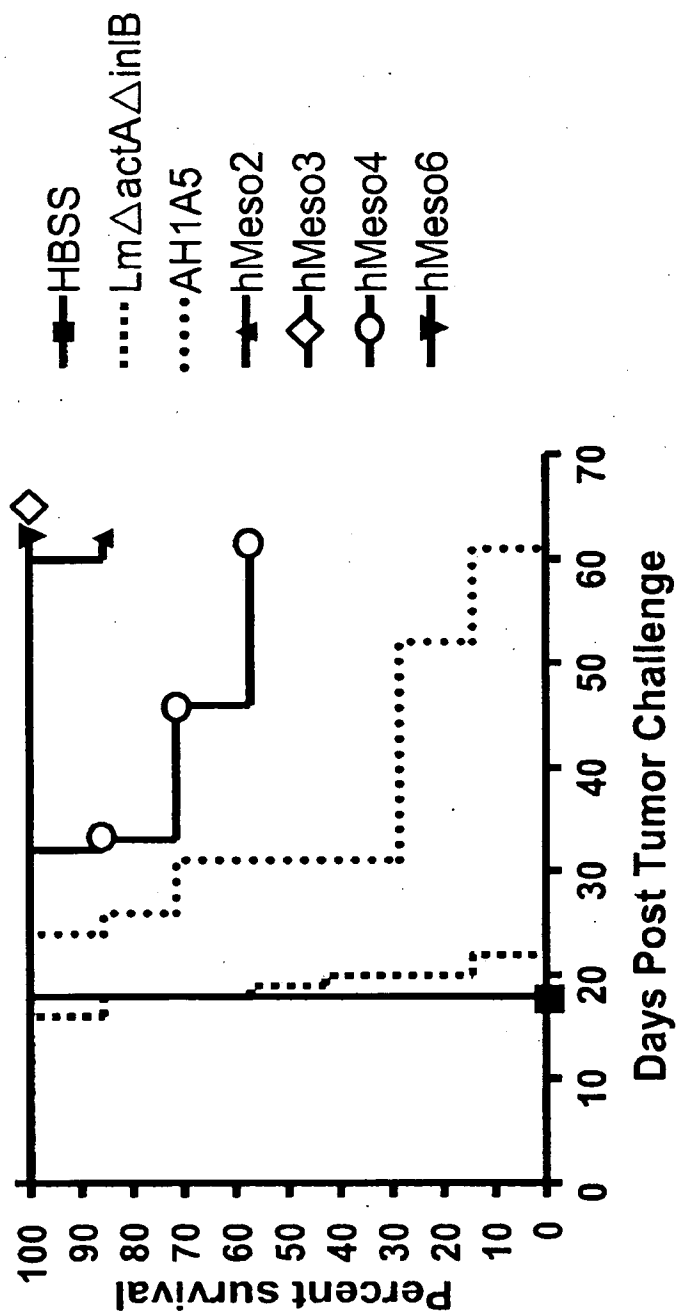
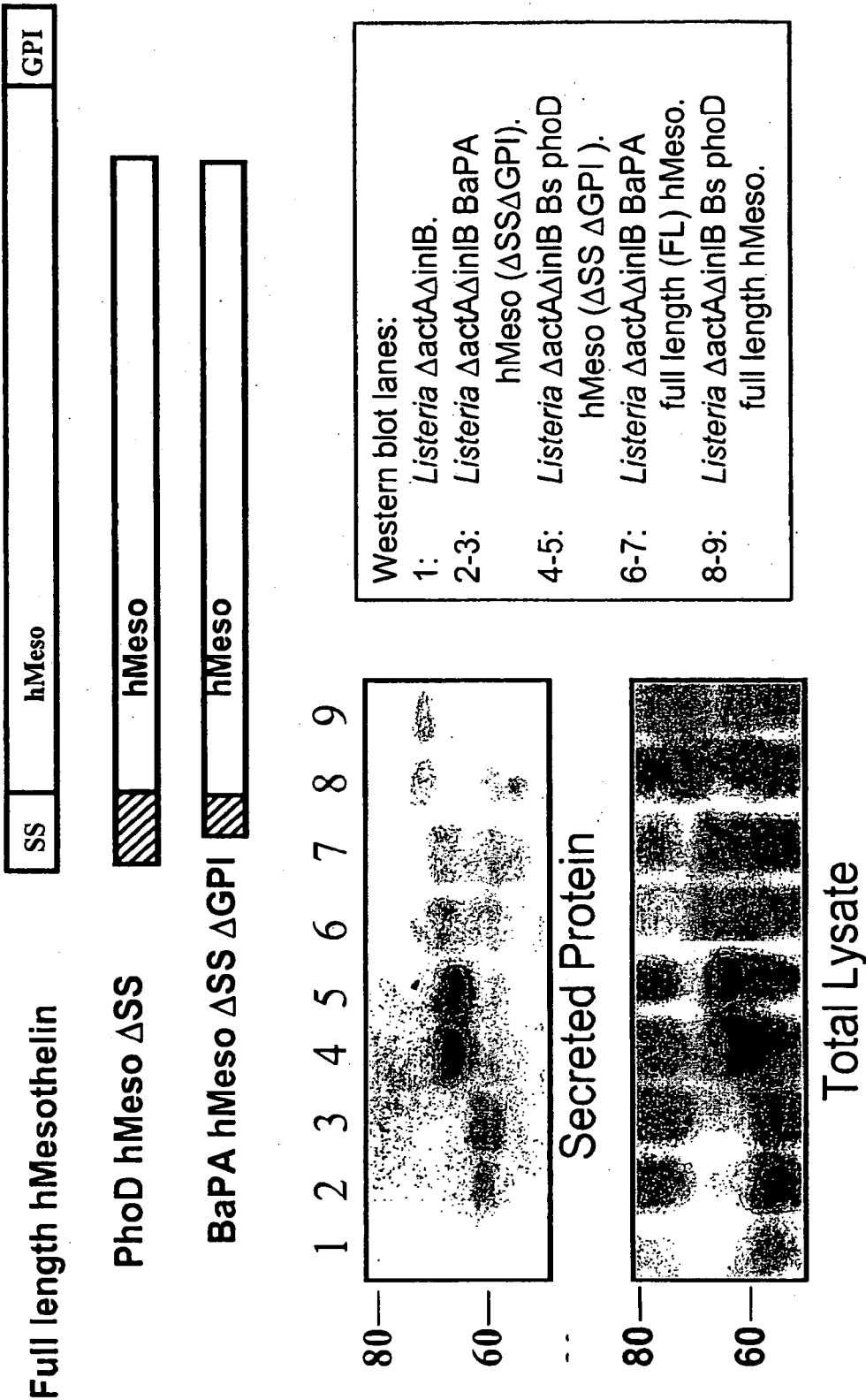
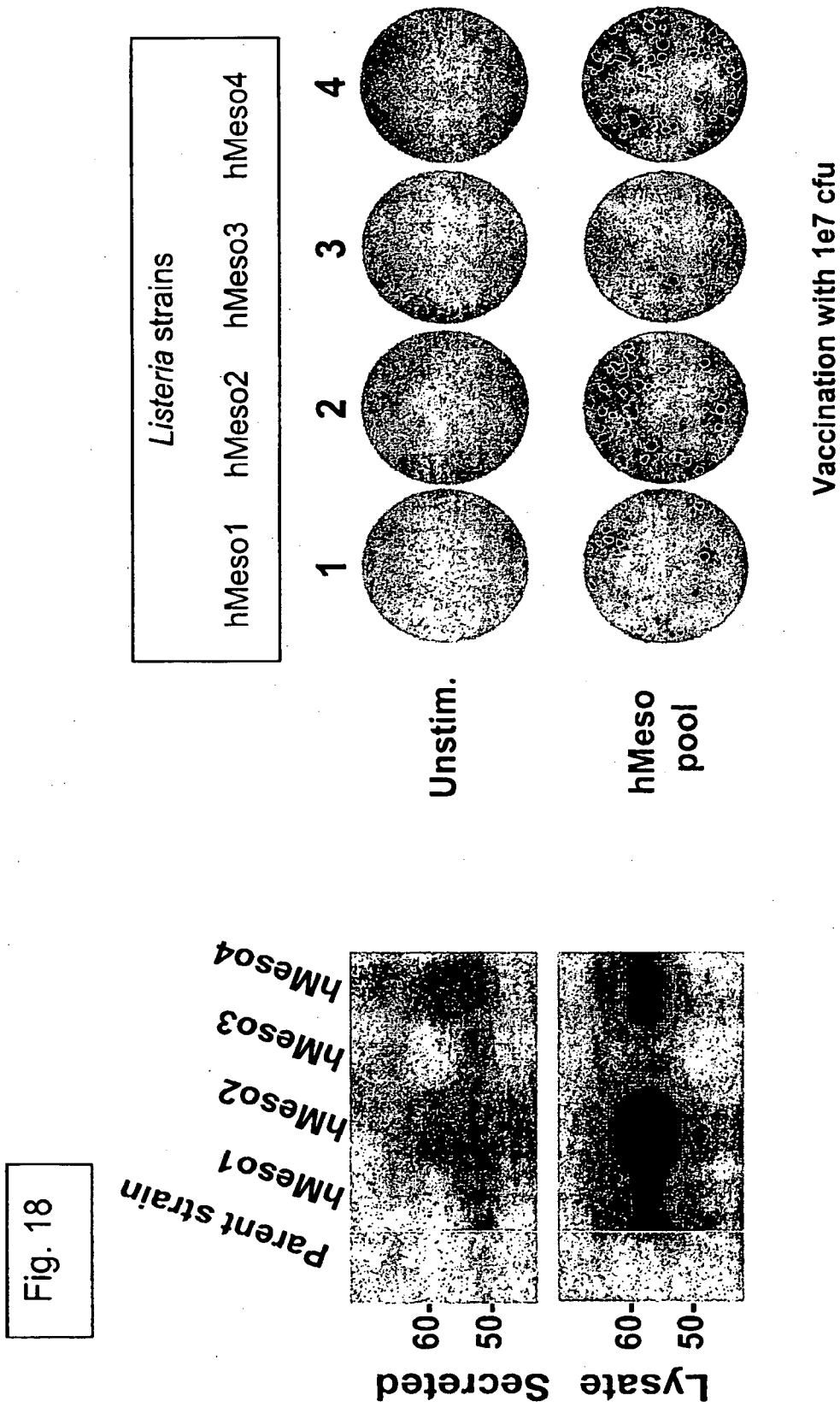


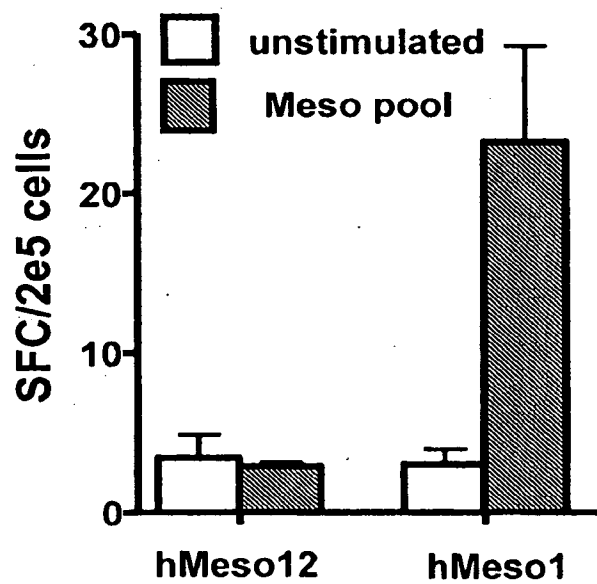
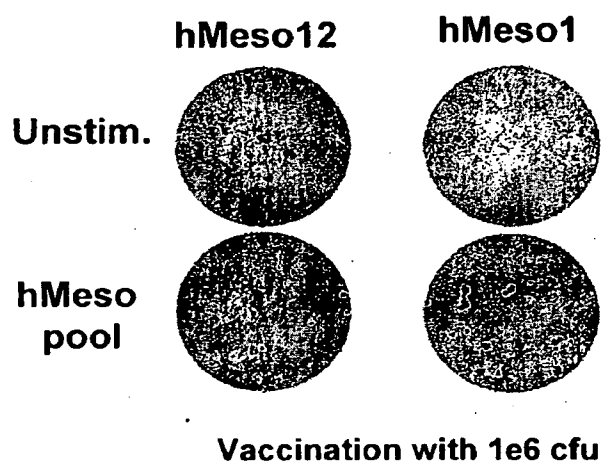
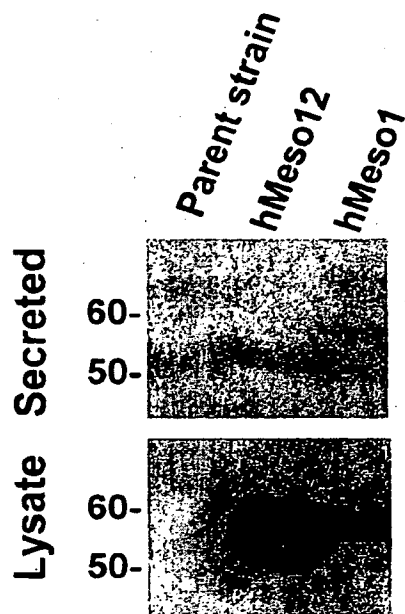
Fig. 17



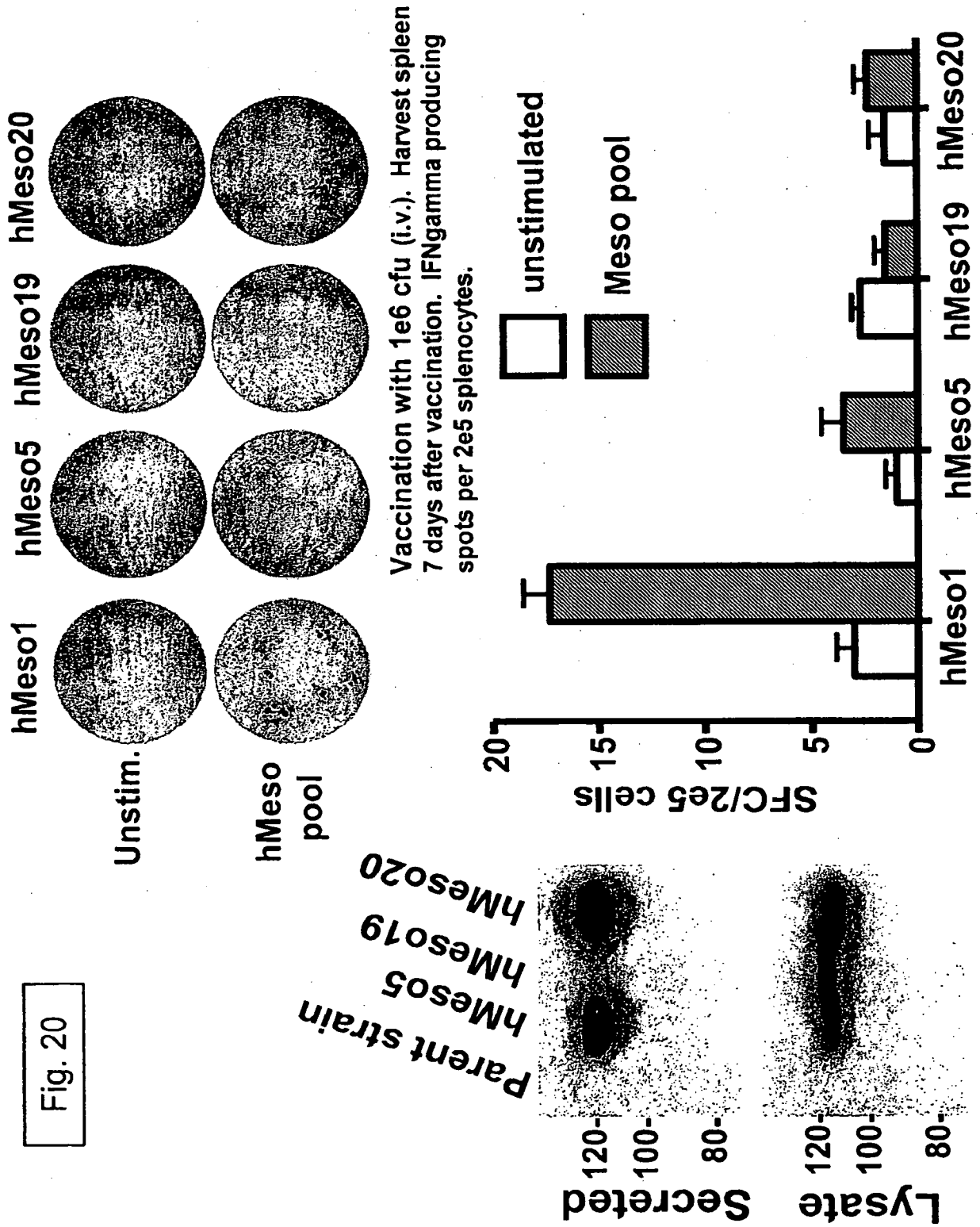


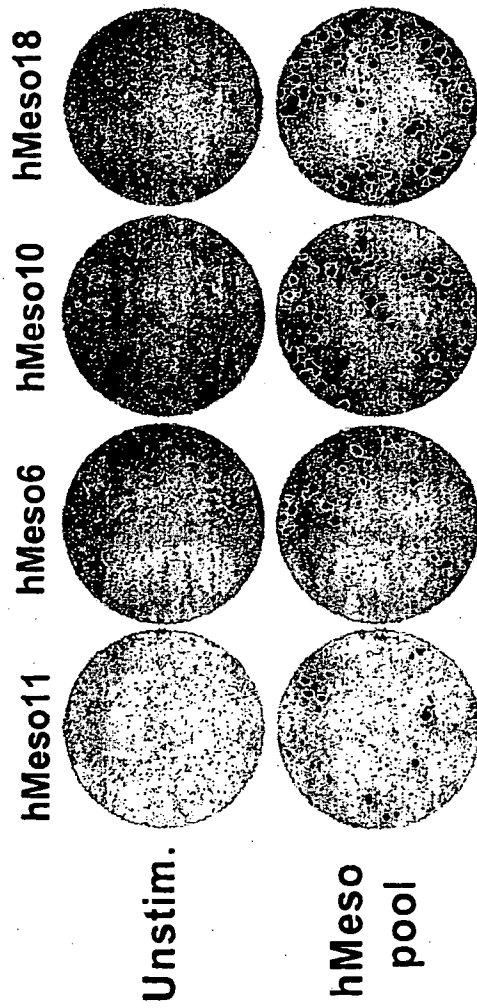
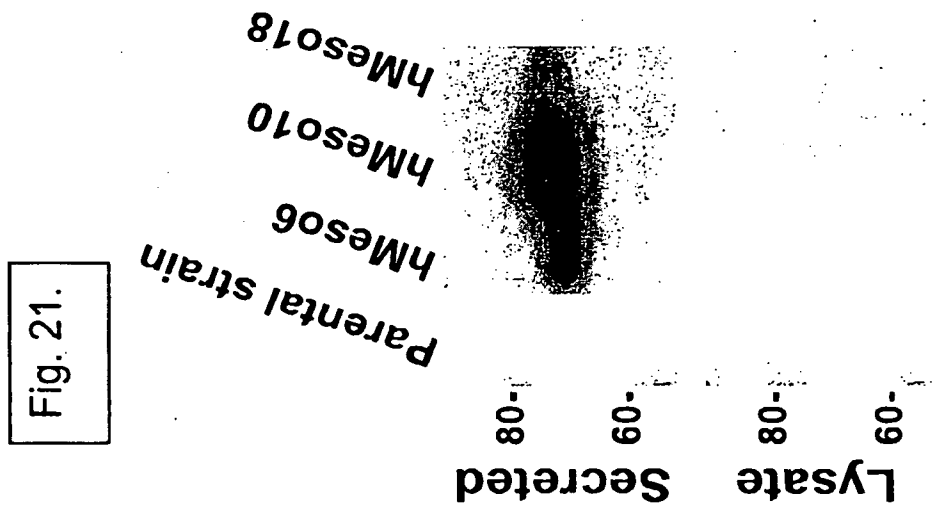
19/58

FIG. 19

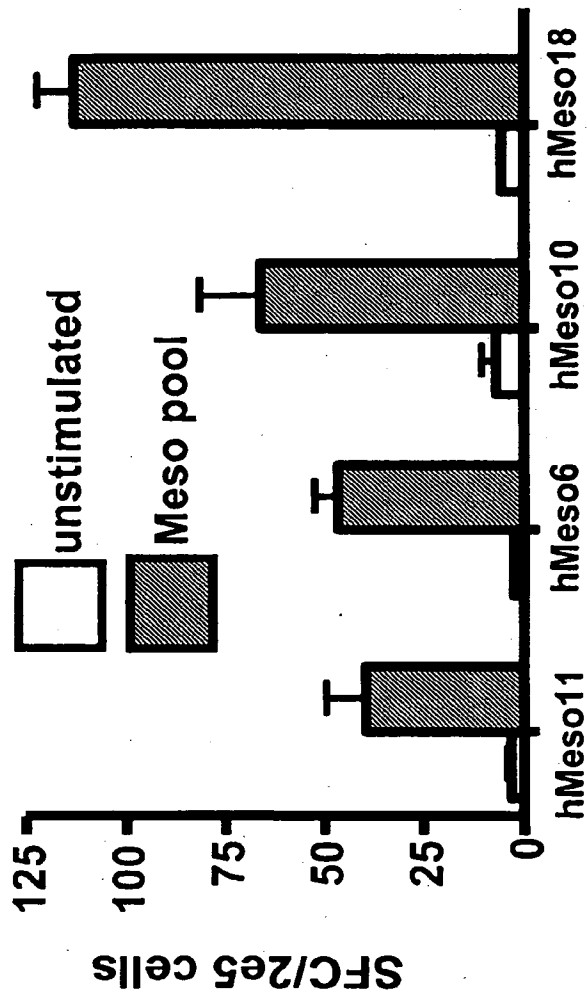


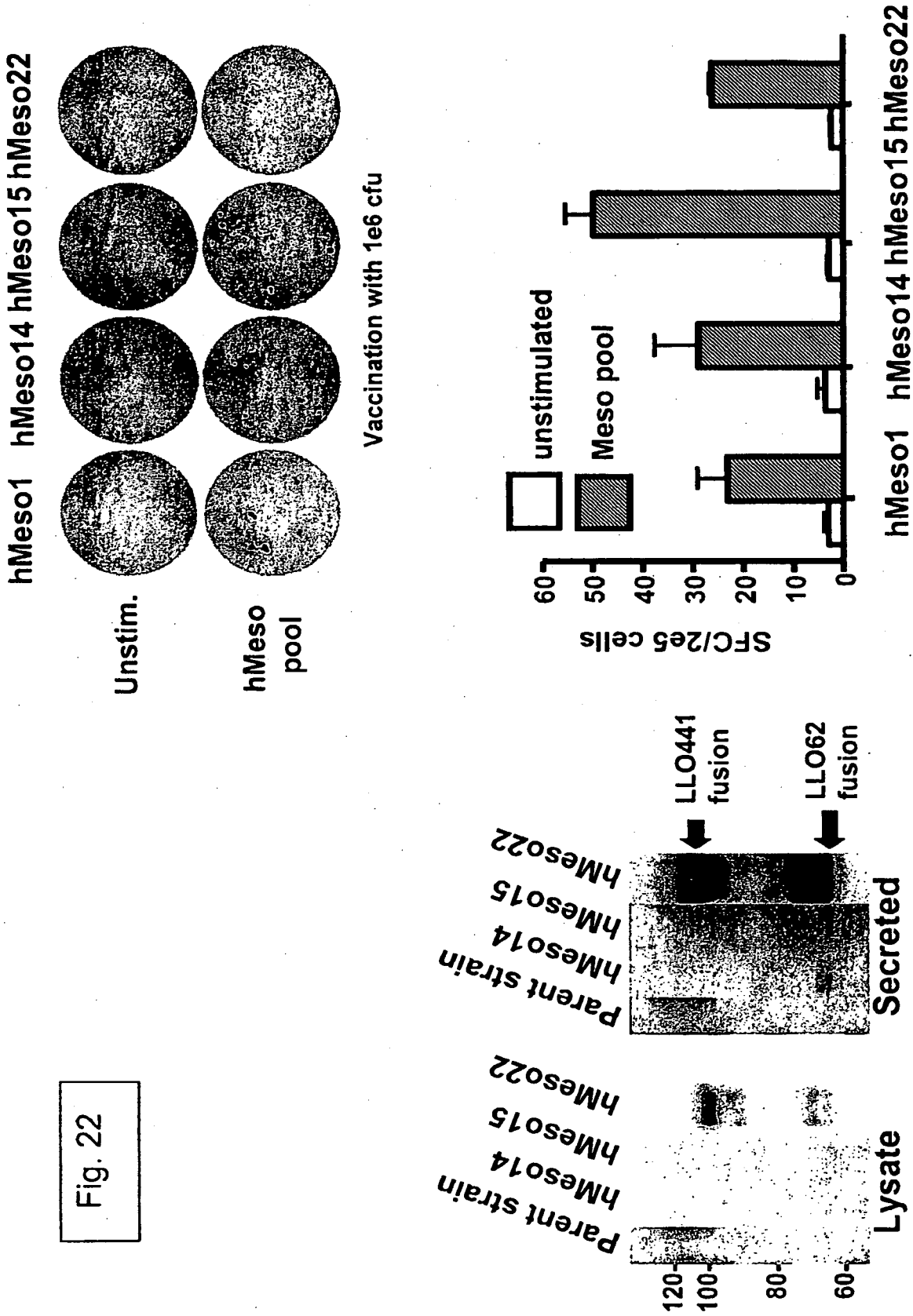
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Vaccination with $1e7$ cfu
Harvest spleen 7 days after vaccination (i.v.).
IFN-gamma producing spots per $2e5$ splenocytes.

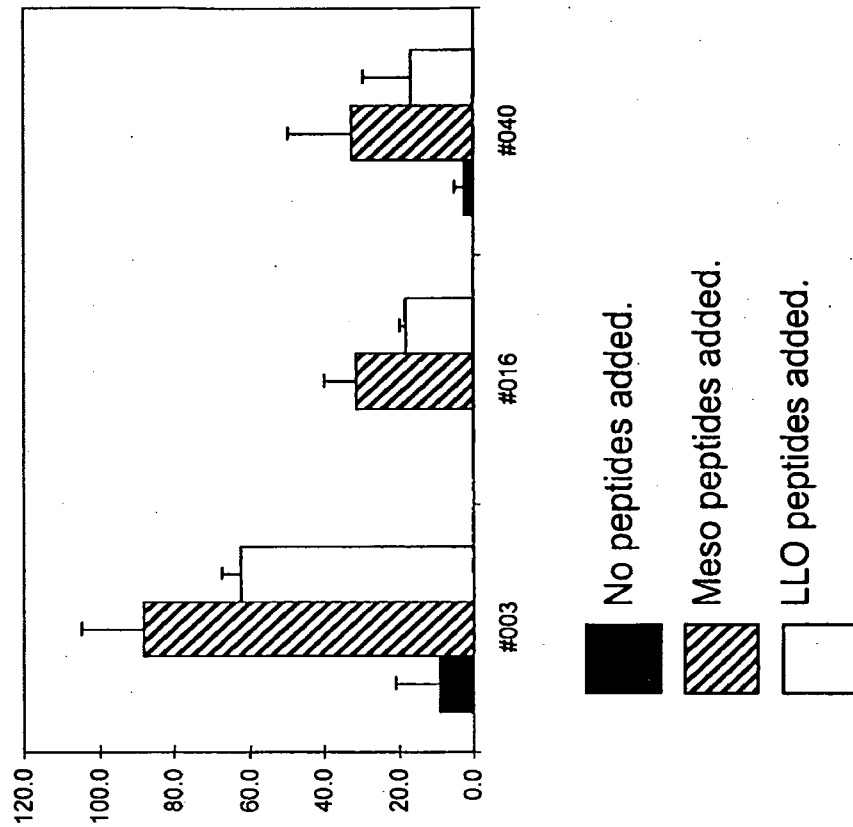
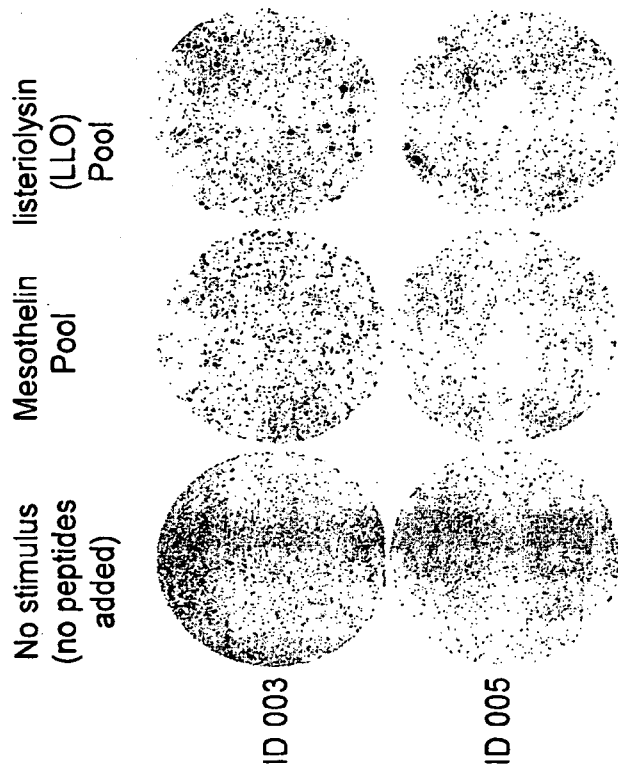




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Interferon-gamma elispot using PBMC from healthy volunteers. (SFU/1E6 PBMCs versus volunteer).

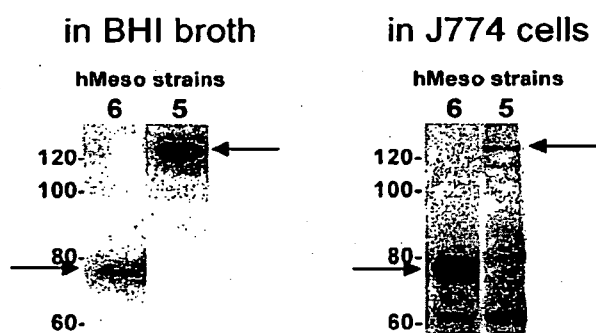
Fig. 23



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Fig. 24

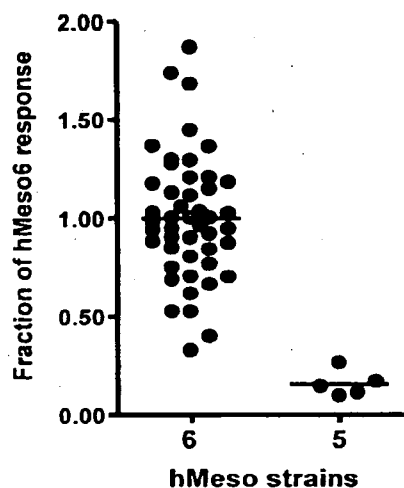
hMeso expression by *Listeria* in
broth or in mammalian cells



hMeso6: *L. monocytogenes* Δ actA Δ inlB
encoding actA-N100-hMeso Δ SS Δ GPI.

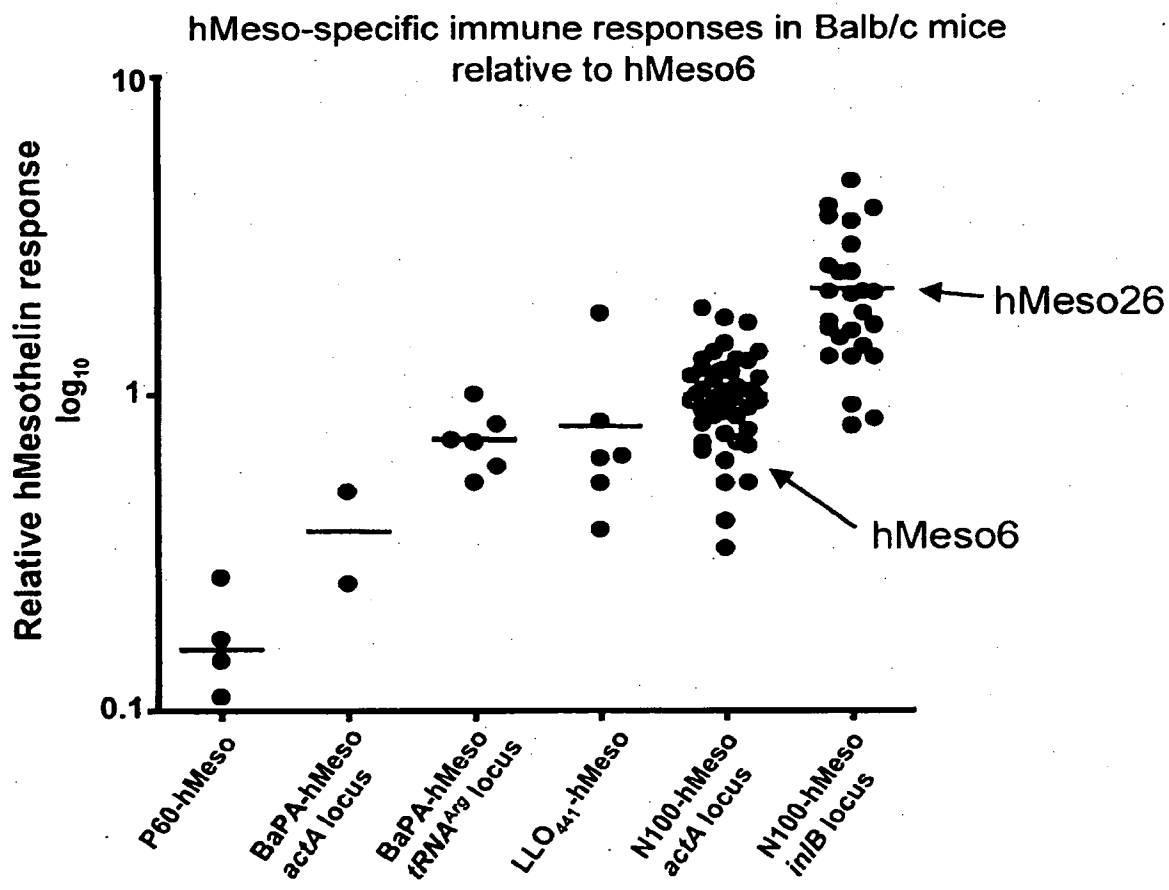
hMeso5: *L. monocytogenes* Δ actA Δ inlB
encoding p60-hMeso Δ SS Δ GPI.

hMeso-specific
immune responses in Balb/c
relative to hMeso6



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Fig. 25



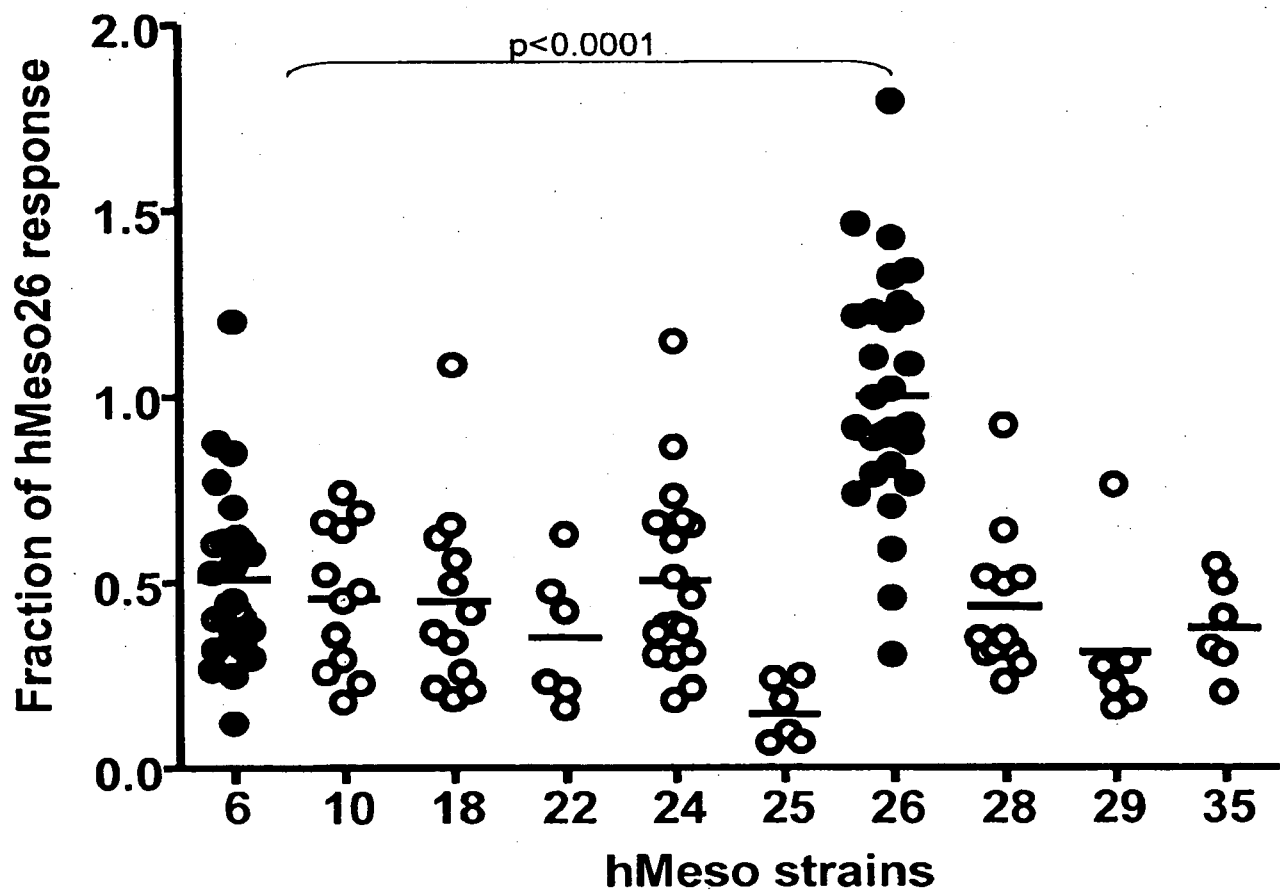
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Fig. 26

Control <i>Listeria</i>	hMeso6	hMeso26
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**hMeso-specific responses in Balb/c mice
relative to hMeso26**



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Fig. 27

hMeso-specific immune responses in Balb/c mice (1e6 cfu)

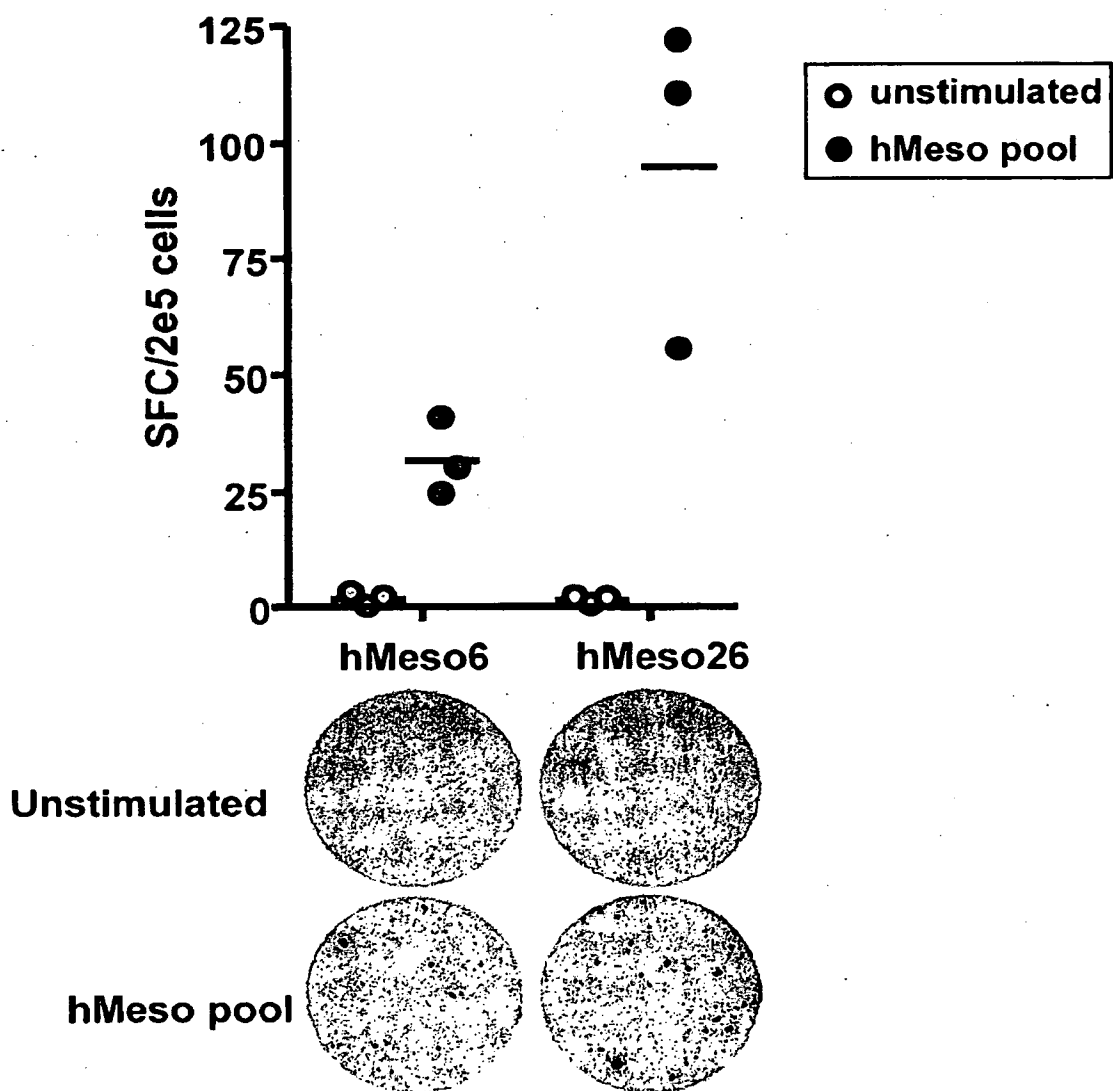
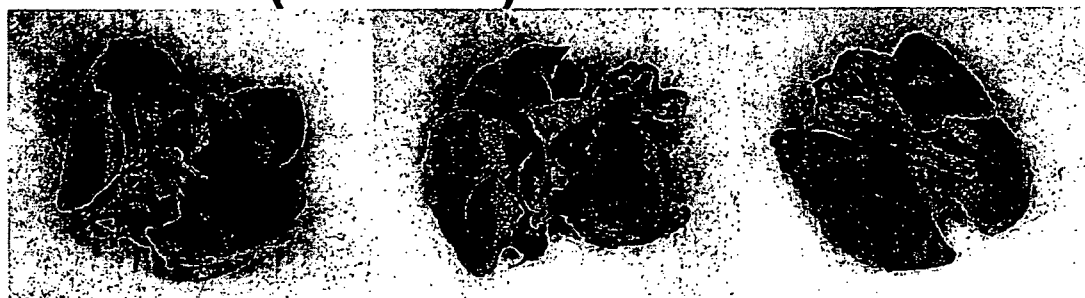
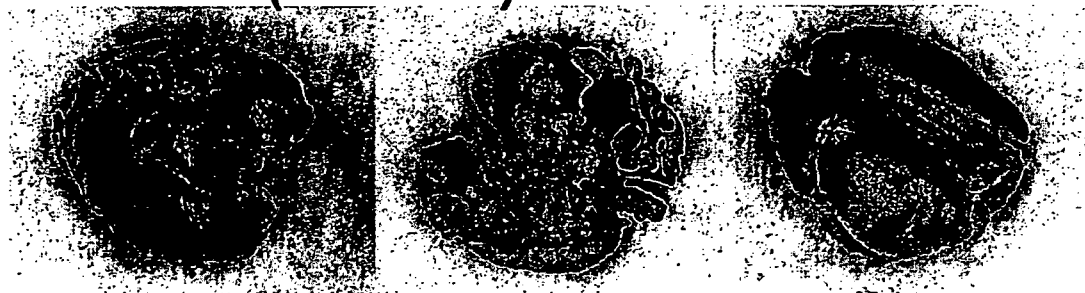
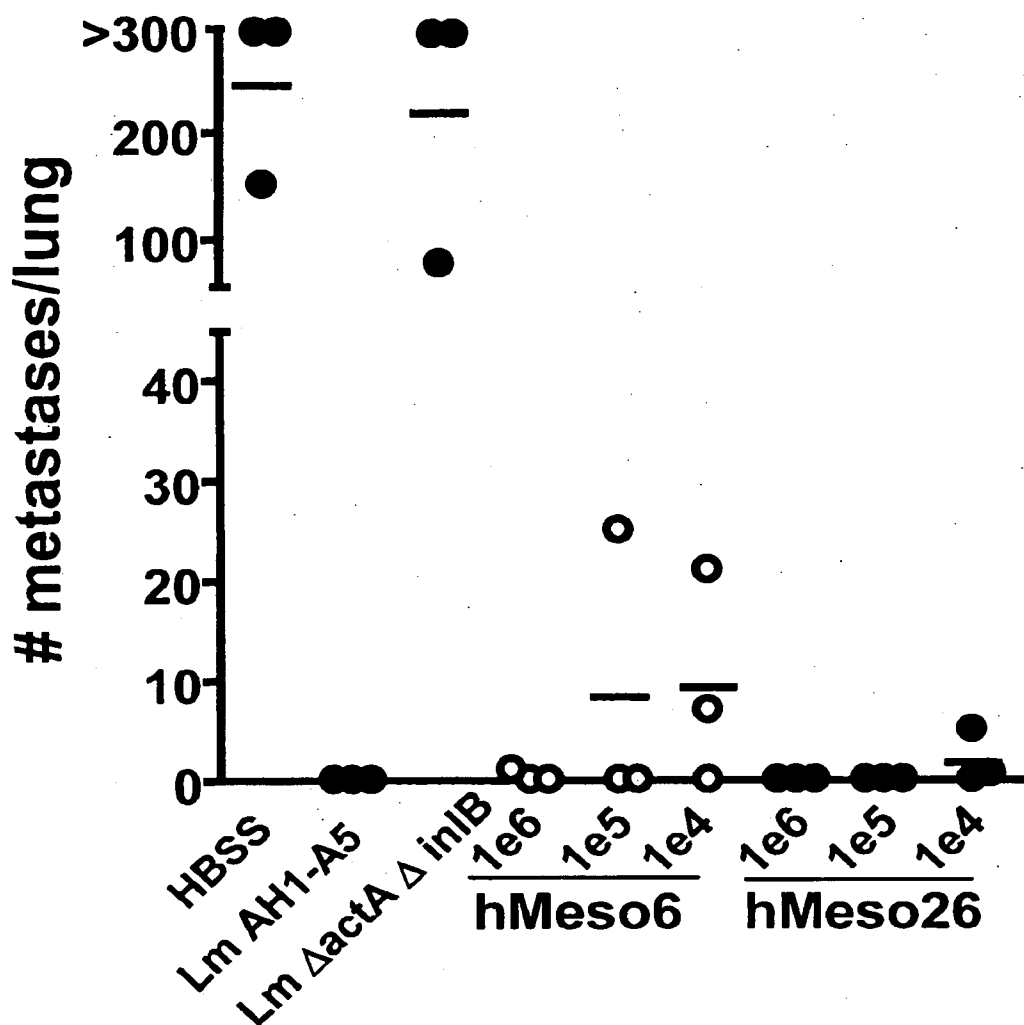


Fig. 28**HBSS****AH1-A5 (1e6 cfu)****hMeso6 (1e4 cfu)****hMeso26 (1e4 cfu)**

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Fig. 29

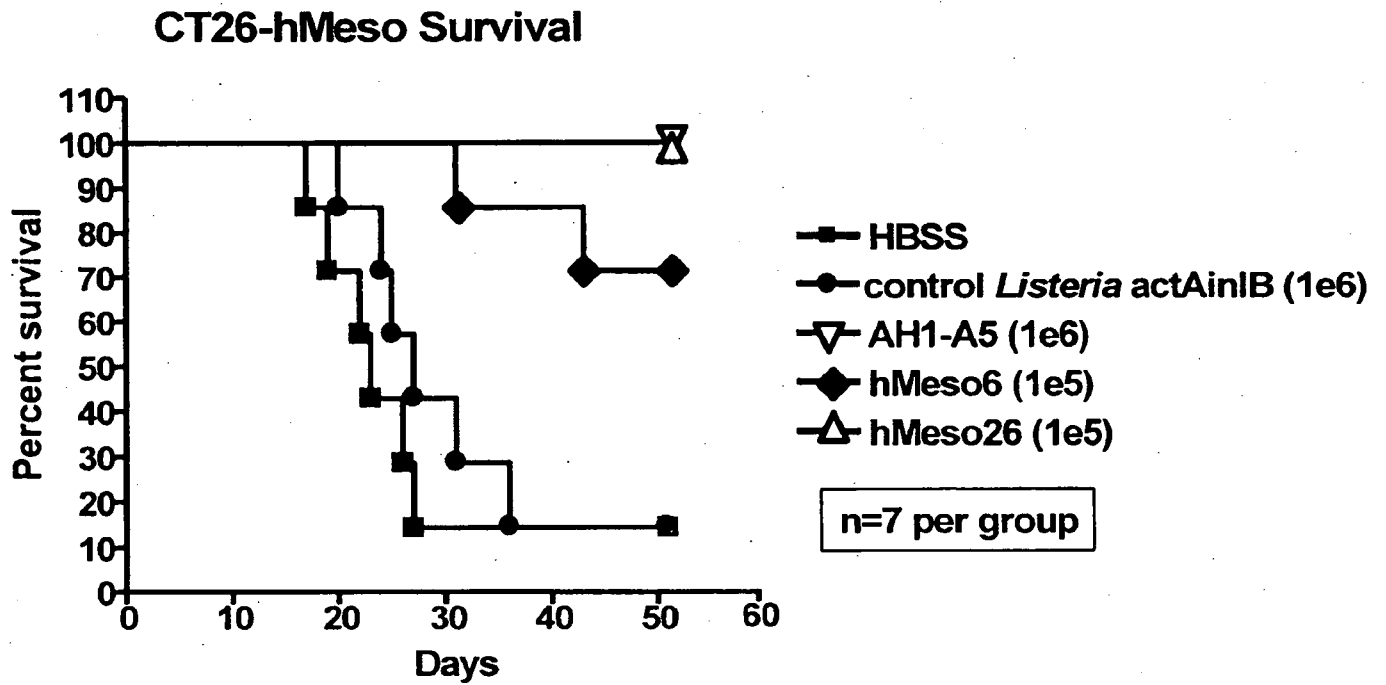
Lung metastases at day19 (inject tumor cells i.v. at t=0 days; *Listeria* i.v. at t=3 days, harvest lungs at t=19days).



Day 0. Inject tumor cells (i.v.).
 Day 3. Inject *Listeria* vaccines (i.v.).
 Day 19. Harvest lungs.

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Fig. 30



Day 0. Inject tumor cells (i.v.).
Day 3. Inject *Listeria* vaccines (i.v.).
Then assess survival.

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FIG. 31

Lm $\Delta actA$ $\Delta inlB$ (control)

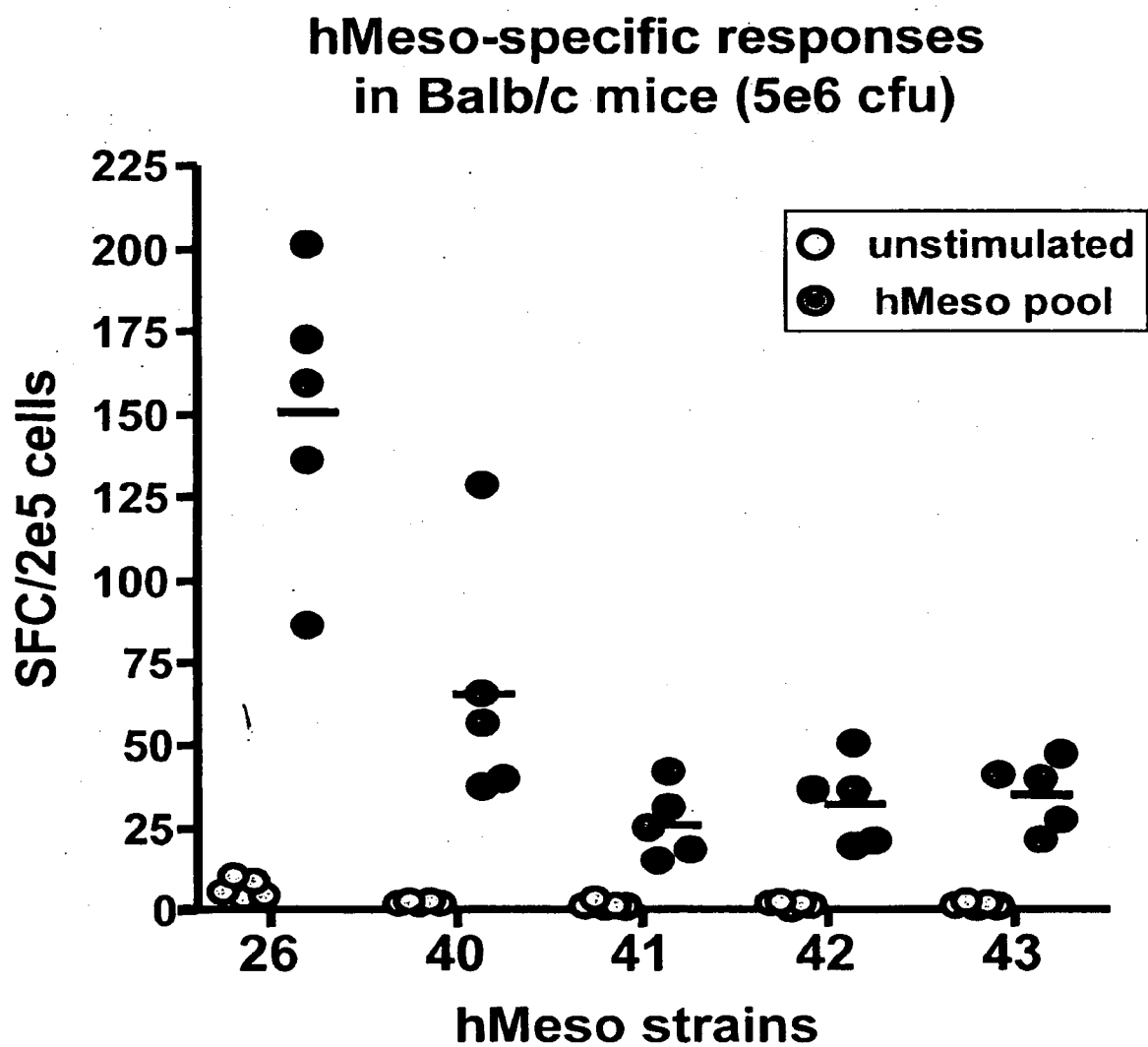
26

40

hMeso
41

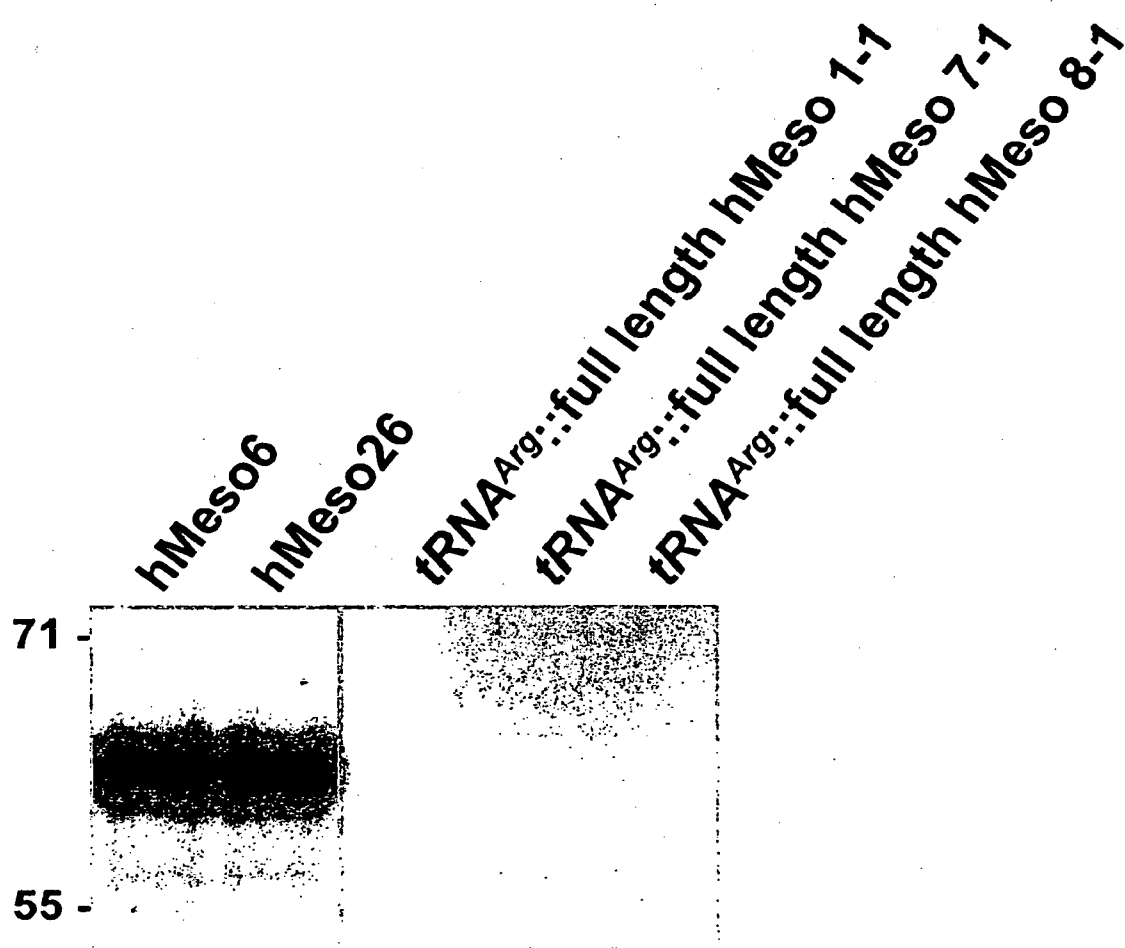
42

43



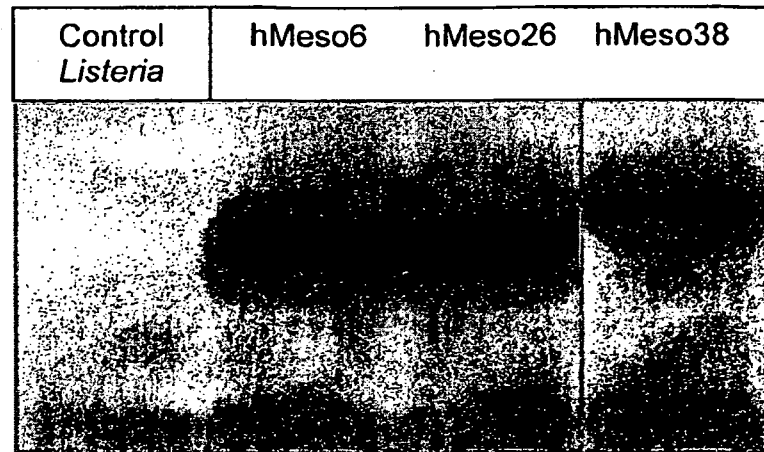
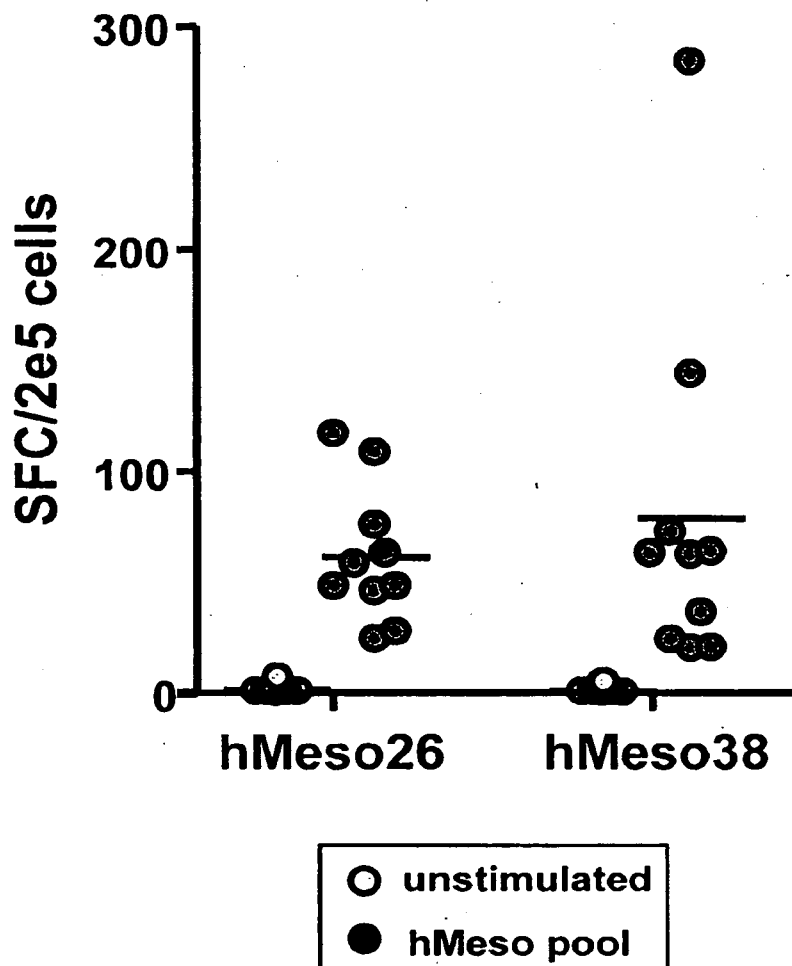
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Fig. 32



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Fig. 33

**hMeso pool responses in Balb/c (5e6 cfu)**

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Fig. 34

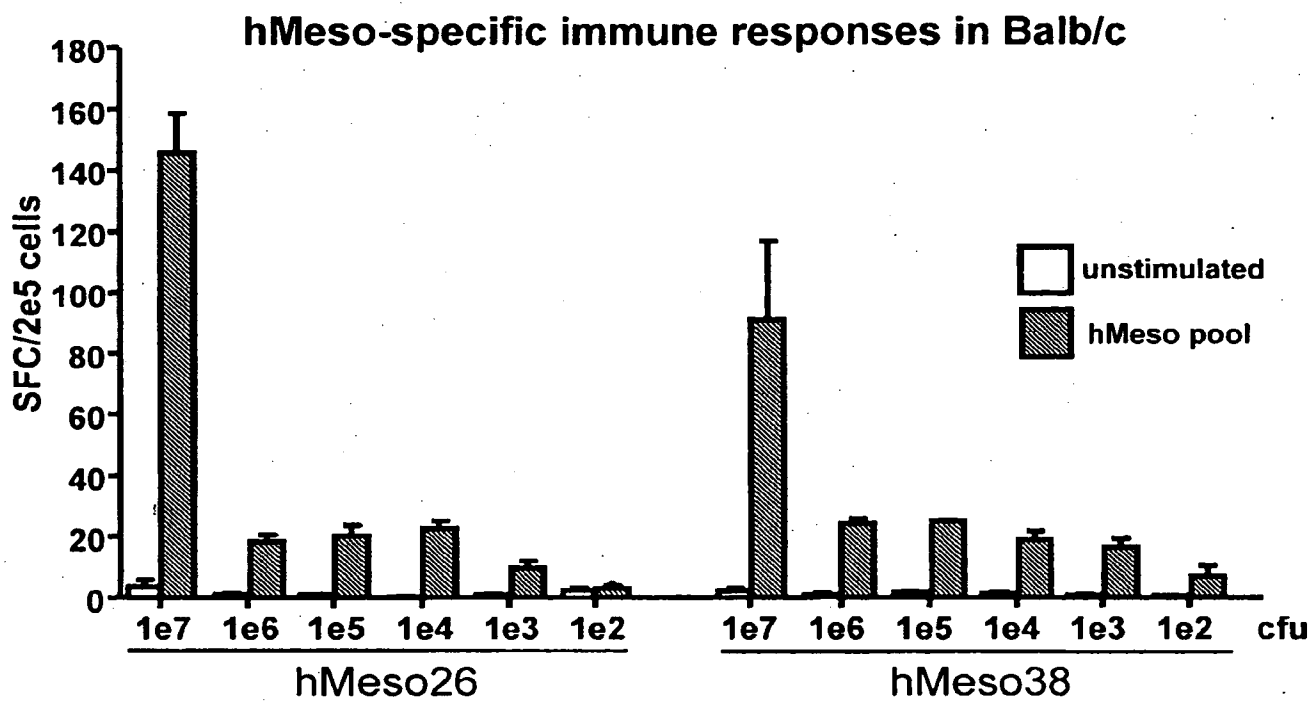
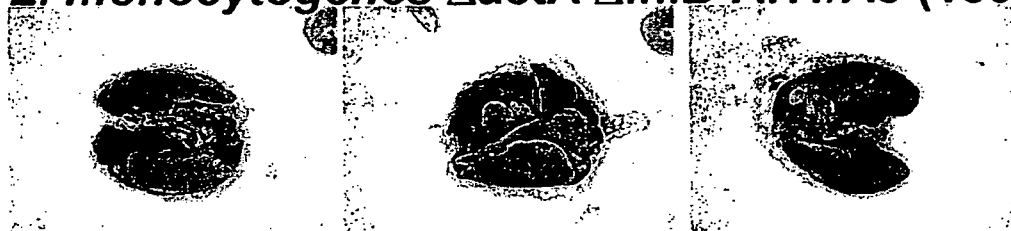
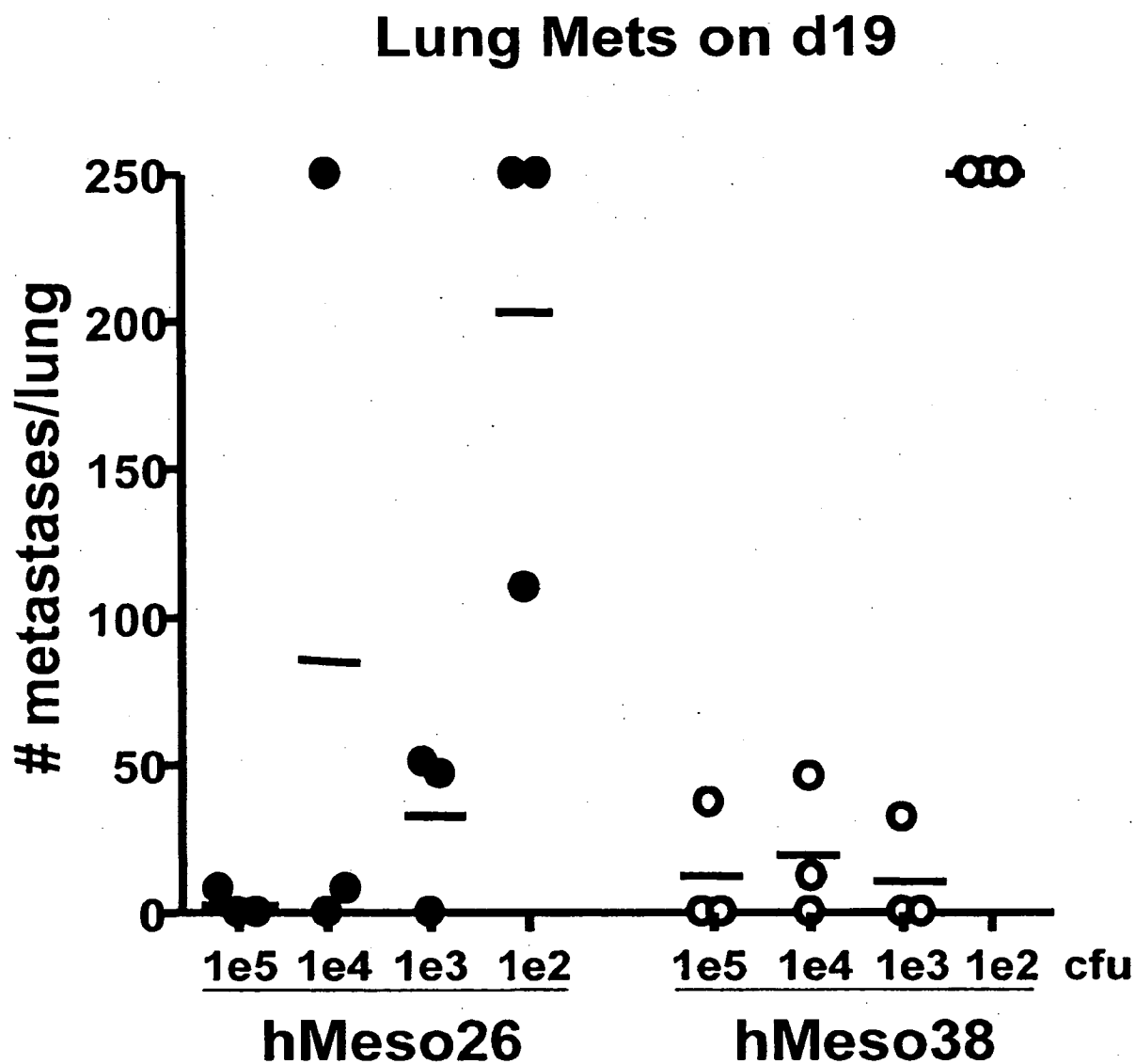


Fig. 35A

Hanks' Buffered Salt Solution (HBSS)***L. monocytogenes* $\Delta actA \Delta inlB$ (1e6 cfu)*****L. monocytogenes* $\Delta actA \Delta inlB$ -AH1/A5 (1e5 cfu)****hMeso 26 (1e5 cfu)****hMeso 38 (1e5 cfu)**

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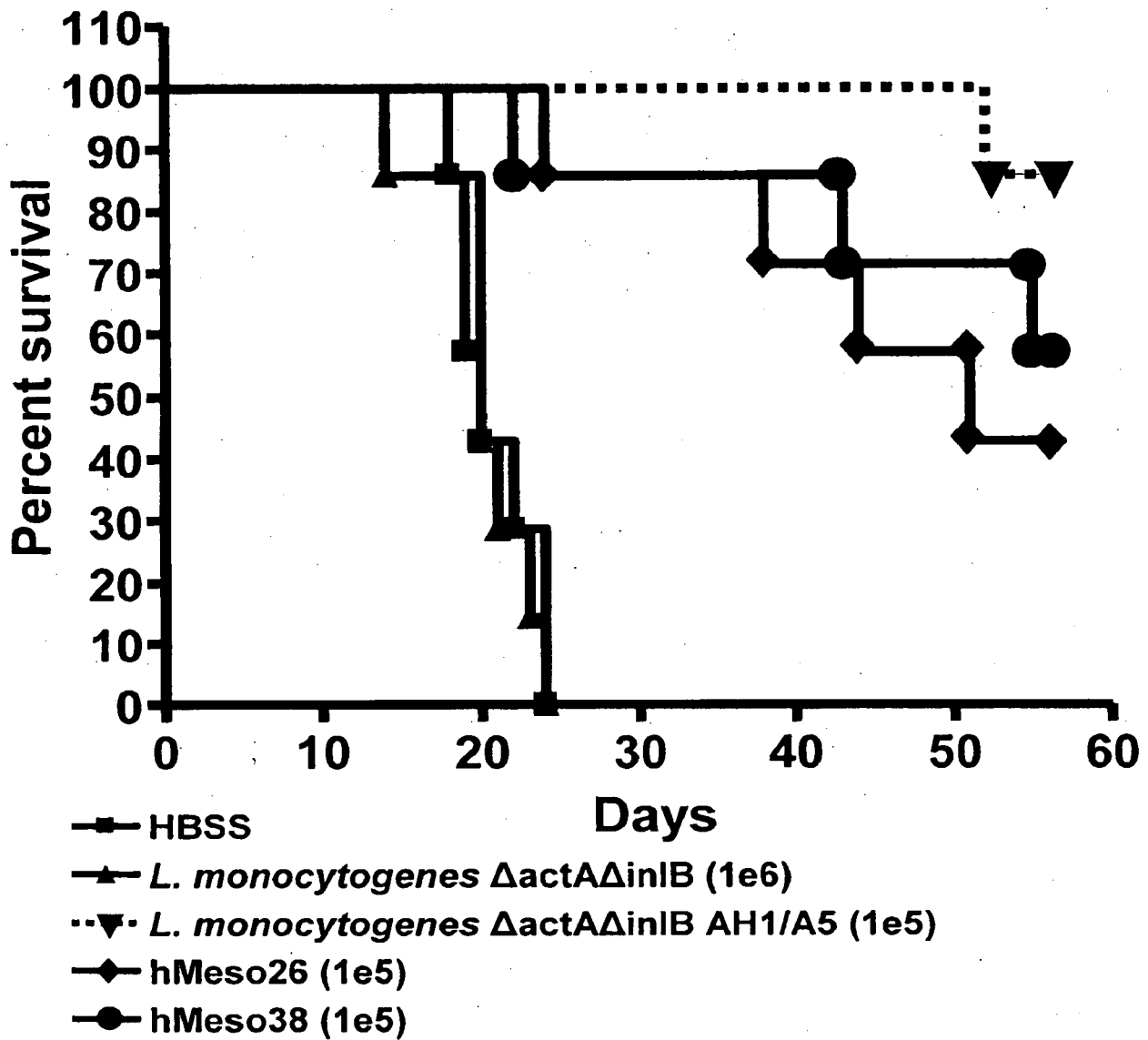
Fig. 35B



Day 0. Inject with tumor cells (i.v.).
Day 3. Inject *Listeria* vaccines (i.v.).
Day 19. Harvest lungs.

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Fig. 36

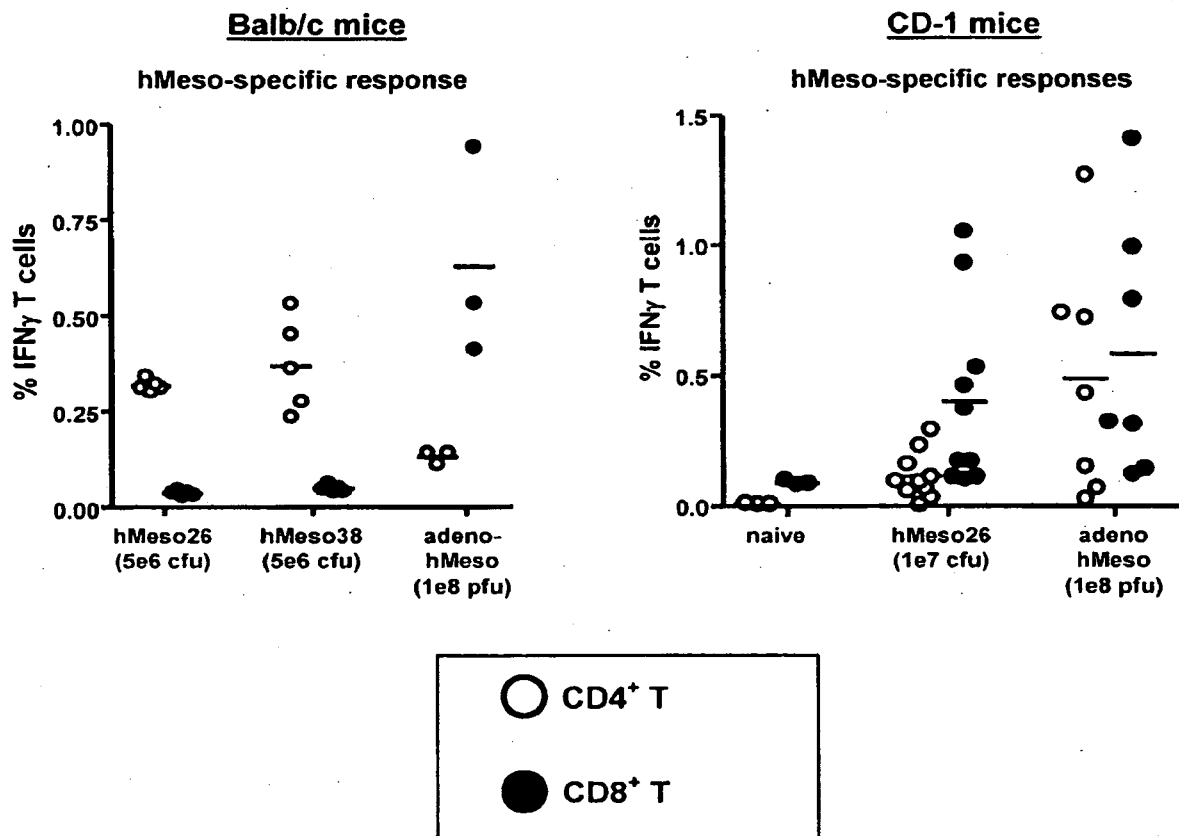
CT26-hMeso Survival

n=7/group

Day 0. Inject tumor cells (i.v.).
 Day 3. Inject *Listeria* vaccines (i.v.).
 Determine survival of mice.

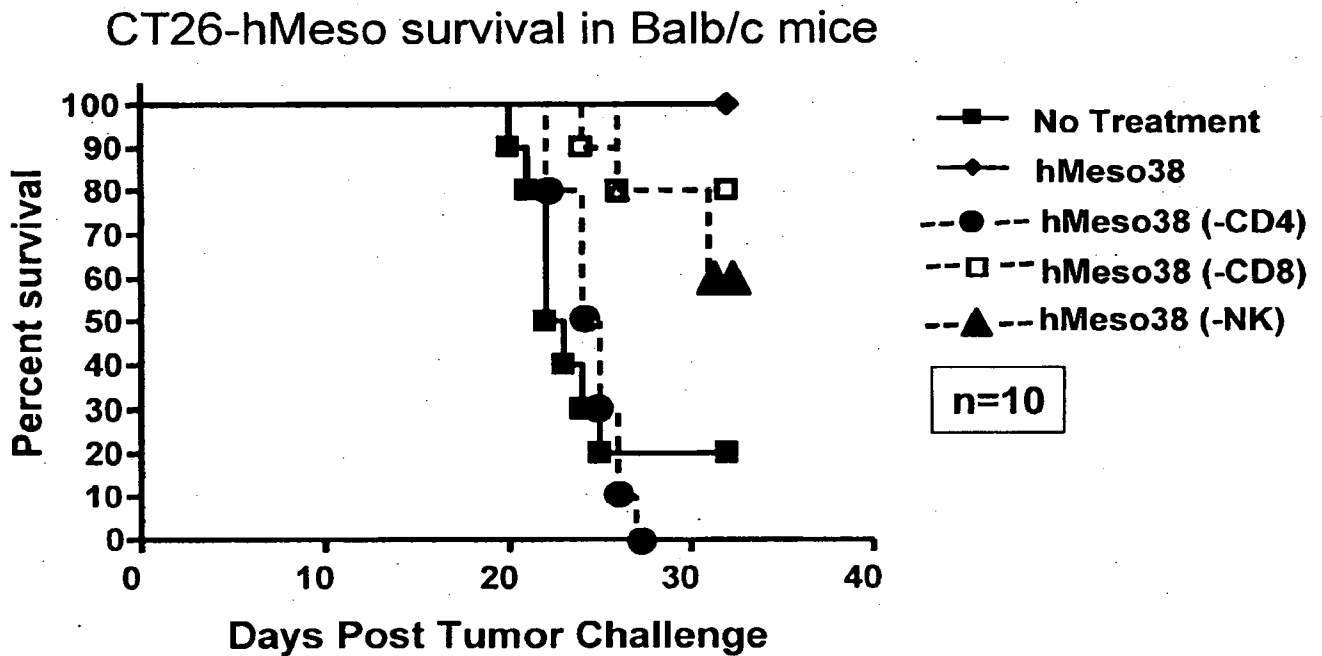
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Fig. 37



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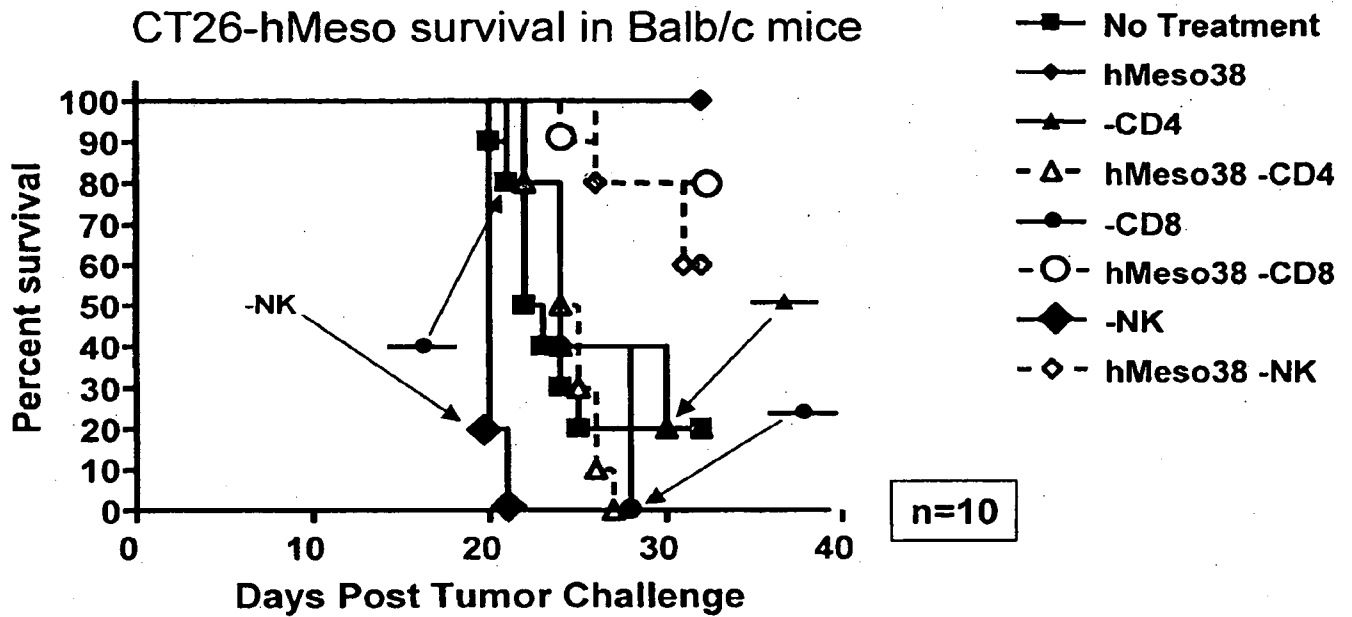
Fig. 38



Days -8, -4, and -1. Inject depleting antibodies.
Day 0. Inject tumor cells (i.v.).
Day 3. Inject *Listeria* vaccines (i.v.).
Weekly antibody boosts.
Test survival of mice.

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Fig. 39



Days -8, -4, -1. Inject depleting antibodies.
 Day 0. Inject tumor cells (i.v.).
 Day 3. Inject *Listeria* vaccines (i.v.).
 Weekly antibody boosts.
 Test survival of mice.

Fig. 40.

L. innocua 1231.

		1		50
U153 int	(1)	MKAAIYIRVSTQEQIEN-YSIQAQTEKLTALCRSKDWDVYDIFIDGGYSG		
lin1231	(1)	MTVGIIYIRVSTEEQVKEGFSISAQKEKLKAYCTAQGWEDFKFYVDEGKSA		
Consensus	(1)	M AIYIRVST EQI FSI AQ EKL A C A WD F FID G SA		100
		51		
U153 int	(50)	SNMNRPALNEMLSKLHE--IDAVVVYRLDRLSRSQRDTITLIEEYFLKNN		
lin1231	(51)	KDMHRPLLQEMISHIKKGLIDTVLVYKLDRLTRSVVDLHNLISIFDEFN-		
Consensus	(51)	M RP LNEMIS I ID VLVYKLDRLSRS D LI F N		150
		101		
U153 int	(98)	VEFVSLSETLDTSSPFGRAMIGILSVFAQLERETIRDRMVMGKIXRIEAG		
lin1231	(100)	CAFKSATEVYDTSSAMGRFFITIISVAQFERENTSERVSFGMAEKVRQG		
Consensus	(101)	F S SE DTSS GR I IIS AQ ERE DRM G KI G		200
		151		
U153 int	(148)	LPLTTAKGRTEFGYDV-IDTKLYINEEEAKQLQMIYDIFEEKSITTLOKR		
lin1231	(150)	EYIPLAP---FGYTKGTGDKLIVNKIEKEIFLQVVMVSTGYSLRQTCEY		
Consensus	(151)	I A FGY D KL IN E I DI SI		250
		201		
U153 int	(197)	LKKLGFKVKS-----YSSYNWLTNDLYCGYVSYADKVHTKGVHEPIIS		
lin1231	(197)	LTNIGLKTRRSNDVWKVSTLIWMLKNPAVYGAIKWNNEIYEN-THEPLID		
Consensus	(201)	L IG K K SS L N G I W IH HEPII		300
		251		
U153 int	(241)	EEQFYRVQEIFSRMGKNPNMNR-DSASLLNNLVVCGKCGLGFVHRRKDTV		
lin1231	(246)	KATFNKVAKILSIRSKSTTSRRGHVHHIFKNRLICPACGKRLSGLRTKYI		
Consensus	(251)	F KV I S K R I N LIC CG R I		350
		301		
U153 int	(290)	SRGK-KYHYRYYSCKTYKHTHELEKCGNKIWRADKLEELIIDRVNNYSFA		
lin1231	(296)	NKNKETFYNNNYRCATCKEHRRAVQIS----EQKIEKAFIDYISNYTLN		
Consensus	(301)	K K FH Y C T K KIE ID I NYS		400
		351		
U153 int	(339)	SRNVDKEDELDLNL--EKLKTEHVKK--KR-----LFDLYISGSYEVS		
lin1231	(342)	KANISSKKLDNNLRKQEMIQKEIISLQKREKFQAWAADLMNDDEFSKL		
Consensus	(351)	NI L E I E I KR DL F		450
		401		
U153 int	(378)	ELDAMMADIDAQIN---YYEAQIEANEELKKNKKIQENLADLATVDFDSL		
lin1231	(392)	MIDTKMEIDAAEDRKKEYDVSLFVSPEDIAK----RNNILRELKINWTSL		
Consensus	(401)	ID M A Y A A EDI K NI I F SL		484
		451		
U153 int	(425)	EFREKQLYLKSLINKIYIDGEQVTIEWL-----		
lin1231	(438)	SPTEKTDFISMFIIEGYVKDDENKAVITKISFL		
Consensus	(451)	EK FI I I D I		

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Fig. 41.

L. innocua 0071.

		1		50
PSA int	(1)	MKIKKLANGKYCVRLRIKVDGEWKEKRLTDTSETNLMYKASKLLKQVQHD		
lin0071	(1)	-MVKKVKGRRYEGSIEQRSKNSWRMRVTVGVDYKGTPIRADRTTRTKNER		
Consensus	(1)	IKKL KY I K WK K KA K K N		
		51		100
PSA int	(51)	S-----SSLKEWNFKIFYTLFMKTFKDGKSS-----Q		
lin0071	(50)	ERERELRNFITELEQNGYTAPARMTFKAFVENEYMPKHAQNNLEVKTWTE		
Consensus	(51)	S L F L K F D		
		101		150
PSA int	(78)	STINLYDLAYNQFVDYFDEKIKLNSIDAVQYQQFINHLSVDYAISTVDTR		
lin0071	(100)	YYKSIVARAYPAFGGVQMDKITTLLHIVNLVAKLQKPGARLDVKPTDSDEK		
Consensus	(101)	I AY F DK I L LD S D K		
		151		200
PSA int	(128)	HR-----K----IRAI FNKAVHLGYMKKNPTIGAHISGQDVAKNKA		
lin0071	(150)	KNKPLSPRSIRNIYFAINSVFETAVEWKVIPINPAEGVRLP--KTTKRPP		
Consensus	(151)	I AIF AV I NP G I K		
		201		250
PSA int	(165)	QFMETDKVHLLLEELAKFHSISRAVIFLAVQTGMRFEIIALTKKDINFT		
lin0071	(198)	TIYTPAEIELLNAAALVKEPLRLQVMIYIALISGCREAELAALVVKHVNLI		
Consensus	(201)	I LL L K MIFIAL SG R EI AL K IN		
		251		300
PSA int	(215)	KRSITVNKAWDYKYTNFTIDTKTKK---SRVIYIDNSTAQYLHSYLNWHT		
lin0071	(248)	EDELTFEQLTVAKAGEGLLLKESTKNDVAGIVSIPAWLTNLIETYISNEV		
Consensus	(251)	IT K I S K A II I N I SYI		
		301		350
PSA int	(262)	DYMKEHAIAKNPLMLLFITYHNKPVDNASCNKALKKICSTINSEPVTLHKL		
lin0071	(298)	LDLKTEGKWANHKLFLADMEGKPIRPDSIYQRWKRFLERHNLFPVIRFHD		
Consensus	(301)	LK A LF KPI S KK N I H L		
		351		400
PSA int	(312)	RHTHTGLCVEAGMDIIYVADRLGHDDINTTLKYYSHLSSNLRQHNQSKVD		
lin0071	(348)	RHTSATLLLNLKGRDIKIIQERLRHKSSVTTSNIYAHVLKDKDAASDFE		
Consensus	(351)	RHT L L G DI I DRL H TT YAHL S D		
		401		424
PSA int	(362)	AFFTLKTDETTNFTTNATKTTE-		
lin0071	(398)	NPF-----		
Consensus	(401)	F		

Fig. 42.

L. innocua 1765.

		1		50
PSA int	(1)	-----MKIKKLANGKYCVRLRIKVDGEWKEKR-----	LT	DTS
lin1765	(1)	MAKNKWQPTKHLGIYEYMTKKGKRYGIRVRYKQGNDYPEINKSGFETIAA		
Consensus	(1)		KY IRLR K DW E	T A
		51		100
PSA int	(33)	ETNLMYKASKLLKQVQHDSSSLKEWNFKEFYTLFMKTFKDGKSSQSTINL		
lin1765	(51)	AKVYKNNIENLKANKKEYVFTNEKLTNTWTFASYMEMFKKKNKSKDTIAN		
Consensus	(51)		L N S FF FM FK S TI	
		101		150
PSA int	(83)	YDLAYNQFVDYFDEKIKLNSIDAVQYQQFINHLSVD-YAISTVDTRHRKI		
lin1765	(101)	KYSIYNNHLEIPFGNYLTDISLDIYEDFLREKIKNGYANNSVKAMHKLM		
Consensus	(101)		YNN LD L I Y FI YA SV HK I	
		151		200
PSA int	(132)	RAIFNKAVHLGYMKKN-PTIGAHISGQDVAKNKAQFMETDKVHLLLEELA		
lin1765	(151)	KSILNAAVRYEKLKRNRLQFAEIEQLEENEVIELKVLETDEFNVFISACR		
Consensus	(151)		KAI N AV L KN A D LETD L I	
		201		250
PSA int	(181)	KFHSISRIVFLAVQTGMRFEIIALTKKDINFTRKRSITVKNKAWDYKYTN		
lin1765	(201)	AFFTKYDFTMIYLAVWGMRRGEVMGVKLKNLTFDDAKQQVRITLDSTRTL		
Consensus	(201)		F S I GMR EIIAL K I F V D T	
		251		300
PSA int	(231)	TFIDTKTKKS---R-VIYIDNSTAQYLHLSYLNWHTDYMKEH--AIKNPLM		
lin1765	(251)	RTPEGKGTCTPAGRRILLIDGEGYRLLKYSVEKAVSIKDHGSVLHQDDF		
Consensus	(251)		D K KS R II ID L L KDH I N	
		301		350
PSA int	(275)	LLFITYHNKPVDNASCNKALKKICSTINSEPVTLHKLRRHTHTGLCVEAGM		
lin1765	(301)	IFRNPTSNRPWAVTRMNDLLRKLEKEYDIK-VYPHLLRRHNFNTQALLAGA		
Consensus	(301)		I NKP N LKKI V H LRH L AG	
		351		400
PSA int	(325)	DIIVADRLGHDDINTTLKYYSHLSSNLRQHNQSKVDAFFTLKTDENTTN		
lin1765	(350)	NSNDLRKFIGHKNS-SMTDHYSHATDEGREK-----LMNTMKDRLSGI-		
Consensus	(351)		L IGH S HYSH S R TLK	
		401	411	
PSA int	(375)	FTTNATKTTE-		
lin1765	(392)	-----		
Consensus	(401)			

Fig. 43.

L. innocua 2601.

	1	50
PSA int	(1)	MKIKKLANGKYCVRLRIKVDGEWKEKRLTDTSETNLMYKASKLLKQVQHD
lin2601	(1)	MKIKKMKNKYTVRLRIKVDGEWKEKRLTDTSETNLMYKASKLLKQVEHD
Consensus	(1)	MKIKKL NGKY VRLRIKVDGEWKEKRLTDTSETNLMYKASKLLKQV HD
	51	100
PSA int	(51)	SSSLKEWNFKEFYTLFMKTFKDGKSSQSTINLYDLAYNQFVDYFDEKIKL
lin2601	(51)	SNSLKEWNFKEFYSLFMKTFKENKSSQSTINLYDLAYNQFVNYFDEKIKL
Consensus	(51)	S SLKEWNFKEFYSLFMKTFKD KSSQSTINLYDLAYNQFV YFDEKIKL
	101	150
PSA int	(101)	NSIDAVQYQQFINHLSVDYAISTVDTRHRKIRAI FNKAVHLGYMKKNPTI
lin2601	(101)	NSIDAVQYQQFINHLALDYAVATIDTRHRKIRAI FNKAVHLGYMKKNPAL
Consensus	(101)	NSIDAVQYQQFINHLALDYAIATIDTRHRKIRAI FNKAVHLGYMKKNP I
	151	200
PSA int	(151)	GAHISGQDVAKNKAQFMETDKVHLLLEELAKFHSISR AVIFLAVQTGMRF
lin2601	(151)	GAHISGHDI AKTKAQYLETDKVHLLLEELAKLHSISR AVIFLAVQTGMRF
Consensus	(151)	GAHISG DIAK KAQFLETDKVHLLLEELAK HSISR AVIFLAVQTGMRF
	201	250
PSA int	(201)	EEIIALTKKDINF TKRSITVNKAWDYKYTNTFIDTKTKKSRVIYIDNSTA
lin2601	(201)	EEIIALTKKDINF TKRSISVNKAWDYKYTNTFTDTTKTKKSRVIYIDNSTV
Consensus	(201)	EEIIALTKKDINF TKRSISVNKAWDYKYTNTF DTCTTKKSRVIYIDNST
	251	300
PSA int	(251)	QYLHSYLNWHTDYMKEHA IKNPLMLLFITYHNKPVDNASCNKALKKICST
lin2601	(251)	QYLQSYLAWHADYMK EHA IENPVMLLFITYHNKPVDNASCNKALKKICTT
Consensus	(251)	QYL SYL WH DYMKEHAI NPLMLLFITYHNKPVDNASCNKALKKICST
	301	350
PSA int	(301)	INSEPTLHKL RHTHTGLCVEAGMDIIYVADRLGHDDINTTLKYYSHLSS
lin2601	(301)	INSETVTLHKL RHTHTGLCVEAGMDIIYVADRLGHDDINTTLKYYSHLSS
Consensus	(301)	INSE VTLHKL RHTHTGLCVEAGMDIIYVADRLGHDDINTTLKYYSHLSS
	351	385
PSA int	(351)	NLRQHNQSKVD AFFTLKTDENTTNFTTNATKTTE-
lin2601	(351)	NLRQQNQSKVD AFFTLKTDENTTKFATNATKTTE-
Consensus	(351)	NLRQ NQSKVD AFFTLKTDENTT F TNATKTTE

Fig. 44.

L.monocytogenes f6854.

	1	50
PSA int	(1) -MKIKKLANGKYCVRLRIKVDGEWK-----EKRLTDTSETNLMYKASKLL	
lmof6854_2703	(1) MASYVNLGNKKYELRVSKGYDARGKQIRKTKNVTVKTVKALKLELSNFEA	
Consensus	(1) LAN KY LRL DA K T L A	
	51	100
PSA int	(45) KQVQHDSSSLKEWNFKEFYTLFMKTFKDGKSSQSTINLYDLAYNQFVDYF	
lmof6854_2703	(51) YVYSSDYTEIKDMRFIDFVEKWRLNYAKRELKGNTIDKYNLFLENWIIPY	
Consensus	(51) D S IKD F DF F F TI Y L NFI F	
	101	150
PSA int	(95) DEKIKLSIDAVQYQQFINHLS-VDYAISTVDTRHRKIRAI FNKAVHLGY	
lmof6854_2703	(101) FERKKISKITTMQLLDYFHEVQKKGVGPSALEGHHRVIRSLFKYATLWGI	
Consensus	(101) EK KI I MQ F L A S LD HR IRAIF A G	
	151	200
PSA int	(144) MKKNPTIGAHISGQDVAKNKAQFMETDKVHLLLEELAKFHSISRIVFLA	
lmof6854_2703	(151) TETDVSLSVKKPTYKVPE--KNIYNRREIEVLIDRIKILQKYQQVMIKLA	
Consensus	(151) SI V N I LLID I MI LA	
	201	250
PSA int	(194) VQTGMRFEIIALTKKDINF TKRSITV NKAWDYKYTN--TFIDTKTKKSR	
lmof6854_2703	(199) LYCGLRRGEVIGLTTKDMYNKNTINVYRAVIKSASEGIKLDETKNKRKR	
Consensus	(201) L GLR EIIALT KDINF K SI V KA S DTK KK R	
	251	300
PSA int	(242) VIYIDNSTAQYLHSYLNWHTDYMKEHAIKNP-----LMLLFITYHN	
lmof6854_2703	(249) IVPAPAGLMQEIKELAKEKQKNKDKLGLLWKGTKDLDGKTVVLI FSHDDG	
Consensus	(251) II Q I AI LMLIF	
	301	350
PSA int	(283) KPVDNASCNKALKKICSTINS--E-PVTLHKL RHTHTGLCVEAGMDIIYV	
lmof6854_2703	(299) TPFTPASVTRMFNRFLEKEENNDLTKISFHDLRHSAASFLEQGINVKVI	
Consensus	(301) P AS K K IS H LRHS LE GI I I	
	351	400
PSA int	(330) ADRLGHDDINTTLKYYSHLSSNLRQHNSKVD AFFT LKTDENTTNETTNA	
lmof6854_2703	(349) QNILGHSDIKVTLNTYAHITEDGYSEAAKTFDNFYKSSK-----	
Consensus	(351) LGH DI TL YAHIS D FF	
	401	
PSA int	(380) TKTTE-	
lmof6854_2703	(388) -----	
Consensus	(401)	

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Fig. 45A

```
1   attaaggact cggggcagga ggggcagaag ttgcgcgcag gccggcgggc gggagcggac
60   accgaggccg gcgtgcaggc gtgcgggtgt gcgggagccg ggctcggggg gatcggaccg
120  agagcgagaa gcgcggc atg gag ctc cag gca gcc cgc gcc tgc ttc gcc
170  ctg ctg tgg ggc tgt gcg ctg gcc gcg gcc gcg gcg cag ggc aag
218  gaa gtg gta ctg ctg gac ttt gct gca gct gga ggg gag ctc ggc tgg
266  ctc aca cac ccg tat ggc aaa ggg tgg gac ctg atg cag aac atc atg
314  aat gac atg ccg atc tac atg tac tcc gtg tgc aac gtg atg tct ggc
362  gac cag gac aac tgg ctc cgc acc aac tgg gtg tac cga gga gag gct
410  gag cgt atc ttc att gag ctc aag ttt act gta cgt gac tgc aac agc
458  ttc cct ggt ggc gcc agc tcc tgc aag gag act ttc aac ctc tac tat
506  gcc gag tcg gac ctg gac tac ggc acc aac ttc cag aag cgc ctg ttc
554  acc aag att gac acc att gcg ccc gat gag atc acc gtc agc agc gac
602  ttc gag gca cgc cac gtg aag ctg aac gtg gag gag cgc tcc gtg ggg
650  ccg ctc acc cgc aaa ggc ttc tac ctg gcc ttc cag gat atc ggt gcc
698  tgt gtg gcg ctg ctc tcc gtc cgt gtc tac tac aag aag tgc ccc gag
746  ctg ctg cag ggc ctg gcc cac ttc cct gag acc atc gcc ggc tct gat
794  gca cct tcc ctg gcc act gtg gcc ggc acc tgt gtg gac cat gcc gtg
842  gtg cca ccg ggg ggt gaa gag ccc cgt atg cac tgt gca gtg gat ggc
890  gag tgg ctg gtg ccc att ggg cag tgc ctg tgc cag gca ggc tac gag
938  aag gtg gag gat gcc tgc cag gcc tgc tcg cct gga ttt ttt aag ttt
986  gag gca tct gag agc ccc tgc ttg gag tgc cct gag cac acg ctg cca
1034 tcc cct gag ggt gcc acc tcc tgc gag tgt gag gaa ggc ttc ttc cgg
1082 gca cct cag gac cca gcg tcg atg cct tgc aca cga ccc ccc tcc gcc
1130 cca cac tac ctc aca gcc gtg ggc atg ggt gcc aag gtg gag ctg cgc
1178 tgg acg ccc cct cag gac agc ggg ggc cgc gag gac att gtc tac agc
1226 gtc acc tgc gaa cag tgc tgg ccc gag tct ggg gaa tgc ggg ccg tgt
1274 gag gcc agt gtg cgc tac tcg gag cct cct cac gga ctg acc cgc acc
1322 agt gtg aca gtg agc gac ctg gag ccc cac atg aac tac acc ttc acc
1370 gtg gag gcc cgc aat ggc gtc tca ggc ctg gta acc agc cgc agc ttc
1418 cgt act gcc agt gtc agc atc aac cag aca gag ccc ccc aag gtg agg
1466 ctg gag ggc cgc agc acc acc tcg ctt agc gtc tcc tgg agc atc ccc
1514 ccg ccg cag cag agc cga gtg tgg aag tac gag gtc act tac cgc aag
1562 aag gga gac tcc aac agc tac aat gtg cgc cgc acc gag ggt ttc tcc
1610 gtg acc ctg gac gac ctg gcc cca gac acc acc tac ctg gtc cag gtg
1658 cag gca ctg acg cag gag ggc cag ggg gcc ggc agc aag gtg cac gaa
1706 ttc cag acg ctg tcc ccg gag gga tct ggc aac ttg gcg gtg att ggc
1754 ggc gtg gct gtc ggt agt gtc ctg ctt ctg gtg ctg gca gga gtt ggc
1802 ttc ttt atc cac cgc agg agg aag aac cag cgt gcc cgc cag tcc ccg
1850 gag gac gtt tac ttc tcc aag tca gaa caa ctg aag ccc ctg aag aca
1898 tac gtg gac ccc cac aca tat gag gac ccc aac cag gct gtg ttg aag
1946 ttc act acc gag atc cat cca tcc tgt gtc act cgg cag aag gtg atc
1994 gga gca gga gag ttt ggg gag gtg tac aag ggc atg ctg aag aca tcc
2042 tcg ggg aag aag gag gtg ccg gtg gcc atc aag acg ctg aaa gcc ggc
2090 tac aca gag aag cag cga gtg gac ttc ctc ggc gag gcc ggc atc atg
2138 ggc cag ttc agc cac cac aac atc atc cgc cta gag ggc gtc atc tcc
2186 aaa tac aag ccc atg atg atc atc act gag tac atg gag aat ggg gcc
2234 ctg gac aag ttc ctt cgg gag aag gat ggc gag ttc agc gtg ctg cag
2282 ctg gtg ggc atg ctg cgg ggc atc gca gct ggc atg aag tac ctg gcc
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Fig. 45B

(sequence continued from Fig. 45A)

```
2330 aac atg aac tat gtg cac cgt gac ctg gct gcc cgc aac atc ctc gtc
2378 aac agc aac ctg gtc tgc aag gtg tct gac ttt ggc ctg tcc cgc gtg
2426 ctg gag gac gac ccc gag gcc acc tac acc acc agt ggc ggc aag atc
2474 ccc atc cgc tgg acc gcc ccg gag gcc att tcc tac cgg aag ttc acc
2522 tct gcc agc gac gtg tgg agc ttt ggc att gtc atg tgg gag gtg atg
2570 acc tat ggc gag cgg ccc tac tgg gag ttg tcc aac cac gag gtg atg
2618 aaa gcc atc aat gat ggc ttc cgg ctc ccc aca ccc atg gac tgc ccc
2666 tcc gcc atc tac cag ctc atg atg cag tgc tgg cag cag gag cgt gcc
2714 cgc cgc ccc aag ttc gct gac atc gtc agc atc ctg gac aag ctc att
2762 cgt gcc cct gac tcc ctc aag acc ctg gct gac ttt gac ccc cgc gtg
2810 tct atc cgg ctc ccc agc acg agc ggc tgc gag ggg gtg ccc ttc cgc
2858 acg gtg tcc gag tgg ctg gag tcc atc aag atg cag cag tat acg gag
2906 cac ttc atg gcg gcc ggc tac act gcc atc gag aag gtg gtg cag atg
2954 acc aac gac gac atc aag agg att ggg gtg cgg ctg ccc ggc cac cag
3002 aag cgc atc gcc tac agc ctg ctg gga ctc aag gac cag gtg aac act
3050 gtg ggg atc ccc atc tga gcctcgacag ggcttgagc cccatcgcc
3098 aagaatactt gaagaaacag agtggcctcc ctgctgtgcc atgctgggcc actggggact
3158 ttattttattt ctagttcttt cctccccctg caacttccgc tgaggggtct cggatgacac
3218 cctggcctga actgaggaga tgaccaggga tgctgggctg ggccctcttt ccctgcgaga
3278 cgcacacagc tgagcactta gcaggcaccg ccacgtccca gcatccctgg agcaggagcc
3338 ccgccacagc cttcggacag acatatagga tattcccaag ccgaccttcc ctccgccttc
3398 tcccacatga ggccatctca ggagatggag ggcttggccc agcgccaagt aaacagggta
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3518 aaagcggctc ctgtccctct agtgccttct ttagaccctc gggcccatc ctcacccctg
3578 actggccaaa cccttgcttt cctgggcctt tgcaagatgc ttggttgtgt tgaggttttt
3638 aaatatatat tttgtacttt gtggagagaa tgtgtgtgtg tggcaggggg ccccgccagg
3698 gctggggaca gaggtgttca aacattcgtg agctggggac tcagggaccg gtgctgcagg
3758 agtgtcctgc ccatgccccca gtcggcccca tctctcatcc ttttggataa gtttctattc
3818 tgtcagtgtt aaagattttg ttttgttgga catttttttc gaatcttaat ttattatttt
3878 ttttatattt attgttagaa aatgacttat ttctgctctg gaataaagtt gcagatgatt
3938 caaaccgaaa aaaaaaaaaa aaaaa 3963
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Fig. 46A

1

Met Glu Leu Gln Ala Ala Arg Ala Cys Phe Ala Leu Leu Trp Gly Cys Ala
 Leu Ala Ala Ala Ala Ala Ala Gln Gly Lys Glu Val Val Leu Leu Asp Phe
 Ala Ala Ala Gly Gly Glu Leu Gly Trp Leu Thr His Pro Tyr Gly Lys Gly
 Trp Asp Leu Met Gln Asn Ile Met Asn Asp Met Pro Ile Tyr Met Tyr Ser
 Val Cys Asn Val Met Ser Gly Asp Gln Asp Asn Trp Leu Arg Thr Asn Trp
 Val Tyr Arg Gly Glu Ala Glu Arg Ile Phe Ile Glu Leu Lys Phe Thr Val
 Arg Asp Cys Asn Ser Phe Pro Gly Gly Ala Ser Ser Cys Lys Glu Thr Phe
 Asn Leu Tyr Tyr Ala Glu Ser Asp Leu Asp Tyr Gly Thr Asn Phe Gln Lys
 Arg Leu Phe Thr Lys Ile Asp Thr Ile Ala Pro Asp Glu Ile Thr Val Ser
 Ser Asp Phe Glu Ala Arg His Val Lys Leu Asn Val Glu Glu Arg Ser Val
 Gly Pro Leu Thr Arg Lys Gly Phe Tyr Leu Ala Phe Gln Asp Ile Gly Ala
 Cys Val Ala Leu Leu Ser Val Arg Val Tyr Tyr Lys Lys Cys Pro Glu Leu
 Leu Gln Gly Leu Ala His Phe Pro Glu Thr Ile Ala Gly Ser Asp Ala Pro
 Ser Leu Ala Thr Val Ala Gly Thr Cys Val Asp His Ala Val Val Pro Pro
 Gly Gly Glu Glu Pro Arg Met His Cys Ala Val Asp Gly Glu Trp Leu Val
 Pro Ile Gly Gln Cys Leu Cys Gln Ala Gly Tyr Glu Lys Val Glu Asp Ala
 Cys Gln Ala Cys Ser Pro Gly Phe Phe Lys Phe Glu Ala Ser Glu Ser Pro
 Cys Leu Glu Cys Pro Glu His Thr Leu Pro Ser Pro Glu Gly Ala Thr Ser
 Cys Glu Cys Glu Glu Gly Phe Phe Arg Ala Pro Gln Asp Pro Ala Ser Met
 Pro Cys Thr Arg Pro Pro Ser Ala Pro His Tyr Leu Thr Ala Val Gly Met
 Gly Ala Lys Val Glu Leu Arg Trp Thr Pro Pro Gln Asp Ser Gly Gly Arg
 Glu Asp Ile Val Tyr Ser Val Thr Cys Glu Gln Cys Trp Pro Glu Ser Gly
 Glu Cys Gly Pro Cys Glu Ala Ser Val Arg Tyr Ser Glu Pro Pro His Gly
 Leu Thr Arg Thr Ser Val Thr Val Ser Asp Leu Glu Pro His Met Asn Tyr
 Thr Phe Thr Val Glu Ala Arg Asn Gly Val Ser Gly Leu Val Thr Ser Arg
 Ser Phe Arg Thr Ala Ser Val Ser Ile Asn Gln Thr Glu Pro Pro Lys Val
 Arg Leu Glu Gly Arg Ser Thr Thr Ser Leu Ser Val Ser Trp Ser Ile Pro
 Pro Pro Gln Gln Ser Arg Val Trp Lys Tyr Glu Val Thr Tyr Arg Lys Lys
 Gly Asp Ser Asn Ser Tyr Asn Val Arg Arg Thr Glu Gly Phe Ser Val Thr
 Leu Asp Asp Leu Ala Pro Asp Thr Thr Tyr Leu Val Gln Val Gln Ala Leu
 Thr Gln Glu Gly Gln Gly Ala Gly Ser Lys Val His Glu Phe Gln Thr Leu
 Ser Pro Glu Gly Ser Gly Asn Leu Ala Val Ile Gly Gly Val Ala Val Gly
 Val Val Leu Leu Leu Val Leu Ala Gly Val Gly Phe Phe Ile His Arg Arg
 Arg Lys Asn Gln Arg Ala Arg Gln Ser Pro Glu Asp Val Tyr Phe Ser Lys
 Ser Glu Gln Leu Lys Pro Leu Lys Thr Tyr Val Asp Pro His Thr Tyr Glu
 Asp Pro Asn Gln Ala Val Leu Lys Phe Thr Thr Glu Ile His Pro Ser Cys
 Val Thr Arg Gln Lys Val Ile Gly Ala Gly Glu Phe Gly Glu Val Tyr Lys
 Gly Met Leu Lys Thr Ser Ser Gly Lys Lys Glu Val Pro Val Ala Ile Lys
 Thr Leu Lys Ala Gly Tyr Thr Glu Lys Gln Arg Val Asp Phe Leu Gly Glu
 Ala Gly Ile Met Gly Gln Phe Ser His His Asn Ile Ile Arg Leu Glu Gly
 Val Ile Ser Lys Tyr Lys Pro Met Met Ile Ile Thr Glu Tyr Met Glu Asn
 Gly Ala Leu Asp Lys Phe Leu Arg Glu Lys Asp Gly Glu Phe Ser Val Leu
 Gln Leu Val Gly Met Leu Arg Gly Ile Ala Ala Gly Met Lys Tyr Leu Ala
 Asn Met Asn Tyr Val His Arg Asp Leu Ala Ala Arg Asn Ile Leu Val Asn
 Ser Asn Leu Val Cys Lys Val Ser Asp Phe Gly Leu Ser Arg Val Leu Glu
 Asp Asp Pro Glu Ala Thr Tyr Thr Thr Ser Gly Gly Lys Ile Pro Ile Arg
 Trp Thr Ala Pro Glu Ala Ile Ser Tyr Arg Lys Phe Thr Ser Ala Ser Asp

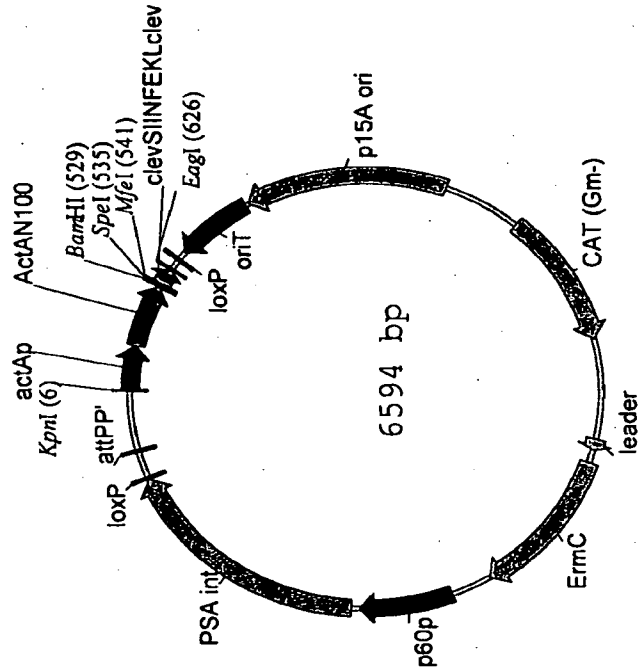
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Fig. 46B

(sequence continued from Fig. 46A)

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Pro Tyr Trp Glu Leu Ser Asn His Glu Val Met Lys Ala Ile Asn Asp Gly
Phe Arg Leu Pro Thr Pro Met Asp Cys Pro Ser Ala Ile Tyr Gln Leu Met
Met Gln Cys Trp Gln Gln Glu Arg Ala Arg Arg Pro Lys Phe Ala Asp Ile
Val Ser Ile Leu Asp Lys Leu Ile Arg Ala Pro Asp Ser Leu Lys Thr Leu
Ala Asp Phe Asp Pro Arg Val Ser Ile Arg Leu Pro Ser Thr Ser Gly Ser
Glu Gly Val Pro Phe Arg Thr Val Ser Glu Trp Leu Glu Ser Ile Lys Met
Gln Gln Tyr Thr Glu His Phe Met Ala Ala Gly Tyr Thr Ala Ile Glu Lys
Val Val Gln Met Thr Asn Asp Asp Ile Lys Arg Ile Gly Val Arg Leu Pro
Gly His Gln Lys Arg Ile Ala Tyr Ser Leu Leu Gly Leu Lys Asp Gln Val
Asn Thr Val Gly Ile Pro Ile
976

Fig. 47A



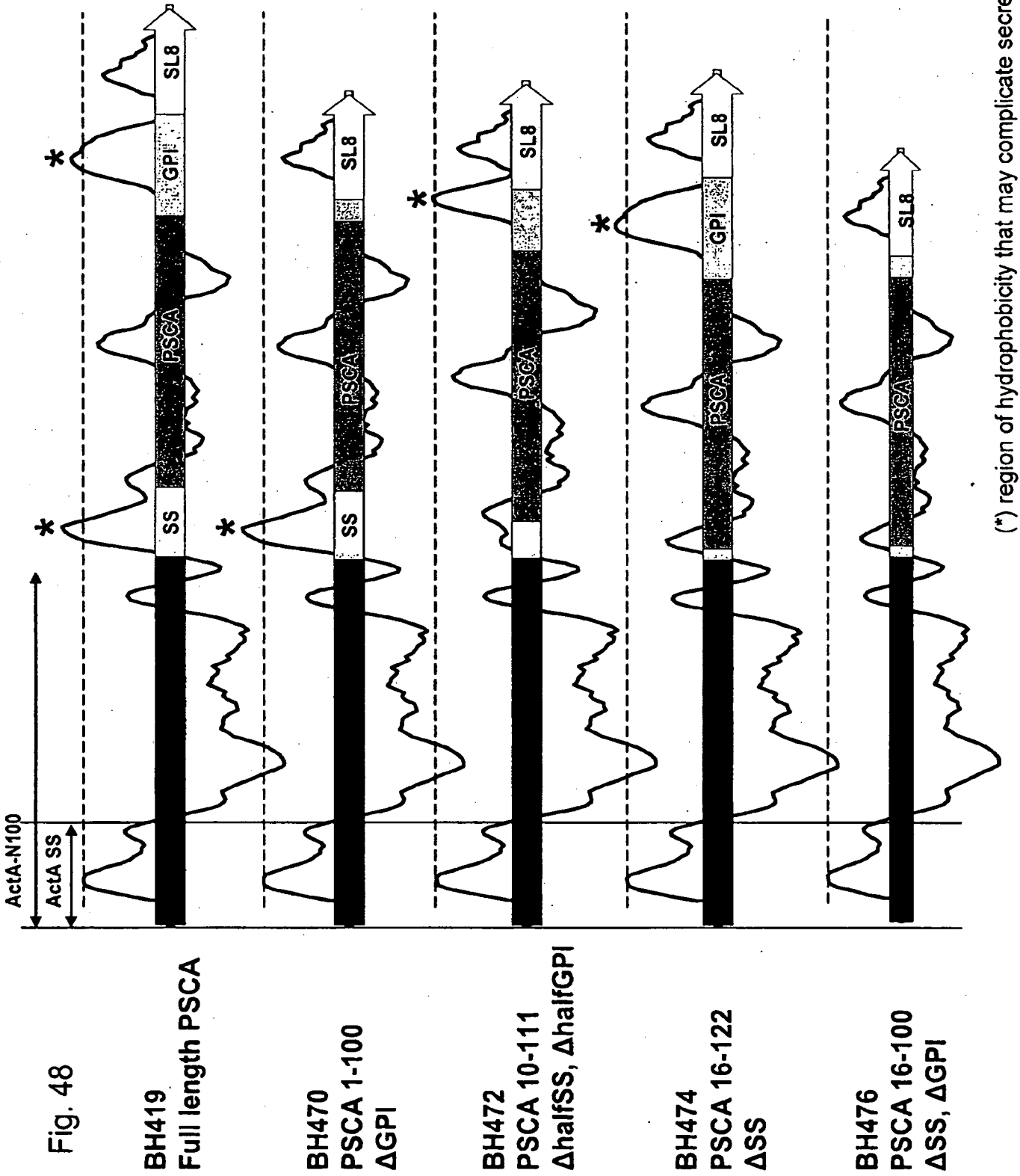
pINT-ActAN100-Bam-Spe-Mfe-SIINFEKL

Fig. 47B

```

                    SpeI
                    -----
                    BamHI           MfeI           BglII
                    -----
K   A   E   K   G   S   T   S   Q   L   A   D   L   V   L   A   K   V   L
513  AAA GCA GAG AAA GGT GGA TCC ACT AGT CAA TTG GCA GAT CTT GTA TTA GCA AAA GTT TTA
      <---- ActAN100-----> |---linker---|
                                     BglII           EagI
                                     -----
Q   L   E   S   I   I   N   F   E   K   L   A   D   L   V   A   *
573  CAA TTA GAA AGT ATT AAT TTT GAA AAA TTA GCA GAT CTT GTA GCA TAA GCG GCC GCC

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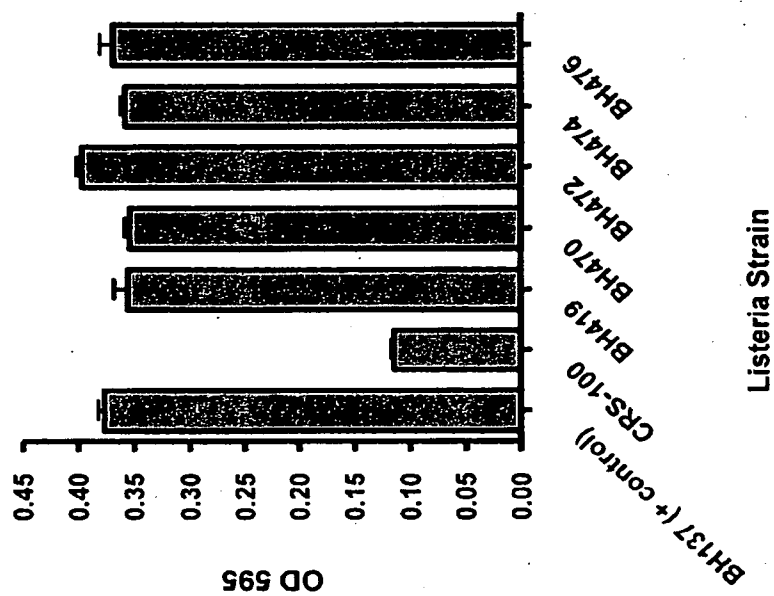


Fig. 49A

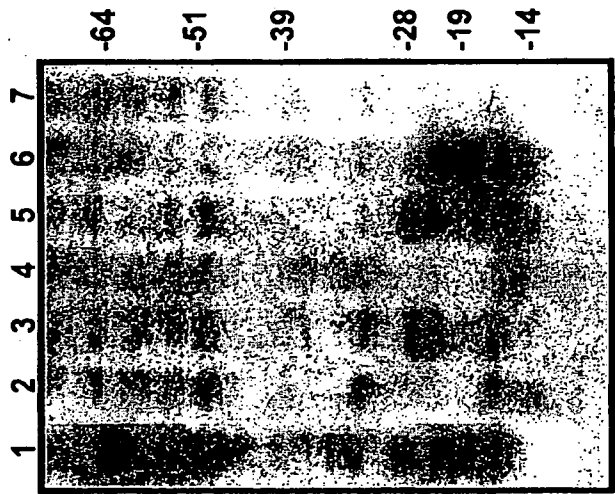
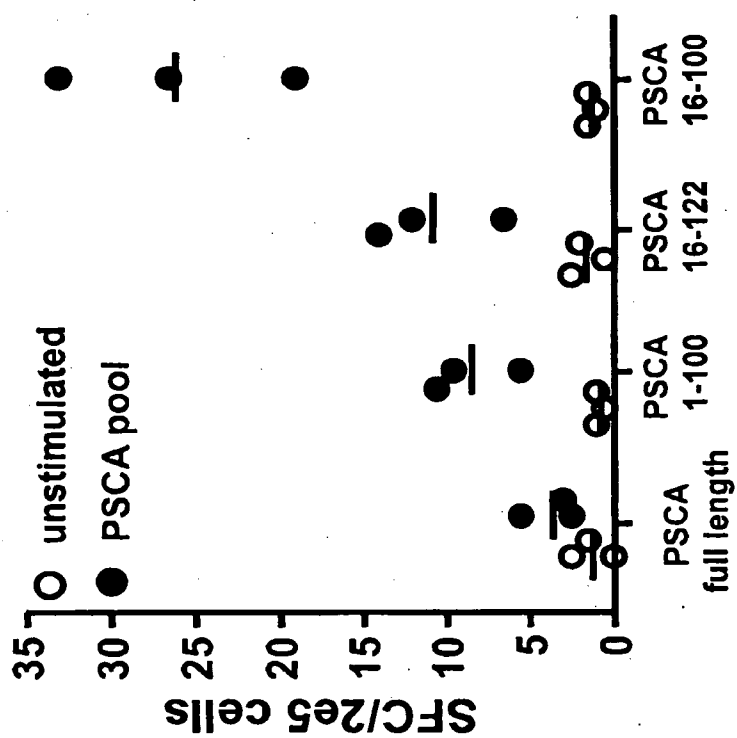


Fig. 49B

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Fig. 50



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Fig. 51A

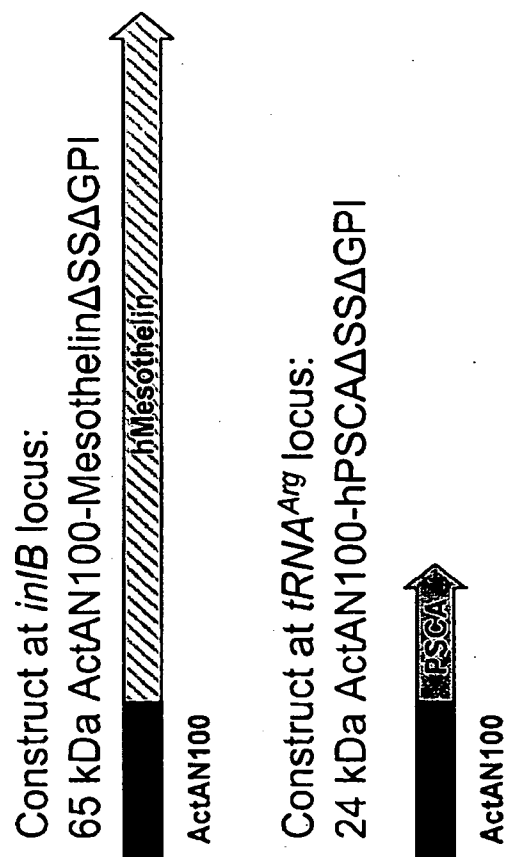
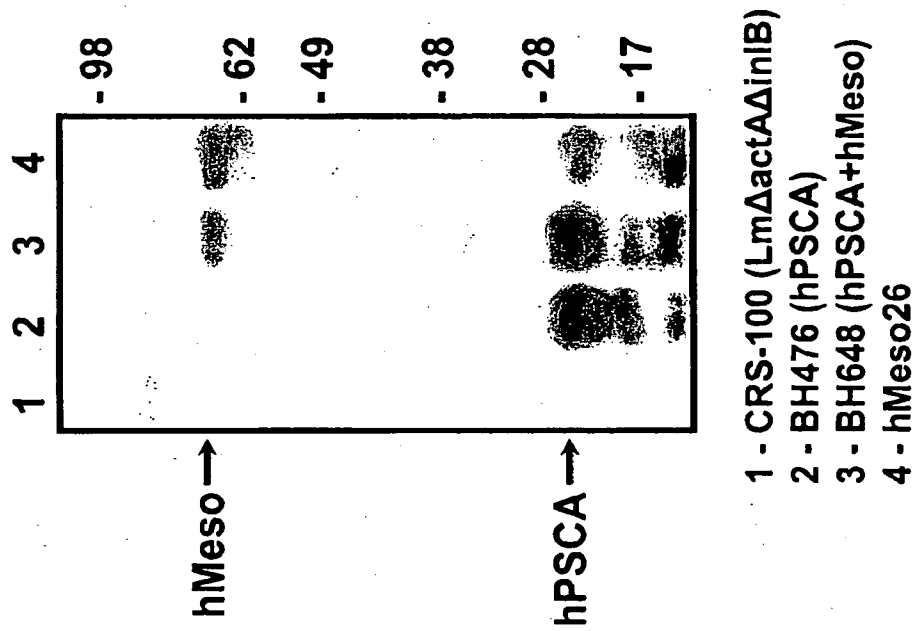


Fig. 51B



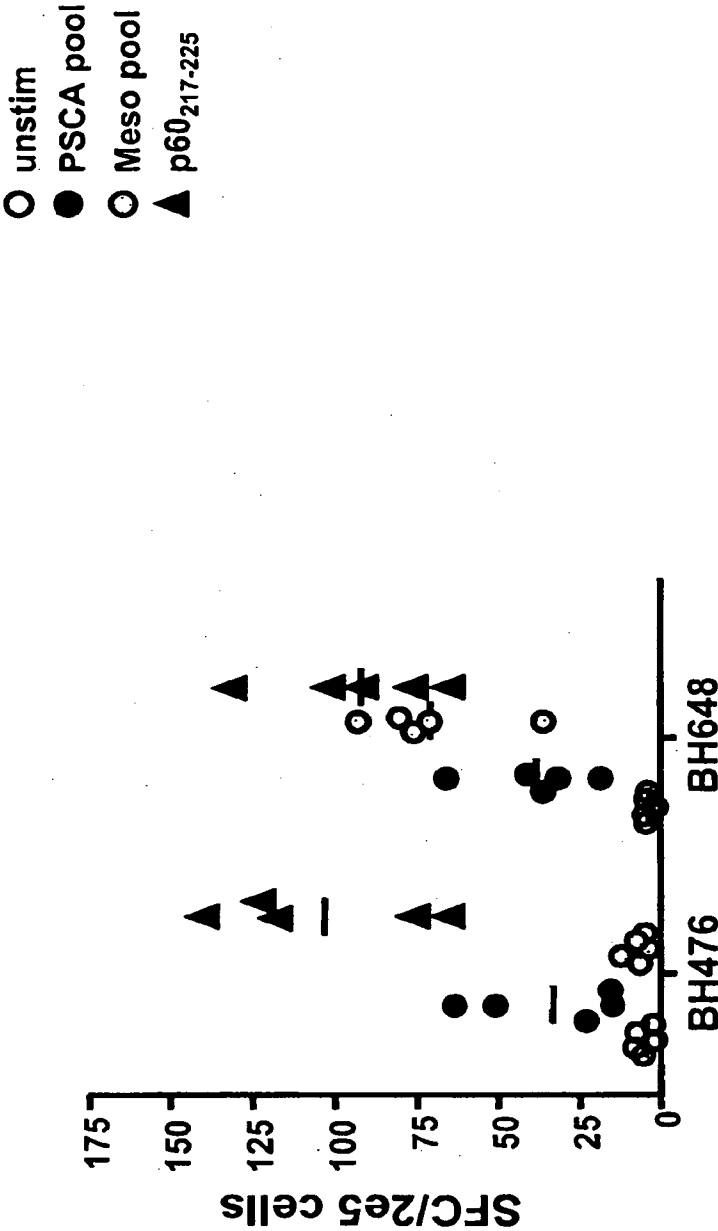


Fig. 52