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(54) **INVASIVE BACTERIAL VECTORS FOR EXPRESSING ALPHAVIRUS REPLICONS**

(76) Inventors: **Jaap Goudsmit**, Amsterdam (NL); **Jerald C. Sadoff**, Washington, DC (US); **Wayne Koff**, Stony Brook, NY (US)

Correspondence Address:
**HALE AND DORR LLP**
**300 PARK AVENUE**
**NEW YORK, NY 10022 (US)**

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(57) **ABSTRACT**

The present invention is directed to a bacterial delivery system for delivering alphavirus replicon DNA into an animal or animal cells with the replicon encoding one or more heterologous genes to be expressed in the animal or the animal cells. The bacteria are invasive bacteria or attenuated invasive bacteria engineered to contain a DNA vector that encodes the alphavirus replicon in a eukaryotic expression cassette. The heterologous gene can encode an antigen, a therapeutic agent, an immunoregulatory agent, an anti-sense RNA, a catalytic RNA, a protein, a peptide, an antibody or an antigen-binding fragment of an antibody. In a preferred embodiment, the heterologous gene encodes one or more antigens useful as a vaccine for HIV. In addition to the bacterial delivery system, the invention provides methods of introducing and expressing the heterologous gene(s) in animal or animal cells and methods of stimulating or inducing an immune response.

# INVASIVE BACTERIAL VECTORS FOR EXPRESSING ALPHAVIRUS REPLICONS

[0001] This application is a continuation of U.S. Ser. No. 09/697,236 filed Oct. 26, 2000.

## FIELD OF THE INVENTION

[0002] The present invention is directed to a bacterial delivery system for delivering alphavirus replicon DNA into an animal or animal cells, the replicon encoding a heterologous gene to be expressed in the animal or the animal cells. The bacteria are invasive bacteria or attenuated invasive bacteria engineered to contain a DNA vector that encodes the alphavirus replicon in a eukaryotic expression cassette. Upon bacterial infection, primary transcription of the DNA vector is driven by the eukaryotic expression vector and produces an alphavirus replicon RNA which is transported the cytoplasm. That RNA is transcribed and translated to express the heterologous gene encoded in the alphavirus replicon. The heterologous gene may encode an antigen, a therapeutic agent, an immunoregulatory agent, an anti-sense RNA, a catalytic RNA, a protein, a peptide or any other molecule desired for delivery to an animal or animal cell. In a preferred embodiment, the heterologous gene encodes an antigen useful as a vaccine for HIV. In addition to the bacterial delivery system, the invention provides methods of introducing and expressing the heterologous gene, methods of stimulating or inducing an immune response and vaccines therefor.

## BACKGROUND OF THE INVENTION

[0003] There are many applications for delivering DNA to animals or animal cells including for gene therapy of acquired or inherited diseases or conditions, for DNA-based vaccination, for understanding genetic structure and for studying the molecular mechanisms underlying gene expression.

[0004] Successful delivery of DNA to animal tissue has been achieved by cationic liposomes (Watanabe et al., Mol. Reprod. Dev., 38:268-274 (1994)), direct injection of naked DNA into animal muscle tissue (Robinson et al., Vacc., 11:957-960 (1993); Hoffman et al., Vacc., 12:1529-1533; (1994); Xiang et al., Virol., 199:132-140 (1994); Webster et al., Vacc., 12:1495-1498 (1994); Davis et al., Vacc., 12:1503-1509 (1994); and Davis et al., Hum. Molec. Gen., 2:1847-1851 (1993)), and embryos (Naito et al., Mol. Reprod. Dev., 39:153-161 (1994); and Burdon et al., Mol. Reprod. Dev., 33:436-442 (1992)), or intradermal injection of DNA using "gene gun" technology (Johnston et al., supra). A limitation of these techniques is that they only efficiently deliver DNA to parenteral sites. At present, effective delivery of eukaryotic expression cassettes to mucosal tissue has been met with limited success. This is presumably due to poor access to these sites, toxicity of the delivery vehicles or instability of the delivery vehicles when delivered orally.

[0005] For DNA-based vaccination, delivery by injection of naked plasmid DNA has shown potential in mouse models for inducing both humoral and cellular immune responses. However, in larger animals, using DNA delivery for vaccination has been hampered by requiring large amounts of DNA or inducing persistent expression of an antigen with the potential for developing tolerance to the antigen. Berglund reported a strategy for inducing or enhancing an immune response by injecting mice with plasmid DNA containing an alphavirus DNA expression vector having a recombinant Semliki Forest Virus (SFV) replicon in a eukaryotic expression cassette [Berglund et al.. (1998) Nature Biotechnology 16:562-565]. The eukaryotic expression cassette controlled expression of the primary nuclear transcription of the SFV replicon. This SFV replicon transcript, encoding the heterologous antigen, was transported to the cytoplasm and amplified by the self-encoded SFV replicase complex. The amplified RNA replicon lead to high level production of an mRNA encoding the heterologous antigen. Similar results were described by Polo and his group [Polo et al.. (1998) Nature Biotechnology 16:517-518; Hariharan et al.. (1998) J. Virol. 72:950-958]. Both groups found strong immune responses could be induced using small amounts of input plasmid DNA. Although this method allows greater expression from the input DNA vector, the method still has the disadvantages associated with parenteral delivery.

[0006] Alternatively, a method to deliver DNA to animals that overcomes the disadvantages of conventional delivery methods is by administering attenuated, invasive bacteria containing a bacterial DNA vector having a eukaryotic expression cassette encoding the gene to be expressed. For example, U.S. Pat. No. 5,877,159 to Powell et al.. describes live bacteria that can invade animal cells without establishing a productive infection or causing disease to thereby introduce a eukaryotic expression cassette encoding an antigen capable of being expressed by the animal cells. While this method allows delivery of the DNA vaccine to mucosal surfaces, including easy administration, a concern for vaccine delivery in developing countries, it does not have the advantage of providing amplifiable mRNA encoding the gene of interest.

[0007] Accordingly, the present invention combines use of a live attenuated invasive bacteria with eukaryotic expression cassettes encoding an alphavirus replicon to provide improved bacterial delivery systems to deliver a heterologous gene, and preferably a gene encoding an antigen, to an animal. Such systems have the advantages of both bacterial delivery systems and alphavirus replicon vectors and are efficacious, cost effective, and safe. The bacterial delivery systems of the invention are particularly useful for delivering DNA for gene therapy and vaccinations.

[0008] All cited references and patents are incorporated herein in their entirety by reference.

## SUMMARY OF THE INVENTION

[0009] In accordance with the invention, one embodiment is directed to a bacterial delivery system which comprises a live invasive bacteria or attenuated, invasive bacteria containing a DNA comprising a eukaryotic expression cassette operably linked to an alphavirus replicon DNA capable of amplification in animal cells, wherein the alpha virus replicon DNA comprises an alphavirus replicon comprises nucleic acid sequences operably linked to a heterologous nucleic acid sequence to control expression thereof. The heterologous nucleic acid sequence can encode an antigen, an antigenic fragment of a protein, a therapeutic agent, an immunoregulatory agent, an anti-sense RNA, a catalytic RNA, a protein, a peptide or any other molecule encodable

by DNA and desired for delivery to an animal or animal cell. The heterologous nucleic acid sequences can be obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HCV, HIV, HSV, FeLV, FIV, HTLV-I, HTLV-II, and CMV. Such viral sequences can encode one or more viral genes or antigenic fragments thereof. The heterologous nucleotide sequence can encode a cytokine, an interleukin, erythropoietin or other immunostimulatory or immunoregulatory protein.

[0010] Another embodiment of this invention is directed to a method for introducing and expressing a gene in an animal comprising infecting said animal with live invasive bacteria, wherein said bacteria contain a DNA comprising a eukaryotic expression cassette, wherein said cassette expresses an alphavirus replicon RNA capable of amplification in cells of said animal, wherein said RNA encodes a heterologous gene product, and wherein said gene product is expressed in said animal. The method is applicable to deliver genes encoding an antigen, an antigenic protein fragment, a therapeutic agent, an immunoregulatory agent, an anti-sense RNA, a catalytic RNA, a protein, a peptide or any other molecule encodable by DNA and desired for delivery to an animal or an animal cell.

[0011] Another aspect of the invention provides a method for inducing an immune response in an animal which comprises infecting said animal live attenuated invasive bacteria, wherein said bacteria contain one or more DNAs comprising a eukaryotic expression cassette, wherein said cassette expresses an alphavirus replicon RNA capable of amplification in said animal, wherein said RNA encodes an antigen, and wherein said antigen is expressed at a level sufficient to stimulate an immune response to said antigen. In a preferred embodiment the antigen is derived from a virus

[0012] Yet another aspect of the invention relates to a method for introducing and expressing a gene in animal cells comprising (a) infecting said animal cells with live invasive bacteria, wherein the bacteria contain one or more DNAs comprising a eukaryotic expression cassette, wherein said cassette expresses an alphavirus replicon RNA capable of amplification in said animal cells and wherein said RNA encodes a heterologous gene product; and (b) culturing those cells under conditions sufficient to express the gene product.

## DETAILED DESCRIPTION OF THE INVENTION

[0013] As used herein, "invasive bacteria" are bacteria that are capable of delivering eukaryotic expression cassettes to animal cells or animal tissue. "Invasive bacteria" include bacteria that are naturally capable of entering the cytoplasm or nucleus of animal cells, as well as bacteria that are genetically engineered to enter the cytoplasm or nucleus of animal cells or cells in animal tissue. As used herein, "attenuated, invasive bacteria" are invasive bacteria as defined herein which are capable of infecting an animal host without establishing a productive infection and/or causing disease in the infected host. Thus, at most an attenuated bacterial strain may cause a self-limiting, clinically-insignificant infection.

[0014] Attenuated bacteria of the invention can be prepared by methods known in the art. For example, attenuating mutations can be introduced into bacterial pathogens using non-specific mutagenesis either chemically, using agents such as N-methyl-N'-nitro-N-nitrosoguanidine, or using recombinant DNA techniques; classic genetic techniques, such as Tn10 mutagenesis, P22-mediated transduction, lambda-phage mediated crossover, and conjugational transfer; or site-directed mutagenesis using recombinant DNA techniques. Recombinant DNA techniques are preferable. Examples of such attenuating mutations include, but are not limited to:

[0015] (i) auxotrophic mutations, such as aro (Hoiseth et al., Nature, 291:238-239 (1981)), gua (McFarland et al., Microbiol. Path., 3:129-141 (1987)), and (Park et al.., J. Bact., 170:3725-3730 (1988), thy (Nnalue et al., Infect. Immun., 55:955-962 (1987)), and asd (Curtiss, supra) mutations;

[0016] (ii) mutations that inactivate global regulatory functions, such as cya (Curtiss et al., Infect. Immun., 55:3035-3043 (1987) ), crp (Curtiss et al. (1987), supra), phoP/phoQ (Groisman et al., Proc. Natl. Acad. Sci., USA, 86:7077-7081 (1989); and Miller et al., Proc. Natl. Acad. Sci., USA, 86:5054-5058 (1989)), $phoP^c$ (Miller et al., J. Bact., 172:2485-2490 (1990)) or ompR (Dorman et al., Infect. Immun., 57:2136-2140 (1989)) mutations;

[0017] (iii) mutations that modify the stress response, such as recA (Buchmeier et al., Mol. Micro., 7:933-936 (1993)), htrA (Johnson et al., Mol. Micro., 5:401-407 (1991)), htpR (Neidhardt et al., Biochem. Biophys. Res. Com., 100:894-900 (1981)), hsp (Neidhardt et al., Ann. Rev. Genet., 18:295-329 (1984)) and groEL (Buchmeier et al., Sci., 248:730-732 (1990)) mutations;

[0018] (iv) mutations in specific virulence factors, such as lsyA (Libby et al., Proc. Natl. Acad. Sci., USA, 91:489-493 (1994)), pag or prg (Miller et al. (1990), supra; and Miller et al. (1989), supra), iscA or virG (d'Hauteville et al., Mol. Micro., 6:833-841 (1992)), plcA (Mengaud et al., Mol. Microbiol., 5:367-72 (1991); Camilli et al., J. Exp. Med, 173:751-754 (1991)), and act (Brundage et al., Proc. Natl. Acad. Sci., USA, 90:11890-11894 (1993)) mutations;

[0019] (v) mutations that affect DNA topology, such as topA (Galan et al., Infect. Immun., 58:1879-1885 (1990)) mutation;

[0020] (vi) mutations that block biogenesis of surface polysaccharides, such as rfb, galE (Hone et al., J. Infect. Dis., 156:164-167 (1987)) or via (Popoff et al., J. Gen. Microbiol., 138:297-304 (1992)) mutations;

[0021] (vii) mutations that modify suicide systems, such as sacB (Recorbet et al., App. Environ. Micro., 59:1361-1366 (1993); Quandt et al., Gene, 127:15-21 (1993)), nuc (Ahrenholtz et al., App. Environ. Micro., 60:3746-3751 (1994)), hok, gef, kil, or phlA (Molin et al., Ann. Rev. Microbiol., 47:139-166 (1993)) mutations;

[0022] (viii) mutations that introduce suicide systems, such as lysogens encoded by P22 (Rennell et al., Virol., 143:280-289 (1985)), lambda murein

transglycosylase (Bienkowska-Szewczyk et al., Mol. Gen. Genet., 184:111-114 (1981)) or S-gene (Reader et al., Virol., 43:623-628 (1971)); and

[0023] (ix) mutations that disrupt or modify the correct cell cycle, such as minB (de Boer et al., Cell, 56:641-649 (1989)) mutation.

[0024] The attenuating mutations can be either constitutively expressed or under the control of inducible promoters, such as the temperature sensitive heat shock family of promoters (Neidhardt et al., 1984, supra), or the anaerobically induced nirB promoter (Harborne et al., Mol. Micro., 6:2805-2813 (1992)) or repressible promoters, such as uapA (Gorfinkiel et al., J. Biol. Chem., 268:23376-23381 (1993)) or gcv (Stauffer et al., J. Bact., 176:6159-6164 (1994)).

[0025] The particular naturally occurring invasive bacteria (or attenuated, invasive bacteria) employed in the present invention is not critical thereto. One of ordinary skill in the art can readily determine which bacterial strains are appropriate for use with the animal or animal cells intended to be infected based on the animal's or cells' susceptibility to infection by different bacterial species. Examples of such naturally-occurring invasive bacteria include, but are not limited to, Salmonella spp. Shigella spp., Listeria spp., Rickettsia spp. and enteroinvasive *Escherichia coli*. Any of these strains can be attenuated if needed using known methods.

[0026] Examples of Shigella strains which can be employed in the present invention include, but are not limited to, *Shigella flexneri* 2a (ATCC No. 29903), *Shigella sonnei* (ATCC No. 29930), and *Shigella disenteriae* (ATCC No. 13313). An attenuated Shigella strain, such as *Shigella flexneri* 2a 2457T Δ aroA Δ virG mutant CVD 1203 (Noriega et al., supra), *Shigella flexneri* M90T Δ icsA mutant (Goldberg et al., Infect. Immun., 62:5664-5668 (1994)), *Shigella flexneri* Y SFL114 aroD mutant (Karnell et al., Vacc., 10:167-174 (1992)), and *Shigella flexneri* Δ aroA Δ aroD mutant (Verma et al., Vacc., 9:6-9 (1991)) are preferably employed in the present invention. Alternatively, new attenuated Shigella spp. strains can be constructed by introducing an attenuating mutation either singularly or in conjunction with one or more additional attenuating mutations.

[0027] Examples of Listeria strains which can be employed in the present invention include *Listeria monocytogenes* (ATCC No. 15313). Attenuated Listeria strains, such as *L. monocytogenes* Δ actA mutant (Brundage et al., supra) or *L. monocytogenes* Δ plcA (Camilli et al., J. Exp. Med., 173:751-754 (1991)) are preferably used in the present invention. Alternatively, new attenuated Listeria strains can be constructed by introducing one or more attenuating mutations as described for Shigella spp. above.

[0028] Examples of Rickettsia strains which can be employed in the present invention include *Ricketsia rickettsiae* (ATCC Nos. VR149 and VR891), *Ricketsia prowaseckii* (ATCC No. VR233), *Ricketsia tsutsugamuchi* (ATCC Nos. VR312, VR150 and VR609), *Ricketsia mooseri* (ATCC No. VR144), *Ricketsia sibirica* (ATCC No. VR151), and *Rochalimaea quitana* (ATCC No. VR358). Attenuated Ricketsia strains are preferably used in the present invention and can be constructed by introducing one or more attenuating mutations as described for Shigella spp. above.

[0029] Examples of enteroinvasive Escherichia strains which can be employed in the present invention include *Escherichia coli* strains 4608-58, 1184-68, 53638-C-17, 13-80, and 6-81 (Sansonetti et aL, Ann. Microbiol. (Inst. Pasteur), 132A:351-355 (1982)). Attenuated enteroinvasive Escherichia strains are preferably used in the present invention and can be constructed by introducing one or more attenuating mutations as described for Shigella spp. above.

[0030] Examples of Salmonella strains which can be employed in the present invention include *Salmonella typhi* (ATCC No. 7251) and *S. typhimurium* (ATCC No. 13311). Attenuated Salmonella strains are preferably used in the present invention and include *S. typhi* aroAaroD (Hone et al., Vacc., 9:810-816 (1991)) and *S. typhimurium* aroA mutant (Mastroeni et al., Micro. Pathol., 13:477-491 (1992))). Alternatively, new attenuated Salmonella strains can be constructed by introducing one or more attenuating mutations as described for Shigella spp. above.

[0031] Examples of additional bacteria which can be genetically engineered to be invasive include, but are not limited to, Yersinia spp., Escherichia spp., Klebsiella spp., Bordetella spp., Neisseria spp., Aeromonas spp., Francisella spp., Corynebacterium spp., Citrobacter spp., Chlamydia spp., Hemophilus spp., Brucella spp., Mycobacterium spp., Legionella spp., Rhodococcus spp., Pseudomonas spp., Helicobacter spp., Salmonella spp., Vibrio spp., Bacillus spp., and Erysipelothrix spp. These organisms can be engineered to mimic the invasion properties of Shigella spp., Listeria spp., Rickettsia spp., or enteroinvasive *E. coli* spp. by inserting genes that enable them to access the cytoplasm of an animal cell. Examples of useful strains from these bacteria are found in U.S. Pat. No. 5,877,159 and also include *Mycobacterium bovis* BCG.

[0032] Examples of such genes include the invasive proteins of Shigella, hemolysin or the invasion plasmid of Escherichia, or listeriolysin O of Listeria, as such techniques are known to result in strains that are capable of entering the cytoplasm of infected animal cells (Formal etal., Infect. immun., 46:465 (1984); Bielecke etal., Nature, 345:175-176 (1990); Small et al., In: Microbiology-1986, pages 121-124, Levine et al., Eds., American Society for Microbiology, Washington, D.C. (1986); and Zychlinsky et al., Molec. Micro., 11:619-627 (1994)). Any gene or combination of genes, from one or more sources, that mediates entry into the cytoplasm of animal cells will suffice. Thus, such genes are not limited to bacterial genes, and include viral genes, such as influenza virus hemagglutinin HA-2 which promotes endosmolysis (Plank et al., J. Biol. Chem., 269:12918-12924 (1994)).

[0033] The above invasive genes can be introduced into the target strain using chromosome or plasmid mobilization (Miller, A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1992); Bothwell et al., supra; and Ausubel et al., supra), bacteriophage-mediated transduction (de Boer, supra; Miller, supra; and Ausubel et al., supra), or chemical (Bothwell et al., supra; Ausubel et al., supra; Felgner et al., supra; and Farhood, supra), electroporation (Bothwel et al., supra; Ausubel et al., supra; and Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) and physical transformation techniques (Johnston et al., supra; and Bothwell, supra). The genes can be incorporated on bacteriophage (de Boer et al.,

Cell, 56:641-649 (1989)), plasmids vectors (Curtiss et al., supra) or spliced into the chromosome (Hone et al., supra) of the target strain.

[0034] Furthermore, the bacteria for use in the invention can be modified to increase their ability to infect mucosal surfaces and tissues in an animal Such modifications permit the bacteria to circumvent natural host barriers. Methods for constructing such bacteria are described in U.S. Pat. No. 5,877,159.

[0035] In accordance with the invention the invasive or attenuated invasive bacteria contain a DNA comprising a eukaryotic expression cassette operably linked to an alphavirus replicon DNA. A eukaryotic expression cassette is usually in the form of a plasmid which contains elements needed for transcription of the alphavirus replicon DNA and transport from the nucleus into the cytoplasm. For example, RNA polymerase II cassettes provide the needed control and regulatory elements. Hence, the elements for transcription include but are not limited to promoters active in eukaryotic cells, enhancers, transcription termination signals including polyadenylation signals or polyA tracts, elements to facilitate nucleocytoplasmic transport, elements to facilitate processing of the 3' alphavirus replicon RNA into an authentic virus-like RNA 3' ends and the like.

[0036] Hence, the particular eukaryotic cassette employed in the present invention is not critical thereto, and can be selected from, e.g., any of the many commercially available cassettes, such as pCEP4 or pRc/RSV obtained from Invitrogen Corporation (San Diego, Calif.), pXT1, pSG5, pPbac or pMbac obtained from Stratagene (La Jolla, Calif.), pPUR or pMAM obtained from ClonTech (Palo Alto, Calif.), and pSV.beta.-gal obtained from Promega Corporation (Madison, Wis.), or synthesized either de novo or by adaptation of a publically or commercially available eukaryotic expression system.

[0037] The individual elements within the eukaryotic expression cassette can be derived from multiple sources and may be selected to confer specificity in sites of action or longevity of the cassettes in the recipient cell. Such manipulation of the eukaryotic expression cassette can be done by any standard molecular biology approach.

[0038] Various promoters well-known to be useful for driving expression of genes in animal cells, such as the viral-derived SV40, CMV immediate early and, RSV promoters or eukaryotic derived β-casein, uteroglobin, β-actin or tyrosinase promoters. The particular promoter is not critical to the invention, unless the object is to obtain tissue-specific expression. In this case, the promoter can be selected which is only active in the desired tissue or selected cell type. Examples of tissue-specific promoters include, but are not limited to, αS1- and β-casein promoters which are specific for mammary tissue (Platenburg et al., Trans. Res., 3:99-108 (1994); and Maga et al., Trans. Res., 3:36-42 (1994)); the phosphoenolpyruvate carboxykinase promoter which is active in liver, kidney, adipose, jejunum and mammary tissue (McGrane et al., J. Reprod. Fert., 41:17-23 (1990)); the tyrosinase promoter which is active in lung and spleen cells, but not testes, brain, heart, liver or kidney (Vile et al., Canc. Res., 54:6228-6234 (1994)); the involucerin promoter which is only active in differentiating keratinocytes of the squamous epithelia (Carroll et al., J. Cell Sci., 103:925-930 (1992)); and the uteroglobin promoter which is active in lung and endometrium (Helftenbein et al., Annal. N.Y. Acad. Sci., 622:69-79 (1991)).

[0039] Alternatively, cell specific enhancer sequences can be used to control expression, for example human neurotropic papovirus JCV enhancer regulates viral transcription in glial cells alone (Remenick et al., J. Virol., 65:5641-5646 (1991)). Yet another way to control tissue specific expression is to use a hormone responsive element (HRE) to specify which cell lineages a promoter will be active in, for example, the MMTV promoter requires the binding of a hormone receptor, such as progesterone receptor, to an upstream HRE before it is activated (Beato, FASEB J., 5:2044-2051 (1991); and Truss et al., J. Steroid Biochem. Mol. Biol., 41:241-248 (1992)).

[0040] Suitable transcription termination elements include the SV 40 transcription termination region and terminators derived therefrom.

[0041] Additional examples of eukaryotic expression cassettes and/or regulatory elements suitable for expressing alphavirus replicon DNA are described in U.S. Pat. Nos. 5,824,538 and 5,877,159.

[0042] The bacteria of the bacterial delivery systems can contain one or more eukaryotic expression cassettes operably linked to an alphavirus replicon. Such cassettes can be provided on the same or different plasmids or DNA molecules contained in the bacteria. For example, in some instances it may be desirable for the eukaryotic expression cassette to be integrated into the bacterial chromosome or other episomal DNA.

[0043] Alphavirus are from the Togavirus family and are well known in the art. There are 26 known viruses and virus subtype classified using the hemagglutination assay. See, e.g., U.S. Pat. No. 5,843,723 for list of the many of the alphaviruses. The commonly studied alphaviruses include Sindbis, SFV, Venezuelan equine encephalitis virus (VEE) and Ross River virus. The morphogenesis of the viruses is fairly uniform and the virions are small enveloped 60-65 nm particles of positive strand RNA. The genomic RNA (49S RNA) of alphaviruses is approximately 11-12 kb in length, and contains a 5' cap and a 3' polyadenylate tail. Infectious enveloped virus is produced by assembly of the viral nucleocapsid proteins onto genomic RNA in the cytoplasm, and budding through the cell membrane embedded with viral-encoded glycoproteins. During viral replication, the genomic 49S RNA serves as template for synthesis of a complementary negative strand. The negative strand in turn serves as template for full-length genomic RNA and for an internally initiated positive-strand 26S subgenomic RNA. The nonstructural proteins are translated from the genomic RNA. Alphaviral structural proteins are translated from the subgenomic 26S RNA. All viral genes are expressed as polyproteins and processed into individual proteins by proteolytic cleavage post-translation.

[0044] As used herein, an "alphavirus replicon" of the present invention is used interchangeably to refer to RNA or DNA comprising those portions of the alphavirus genome RNA essential for transcription and export of a primary RNA transcript from the cell nucleus to the cytoplasm, for cytoplasmic amplification of the transported RNA and for subgenomic RNA expression of a heterologous nucleic acid sequence. Thus, the replicon encodes and expresses those

non-structural proteins needed for cytoplasmic amplification of the alphavirus RNA and expression of the subgenomic RNA. It is further preferable that the alphavirus replicon can not be encapsidated to produce alphavirus particles or virions. This is achieved in replicons which lack one or more of the alphavirus structural genes, and preferably all of the structural genes. In a preferred embodiment, alphavirus replicons of the invention are capable of being transcribed from a eukaryotic expression cassette and processed into RNA molecules with authentic alphavirus-like 5' and 3' ends.

[0045] Alphavirus replicons and expression vectors containing them are well known in the art and many vectors containing a wide range of alphavirus replicons have been described. Examples of such replicons can be found, e.g., in U.S. Pat. Nos. 5,739,026; 5,766,602; 5,789,245; 5,792,462; 5,814,482; and 5,843,723 and in Polo, supra, and Berglund, supra. While many of the features of these alphavirus replicons are useful for the present invention not all of them are essential for the reasons set forth above. So long as a portion of the alphavirus replicon does not interfere with production of the primary RNA, cytoplasmic amplification thereof and expression of the heterologous nucleic acid sequence, such portions can remain as part of the replicon. Those skilled in the art can readily determine the nature of and remove any unnecessary or interfering sequences.

[0046] The patents and references set forth above also describe representative methods for constructing and producing the alphavirus replicons of the inventions. Alphavirus replicons can be prepared from any alphavirus or any mixture of alphavirus nucleic acid sequences. In this regard the preferred alphavirus replicons are derived from Sindbis virus, SFV, VEE or Ross River virus.

[0047] The alphavirus replicons can be incorporated as DNA into eukaryotic expression cassettes using recombinant DNA techniques conventional in the art.

[0048] In accordance with the invention, the alphavirus replicon comprises a nucleic acid sequence operably linked to a heterologous nucleic acid sequence to control expression thereof. The heterologous nucleic acid sequence can encode an antigen, an antigenic fragment of a protein, a therapeutic agent, an immunoregulatory agent, an anti-sense RNA, a catalytic RNA, a protein, a peptide or any other molecule encodable by DNA and desired for delivery to an animal or animal cell. The heterologous nucleic acid sequences can be obtained from a virus selected from the group consisting of influenza virus, respiratory syncytial virus, HPV, HBV, HCV, HIV, HSV, FeLV, FIV, HTLV-I, HTLV-II, and CMV. Such viral sequences can encode one or more viral genes or antigenic fragments thereof. The heterologous nucleotide sequence can also encode a cytokine, an interleukin, erythropoietin or other immunostimulatory or immunoregulatory protein.

[0049] As used herein, heterologous refers to the relationship between the source of the alphavirus replicon and the source of the heterologous nucleic acid sequence. Thus, the heterologous nucleic acid gene will not be encode an alphavirus gene but could encode a gene that is either foreign or endogenous to the animal cells that have been infected with the bacterial delivery system of the invention. As used herein, "foreign gene or nucleic acid sequence" means a gene or a nucleic acid sequence encoding a protein or fragment thereof or anti-sense RNA or catalytic RNA, which is foreign to the recipient animal cell or tissue, such as a vaccine antigen, immunoregulatory agent, or therapeutic agent. An "endogenous gene or nucleic acid sequence" means a gene or a nucleic acid sequence encoding a protein or part thereof or anti-sense RNA or catalytic RNA which is naturally present in the recipient animal cell or tissue.

[0050] The antigen may be a protein or antigenic fragment thereof from viral pathogens, bacterial pathogens, and parasitic pathogens. Alternatively, the antigen may be a synthetic gene, constructed using recombinant DNA methods, which encode antigens or parts thereof from viral, bacterial, parasitic pathogens. These pathogens can be infectious in humans, domestic animals or wild animal hosts.

[0051] The antigen can be any molecule that is expressed by any viral, bacterial, parasitic pathogen prior to or during entry into, colonization of, or replication in their animal host.

[0052] Single or multiple eukaryotic expression cassettes can be delivered that express any combination of viral, bacterial, parasitic antigens, or synthetic genes encoding all or parts or any combination of viral, bacterial, parasitic antigens.

[0053] The viral pathogens, from which the viral antigens are derived, include, but are not limited to, Orthomyxoviruses, such as influenza virus; Retroviruses, such as RSV and SIV, Herpesviruses, such as EBV; CMV or *herpes simplex* virus; Lentiviruses, such as human immunodeficiency virus; Rhabdoviruses, such as rabies; Picornoviruses, such as poliovirus; Poxviruses, such as vaccinia; Rotavirus; and Parvoviruses. Examples of protective antigens of viral pathogens include the HIV antigens Nef, p24, gp 120, gp41, gp 160, Tat, Rev, and Pol et al., Nature, 313:277-280 (1985)) and T cell and B cell epitopes of gp120 (Palker et al., J. Immunol., 142:3612-3619 (1989)); the *hepatitis B* surface antigen (Wu et al., Proc. Natl. Acad. Sci., USA, 86:4726-4730 (1989)); rotavirus antigens, such as VP4 (Mackow et al., Proc. Natl. Acad. Sci., USA, 87:518-522 (1990)) and VP7 (Green et al., J. Virol., 62:1819-1823 (1988)), influenza virus antigens such as hemagglutinin or nucleoprotein (Robinson et al.., Supra; Webster et al., Supra) and *herpes simplex* virus thymidine kinase (Whitley et al., In: New Generation Vaccines, pages 825-854). In the case of HIV the antigens can be from any structural, accessory or regulatory gene, and includes combinations or chimeras of such genes in multiple or single alphavirus replicons.

[0054] The bacterial pathogens, from which the bacterial antigens are derived, include but are not limited to, Mycobacterium spp., *Helicobacter pylori,* Salmonella spp., Shigella spp., *E. coli,* Rickettsia spp., Listeria spp., *Legionella pneumoniae,* Pseudomonas spp., Vibrio spp., and *Borellia burgdorferi.*

[0055] Examples of protective antigens of bacterial pathogens include the *Shigella sonnei* form 1 antigen (Formal et al., Infect. Immun., 34:746-750 (1981)); the O-antigen of *V. cholerae* Inaba strain 569B (Forrest et al., J. Infect. Dis., 159:145-146 (1989); protective antigens of enterotoxigenic *E. coli,* such as the CFA/I fimbrial antigen (Yamamoto et al., Infect. Immun., 50:925-928 (1985)) and the nontoxic B-subunit of the heat-labile toxin (Clements et al., 46:564-569 (1984)); pertactin of *Bordetella pertussis* (Roberts et al.,

Vacc., 10:43-48 (1992)), adenylate cyclase-hemolysin of *B. pertussis* (Guiso et al., Micro. Path., 11:423-431 (1991)), and fragment C of tetanus toxin of *Clostridium tetani* (Fairweather et al., Infect. Immun., 58:1323-1326 (1990)).

[0056] The parasitic pathogens, from which the parasitic antigens are derived, include but are not limited to, Plasmodium spp., Trypanosome spp., Giardia spp., Boophilus spp., Babesia spp., Entamoeba spp., Eimeria spp., Leishmania spp., Schistosome spp., Brugia spp., Fascida spp., Dirofilaria spp., Wuchereria spp., and Onchocerea spp.

[0057] Examples of protective antigens of parasitic pathogens include the circumsporozoite antigens of Plasmodium spp. (Sadoff et al., Science, 240:336-337 (1988)), such as the circumsporozoite antigen of *P. bergerii* or the circumsporozoite antigen of *P. falciparum;* the merozoite surface antigen of Plasmodium spp. (Spetzler et al., Int. J. Pept. Prot. Res., 43:351-358 (1994)); the galactose specific lectin of *Entamoeba histolytica* (Mann et al., Proc. Natl. Acad. Sci., USA, 88:3248-3252 (1991)), gp63 of Leishmania spp. (Russell et al., J. Immunol., 140:1274-1278 (1988)), paramyosin of *Brugia malayi* (Li et al., Mol. Biochem. Parasitol., 49:315-323 (1991)), the triose-phosphate isomerase of *Schistosoma mansoni* (Shoemaker et al., Proc. Natl. Acad. Sci., USA, 89:1842-1846 (1992)); the secreted globin-like protein of *Trichostrongylus colubriformis* (Frenkel et al., Mol. Biochem. Parasitol., 50:27-36 (1992)); the glutathione-S-transferase's of *Frasciola hepatica* (Hillyer et al., Exp. Parasitol., 75:176-186 (1992)), *Schistosoma bovis* and *S. japonicum* (Bashir et al., Trop. Geog. Med., 46:255-258 (1994)); and KLH of *Schistosoma bovis* and *S. japonicum* (Bashir et al., supra).

[0058] In the present invention, the live invasive bacteria can also deliver eukaryotic expression cassettes encoding a therapeutic agent to animal cells or animal tissue. For example, the eukaryotic expression cassettes can encode tumor-specific, transplant, or autoimmune antigens or parts thereof. Alternatively, the eukaryotic expression cassettes can encode synthetic genes, which encode tumor-specific, transplant, or autoimmune antigens or parts thereof. Examples of tumor specific antigens include prostate specific antigen (Gattuso et al., Human Pathol., 26:123-126 (1995)), TAG-72 and CEA (Guadagni et al., Int. J. Biol. Markers, 9:53-60 (1994)), MAGE-1 and tyrosinase (Coulie et al., J. Immunothera., 14:104-109 (1993)). Recently it has been shown in mice that immunization with non-malignant cells expressing a tumor antigen provides a vaccine effect, and also helps the animal mount an immune response to clear malignant tumor cells displaying the same antigen (Koeppen et al., Anal. N.Y. Acad. Sci., 690:244-255 (1993)). Examples of transplant antigens include the CD3 receptor on T cells (Alegre et al., Digest. Dis. Sci., 40:58-64 (1995)). Treatment with an antibody to CD3 receptor has been shown to rapidly clear circulating T cells and reverse most rejection episodes (Alegre et al., supra). Examples of autoimmune antigens include IAS .beta. chain (Topham et al., Proc. Natl. Acad. Sci., USA, 91:8005-8009 (1994)). Vaccination of mice with an 18 amino acid peptide from IAS beta. chain has been demonstrated to provide protection and treatment to mice with experimental autoimmune encephalomyelitis (Topham et al., supra).

[0059] Alternatively, in the present invention, live invasive bacteria can deliver eukaryotic expression cassettes encoding immunoregulatory molecules. These immunoregulatory molecules include, but are not limited to, growth factors, such as M-CSF, GM-CSF; and cytokines, such as IL-2, IL-4, L-5, IL-6, IL-10, IL-12 or IFN-gamma. Recently, delivery of cytokines expression cassettes to tumor tissue has been shown to stimulate potent systemic immunity and enhanced tumor antigen presentation without producing a systemic cytokine toxicity (Golumbek et al., Canc. Res., 53:5841-5844 (1993); Golumbek et al., Immun. Res., 12:183-192 (1993); Pardoll, Curr. Opin. Oncol., 4:1124-1129 (1992); and Pardoll, Curr. Opin. Immunol., 4:619-623 (1992)).

[0060] The antisense RNA and catalytic RNA species delivered to animal cells can be targeted against any molecule present within the recipient cell or likely to be present within the recipient cell. These include but are not limited to RNA species encoding cell regulatory molecules, such as interleukin-6 (Mahieu et al., Blood, 84:3758-3765 (1994)), oncogenes such as ras (Kashani-Sabet et al., Antisen. Res. Devel., 2:3-15 (1992)), causative agents of cancer such as human papillomavirus (Steele et al., Canc. Res., 52:4706-4711 (1992)), enzymes, viral RNA's and pathogen derived RNA's such as HIV-1 (Meyer et al., Gene, 129:263-268 (1993); Chatterjee et al., Sci., 258:1485-1488 (1992); and Yamada et al., Virol., 205:121-126 (1994)). The RNAs can also be targeted at non-transcribed DNA sequences, such as promoter or enhancer regions, or to any other molecule present in the recipient cells, such as but not limited to, enzymes involved in DNA synthesis or tRNA molecules (Scanlon et al., Proc. Natl. Acad. Sci. USA, 88:10591-10595 (1991); and Baier et al., Mol. Immunol., 31:923-932 (1994)).

[0061] In the present invention, live invasive bacteria can also deliver eukaryotic expression cassettes encoding proteins to animal tissue from which they can later be harvested or purified. An example is the delivery of a eukaryotic expression cassette under the control of a mammary specific viral promoter, such as derived from mouse mammary tumor virus (ATCC No. VR731), encoding α-antitrypsin to mammary tissue of a goat or sheep.

[0062] Alternatively an invasive bacteria carrying a eukaryotic expression cassette can be introduced to a tissue site such that it would not spread from such a site. This could be accomplished by any of several methods including delivery of a very limited dose, delivery of a severely attenuated auxotrophic strain, such as an asd mutant (Curtiss et al., supra) that will be rapidly inactivated or die, or delivery of a bacterial strain that contains attenuating lesions, such as a suicide systems (Rennell et al., supra; and Reader et al., supra) under the control of a strong promoter, such as the anaerobic nirB promoter (Harborne et al., supra) which will be switched on within the recipient host tissue. Additionally, through use of different species and/or serotypes multiple doses of invasive bacteria, the eukaryotic expression cassette of interest can be given to an animal so as to manipulate expression levels or product type. This approach obviates the need for specially raised transgenic animals containing tissue specific promoters and having tight control of expression, as is currently the case (Janne et al., Int. J. Biochem., 26:859-870 (1994); Mullins et al., Hyperten., 22:630-633 (1993); and Persuy et al., Eur. J. Bichem., 205:887-893 (1992)).

[0063] As a further alternative, single or multiple eukaryotic expression cassettes encoding tumor-specific, transplant, and/or autoimmune antigens, can be delivered in any single or multiple combination with eukaryotic expression cassettes encoding immunoregulatory molecules or other proteins.

[0064] The invasive bacteria containing the eukaryotic expression cassette can be used to infect animal cells that are cultured in vitro. The animal cells can be further cultured in vitro, and the cells carrying the desired genetic trait can be enriched by selection for or against any selectable marker introduced to the recipient cell at the time of bactofection. Such markers may include antibiotic resistance genes, e.g., hygromycin, or neomycin, selectable cell surface markers, or any other phenotypic or genotypic element introduced or altered by bactofection. These in vitro-infected cells or the in vitro-enriched cells can then be introduced into animals intravenously, intramuscularly, intradermally, or intraperitoneally, or by any inoculation route that allows the cells to enter the host tissue.

[0065] Alternatively, the invasive (or invasive, attenuated) bacteria containing the eukaryotic expression cassettes can be introduced to infect the animal by intravenous, intramuscular, intradermal, intraperitoneally, peroral, intranasal, intraocular, intrarectal, intravaginal, oral, immersion and intraurethral inoculation routes.

[0066] The amount of the live invasive (or invasive, attenuated) bacteria of the present invention to be administered will vary depending on the species of the subject, as well as the disease or condition that is being treated. Generally, the dosage employed will be about $10^3$ to $10^{11}$ viable organisms, preferably about $10^5$ to $10^9$ viable organisms. Alternatively, when bactofecting individual cells, the dosage of viable organisms to administered will be at a multiplicity of infection ranging from about 0.1 to $10^6$, preferably about $10^2$ to $10^4$.

[0067] The invasive bacteria of the present invention are generally administered along with a pharmaceutically acceptable carrier or diluent.

[0068] The particular pharmaceutically acceptable carrier or diluent employed is not critical to the present invention. Examples of diluents include a phosphate buffered saline, buffer for buffering against gastric acid in the stomach, such as citrate buffer (pH 7.0) containing sucrose, bicarbonate buffer (pH 7.0) alone (Levine et al., J. Clin. Invest., 79:888-902 (1987); and Black et al. J. Infect. Dis., 155:1260-1265 (1987)), or bicarbonate buffer (pH 7.0) containing ascorbic acid, lactose, and optionally aspartame (Levine et al., Lancet, II:467-470 (1988)). Examples of carriers include proteins, e.g., as found in skim milk, sugars, e.g., sucrose, or polyvinylpyrrolidone. Typically these carriers would be used at a concentration of about 0.1-90% (w/v) but preferably at a range of 1-10% (w/v).

[0069] When infecting animal cells, the methods of the invention can be used in mammalian, avian, insect cells and the like. Preferably the mammalian cells are selected from the group consisting of human, bovine, ovine, porcine, feline, buffalo, canine, goat, equine, donkey, deer, and primate cells.

[0070] When infecting animals, the methods of the invention are preferably used in mammals and birds. The preferred mammal is a human.

What is claimed is:

**1**. A bacterial delivery system which comprises live, attenuated invasive Salmonella bacteria containing a DNA comprising a eukaryotic expression cassette operably linked to an alphavirus replicon DNA capable of amplification as RNA in animal cells, wherein the alphavirus replicon DNA comprises at least one nucleic acid control sequence operably linked to a heterologous nucleic acid sequence to control expression of said heterologous genes.

**2**. The bacterial delivery system of claim 1, wherein said Salmonella is *Salmonella typhi*.

**3**. The bacterial delivery system of claim 2, wherein said bacteria are attenuated via an attenuating mutation in an aro gene, an asd gene, an htrA gene or in a combination of one or more of these genes.

**4**. The bacterial delivery system of claim 3, wherein said bacteria have an attenuating mutation in an aro gene and an asd gene.

**5**. The bacterial delivery system of claim 4, wherein said aro gene is aroA and/or aroD.

**6**. The bacterial delivery system of claim 3, wherein said bacteria have an attenuating mutation in an aro gene and an htrA gene.

**7**. The bacterial delivery system of claim 6, wherein said aro gene is aroA and/or aroD.

**8**. The bacterial delivery system of claim 1, wherein said heterologous nucleic acid sequence comprises one or more coding regions of a gene and wherein each coding region of said heterologous nucleic acid sequence can be expressed separately or as an operon.

**9**. The bacterial delivery system of claim 8, wherein said heterologous nucleic acid sequence encodes an antigen or an antigenic fragment of a protein from a viral pathogen.

**10**. The bacterial delivery system of claim 9, wherein said viral pathogen is HIV.

**11**. The bacterial delivery system of any one of claims **1-8**, wherein said heterologous nucleic acid sequence encodes one or more HIV genes selected from the group consisting of env, gag, pol, or an antigenic fragment of a protein encoded by any one of said genes, wherein said genes are from an HIV isolate and/or from a consensus sequence of HIV isolates.

**12**. The bacterial delivery system of claim 11, wherein said heterologous nucleic acid sequence encodes at least one antigen or antigenic fragment from each of the HIV genes env, gag, pol, nef, tat, and rev.

**13**. A method for introducing and expressing a gene in an animal which comprises infecting said animal with the bacterial delivery system of claim 11, and thereby obtaining expression of a gene product encoded by said heterologous nucleic acid sequence in said animal.

**14**. The method of claim 13 wherein infecting occurs by an intranasal delivery route.

**15**. A method for inducing an immune response in an animal which comprises infecting said animal with live, attenuated invasive Salmonella bacteria containing a DNA comprising a eukaryotic expression cassette operably linked to an alphavirus replicon DNA capable of amplification as RNA in animal cells, wherein the alphavirus replicon DNA encodes at least one antigen or antigenic fragment of a protein, and wherein said antigen or said fragment is expressed at a level sufficient to stimulate an immune response to said antigen or said fragment.

**16**. The method of claim 15, wherein said Salmonella is *Salmonella typhi.*

**17**. The method of claim 16, wherein said bacteria are attenuated via an attenuating mutation in an aro gene, an asd gene, an htrA gene or in a combination of one or more of these genes.

**18**. The method of claim 17, wherein said bacteria have an attenuating mutation in an aro gene and an asd gene.

**19**. The method of claim 18, wherein said aro gene is aroA and/or aroD.

**20**. The method of claim 17, wherein said bacteria have an attenuating mutation in an aro gene and an htrA gene.

**21**. The method of claim 20, wherein said aro gene is aroA and/or aroD.

**22**. The method of claim 15, wherein said antigen or said antigenic fragment is a tumor antigen, a transplantation antigen or an autoimmune antigen.

**23**. The method of any one of claims **15-21**, wherein said antigen or said antigenic fragment of a protein is from a viral pathogen, a bacterial pathogen or a parasitic pathogen.

**24**. The method of claim 23, wherein said viral pathogen is HIV.

**25**. The method of any one of claims **15-21**, wherein said antigen or antigenic fragment is encoded by one or more HIV genes selected from the group consisting of env, gag, pol, nef, tat, or rev, wherein said HIV genes are from an HIV isolate and/or from a consensus sequence of HIV isolates.

**26**. The method of claim 25, wherein said alphavirus replicon DNA encodes at least one antigen or antigenic fragment from each of the HIV genes env, gag, pol, nef, tat, and rev.

**27**. The method of any one of claims **15-21**, wherein infecting occurs by an intranasal delivery route.

**28**. The method of claim 25, wherein infecting occurs by an intranasal delivery route.

**29**. The method of claim 26, wherein infecting occurs by an intranasal delivery route.

**30**. A bacterial delivery system which comprises live, attenuated invasive Shigella bacteria containing a DNA comprising a eukaryotic expression cassette operably linked to an alphavirus replicon DNA capable of amplification as RNA in animal cells, wherein the alphavirus replicon DNA comprises at least one nucleic acid control sequence operably linked to a heterologous nucleic acid sequence to control expression of said heterologous genes.

**31**. The bacterial delivery system of claim 30, wherein said Shigella is *Shigella flexneri* or *Shigella flexneri* 2a

**32**. The bacterial delivery system of claim 31, wherein said bacteria are attenuated via an attenuating mutation in an aro gene, a gua gene, a virG gene or in a combination of one or more of these genes.

**33**. The bacterial delivery system of claim 32, wherein said bacteria have an attenuating mutation in an aro gene and a virg gene.

**34**. The bacterial delivery system of claim 33, wherein said aro gene is aroA and/or aroD.

**35**. The bacterial delivery system of claim 32, wherein said bacteria have an attenuating mutation in a gua gene and a virG gene.

**36**. The bacterial delivery system of claim 35, wherein said aro gene is aroA and/or aroD.

**37**. The bacterial delivery system of claim 30, wherein said heterologous nucleic acid sequence comprises one or more coding regions of a gene and wherein each coding region of said heterologous nucleic acid sequence can be expressed separately or as an operon.

**38**. The bacterial delivery system of claim 37, wherein said heterologous nucleic acid sequence encodes an antigen or an antigenic fragment of a protein from a viral pathogen, a bacterial pathogen or a parasitic pathogen.

**39**. The bacterial delivery system of claim 38, wherein said viral pathogen is HIV.

**40**. The bacterial delivery system of any one of claims **30-37**, wherein said heterologous nucleic acid sequence encodes one or more HIV genes selected from the group consisting of env, gag, pol, or an antigenic fragment of a protein encoded by any one of said genes, wherein said genes are from an HIV isolate AND/or from a consensus sequence of HIV isolates.

**41**. The bacterial delivery system of claim 40, wherein said heterologous nucleic acid sequence encodes at least one antigen or antigenic fragment from each of the HIV genes env, gag, pol, nef, tat, and rev.

**42**. A method for introducing and expressing a gene in an animal which comprises infecting said animal with the bacterial delivery system of claim 40, and thereby obtaining expression of a gene product encoded by said heterologous nucleic acid sequence in said animal.

**43**. The method of claim 42, wherein infecting occurs by an intranasal delivery route.

**44**. A method for inducing an immune response in an animal which comprises infecting said animal with live, attenuated invasive Shigella bacteria containing a DNA comprising a eukaryotic expression cassette operably linked to an alphavirus replicon DNA capable of amplification as RNA in animal cells, wherein the alphavirus replicon DNA encodes at least one antigen or antigenic fragment of a protein, and wherein said antigen or said fragment is expressed at a level sufficient to stimulate an immune response to said antigen or said fragment.

**45**. The method of claim 44, wherein said Shigella is *Shigella flexneri* or *Shigella flexneri* 2a.

**46**. The method of claim 45, wherein said bacteria are attenuated via an attenuating mutation in an aro gene, a gua gene, a virG gene or in a combination of one or more of these genes.

**47**. The method of claim 46, wherein said bacteria have an attenuating mutation in an aro gene and a virG gene.

**48**. The method of claim 47, wherein said aro gene is aroA and/or aroD.

**49**. The method of claim 48, wherein said bacteria have an attenuating mutation in a gua gene and a virG gene.

**50**. The method of claim 49, wherein said aro gene is aroA and/or aroD.

**51**. The method of claim 44, wherein said antigen or said antigenic fragment is a tumor antigen, a transplantation antigen or an autoimmune antigen.

**52**. The method of any one of claims **44-50**, wherein said antigen or said antigenic fragment of a protein is from a viral pathogen, a bacterial pathogen or a parasitic pathogen.

**53**. The method of claim 52, wherein said viral pathogen is HIV.

**54**. The method of claim of any one of claims **44-50**, wherein said antigen or antigenic fragment is encoded by one or more HIV genes selected from the group consisting of env, gag, pol, nef, tat, or rev, wherein said HIV genes are from an HIV isolate and/or from a consensus sequence of HIV isolates.

**55**. The method of claim 54, wherein said alphavirus replicon DNA encodes at least one antigen or antigenic fragment from each of the HIV genes env, gag, pol, nef, tat, and rev.

**56**. The method of any one of claims **44-50**, wherein infecting occurs by an intranasal delivery route.

**57**. The method of claim 54, wherein infecting occurs by an intranasal delivery route.

**58**. The method of claim 55, wherein infecting occurs by an intranasal delivery route.

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