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(54) Title: GENERAL SECRETORY PATHWAY (GSP) MUTANT LISTERIA SPP., AND METHODS FOR MAKING AND USING THE SAME

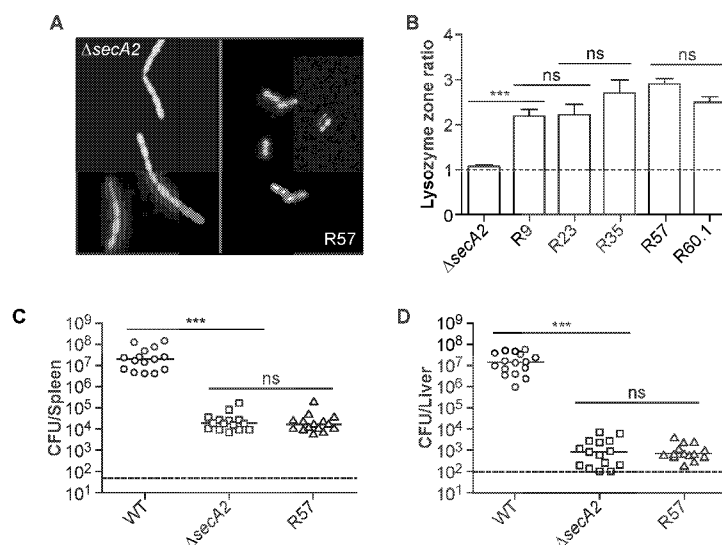


FIG. 3

(57) Abstract: General secretory pathway (GSP) mutant Listeria bacteria are provided. Aspects of the bacteria include the presence of a GSP mutation, e.g., a SecY and/or SecA mutation. Also provided are methods of making and using the Listeria bacteria comprising a GSP mutation as vectors and vaccines expressing a heterologous nucleic acid.

GENERAL SECRETORY PATHWAY (GSP) MUTANT *LISTERIA SPP.*, AND
METHODS FOR MAKING AND USING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

5 Pursuant to 35 U.S.C. § 119 (e), this application claims priority to the filing date of United States Provisional Patent Application No. 62/075,756, filed November 5, 2014; the disclosure of which application is herein incorporated by reference.

GOVERNMENT RIGHTS

10 This invention was made with government support under contracts AI63302 and AI27655 awarded by the National Institutes of Health. The government has certain rights in the invention.

INTRODUCTION

15 The use of vaccines is a cost-effective medical tool for the management of infectious diseases, including infectious diseases caused by bacteria, viruses, parasites, and fungi. In addition to effecting protection against infectious diseases, effort is also being expended to develop vaccines that stimulate the host's immune system to intervene in tumor growth.

20 Host immune responses include both the humoral immune response involving antibody production and the cell-mediated immune response. Protective immunization via vaccine has usually been designed to induce the formation of humoral antibodies directed against infectious agents, tumor cells, or the action of toxins. However, the control of certain diseases characterized by the presence of tumor cells or by chronic
25 infection of cells with infectious agents, often requires a cell-mediated immune response either in place of, or in addition to the generation of antibody. While the humoral immune response may be induced using live infectious agents and agents that have been inactivated, a cellular immune response is most effectively induced through the use of live agents as vaccines. Such live agents include live infectious agents which may gain
30 access to the cytoplasm of host cells where the proteins encoded by these agents are processed into epitopes which when presented to the cellular immune system, induce a protective response.

Microorganisms, particularly *Salmonella* and *Shigella*, which have been attenuated using a variety of mechanisms have been examined for their ability to encode
35 and express heterologous antigens. Such bacteria may be useful as live attenuated

bacterial vaccines which serve to induce a cellular immune response directed against a desired heterologous antigen.

Listeria monocytogenes is a Gram-positive, food-borne human and animal pathogen responsible for serious infections in immunocompromised individuals and pregnant women. Severe *L. monocytogenes* infections in humans are characterized by meningitis, meningoencephalitis, septicemia, and fetal death. *L. monocytogenes* is ubiquitous in nature and, in addition, can be isolated from a wide variety of warm-blooded animals.

L. monocytogenes elicits a predominantly cellular immune response when inoculated into an animal. As such, *L. monocytogenes* has been used widely as an experimental model to study many aspects of infection and immunity. Importantly, infection of mice with sublethal doses of *L. monocytogenes* results in the induction of long-lived cell-mediated immunity (CMI). In preclinical studies, attenuated strains of *L. monocytogenes* have shown tremendous potential as recombinant vaccine vectors. More importantly, attenuated recombinant strains have shown clinical efficacy as therapeutic vaccines for cancer immunotherapy. In principle, many different antigens can be expressed and secreted, but in practice, many antigens have proven difficult to express or secrete or expression/secretion has had detrimental impact on the vaccine strain.

SUMMARY

General secretory pathway (GSP) mutant *Listeria* bacteria are provided. Aspects of the bacteria include the presence of a general secretory pathway (GSP) mutation, e.g., a SecY and/or SecA mutant. Also provided are methods of making and using the bacteria, e.g., as vectors and vaccines.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Swarming motility of suppressor mutants. Motility assessed on semi-solid LB agar following incubation at 30°C after 48hr. Motility is expressed as a percentage of swarming area of the WT strain seeded on the same plate. Error bars represent standard deviations of the mean zone ratio between three independent experiments. Images show motility zones for the suppressor mutants indicated.

Figure 2: Plaque size of secA2 suppressor mutants. L2 fibroblasts were infected with secA2 suppressor mutants and mean plaque size was measured 3 days post infection. Plaque area of each mutant is shown as a percentage compared to WT strain. Error bars represent standard deviations of the mean zone ratio between three independent experiments. Images show plaques for the suppressor mutants indicated.

Figures 3A-3D: Characterization of the R57 revertant. Figure 3A) Fluorescent microscopy of $\Delta secA2$ and R57. Mid-exponential phase cells were stained with SYTO9 green. Figure 3B). Disk diffusion susceptibility to 1mg of lysozyme expressed as ratio of WT, where a ratio >1 indicates increased susceptibility to lysozyme. Error bars represent standard deviations of the mean zone ratio. A Student's t- test was used to analyze statistical significance where ***, $P < 0.0001$ and ns- $P \geq 0.05$. Bacterial burdens in Figure 3C) spleens and Figure 3D) livers 48hr post infection with 1×10^5 CFU in CD1 mice. The dashed-line represents the limit of detection. Results show CFU from three independent experiments. Statistical significance was evaluated using a Mann Whitney test. *** $P < 0.0001$, ns - $P \geq 0.05$.

Figures 4A-4D: Characterization of *lmo2769* mutant in a $\Delta secA2$ background. Figure 4A) Swarming motility of mutants and strains complemented with the *lmo2767* operon was assessed on semi-solid LB agar following incubation at 30°C after 48hr. Motility is expressed as a percentage of the swarming area of the WT strain and set at 100% (represented by a red dotted line). Error bars represent standard deviations of the mean zone ratio between three independent experiments. Figure 4B) Disk diffusion susceptibility to 1mg of lysozyme expressed as a ratio of WT. Error bars represent standard deviations of the mean zone ratio. Figure 4C) and Figure 4D) Plaque area of each mutant is shown as a percentage when compared to the WT strain set at 100% (represented by a red dotted line). Error bars represent standard deviations of the mean plaque size ratio between three independent experiments. A Student's t- test was used to analyze statistical significance where ***, $P < 0.0001$; *, $P < 0.05$ and ns- $P \geq 0.05$.

Figures 5A-5F: Characterization of the *prlA1* mutation. Figure 5A) Swarming motility of mutants and strains complemented with *lmo2767* operon was assessed on semi-solid LB agar following incubation at 30°C after 48hr. Motility is expressed as a percentage of swarming area of the WT strain and set at 100% (represented by a red dotted line). Error bars represent standard deviations of the mean zone ratio between three independent experiments. Figure 5B) Plaque area of each mutant is shown as a percentage when compared to the WT strain set at 100% (represented by a red dotted line). Error bars represent standard deviations of the mean plaque size ratio between three independent experiments. A Student's t- test was used to analyze statistical significance where ***, $P < 0.0001$; *, $P < 0.05$ and ns- $P \geq 0.05$. Figures 5C & 5D) Secreted levels of P60 in supernatants from mid-log cultured cells, quantified by western blotting with inserted images of gels. Bacterial burdens in Figure 5E) spleens and Figure 5F) livers 48hr post IV infection of 1×10^5 CFU in mice, showing data from three independent experiments. The dashed-line represents the limit of detection. Statistical

significance was evaluated using a Mann Whitney test. *** $P < 0.0001$, * $P < 0.05$, ns - $P \geq 0.05$.

Figures 6A-6F: Figures 6A and 6D) Disk diffusion susceptibility to 10 μg of vancomycin, Figures 6B and 6E) 120 μg of penicillin and Figures 6C and 6F) 1mg of lysozyme expressed as ratio of WT, where a ratio >1 indicates increased susceptibility. Error bars represent standard deviations of the mean zone ratio of WT. Student's t- test was used to analyze statistical significance where ***, $P < 0.0001$ and ns- $P \geq 0.05$.

Figures 7A-7F: In vivo virulence of assorted mutants. Bacterial burdens in spleens and livers 48hr post infection with 1×10^5 CFU in CD1 mice. The dashed-line represents the limit of detection. Statistical significance was evaluated using Mann Whitney test. *** $P < 0.0001$, ns - $P \geq 0.05$.

Figures 8A-8E: Swarming motility of *Imo1721* Figure 8A) and *Imo2637::Tn* Figure 8B) mutants was assessed on semi-solid LB agar following incubation at 30°C after 48hr. Motility is expressed as a percentage of swarming area of the WT strain. Error bars represent standard deviations of the mean zone ratio between three independent experiments. Figure 8C) Plaque area of each mutant is shown as a percentage when compared to the WT strain. Error bars represent standard deviations from the mean between three independent experiments. Student's t- test was used to analyze statistical significance where ***, $P < 0.0001$; *, $P < 0.05$ and ns- $P \geq 0.05$. Figure 8D) An image of a western blot showing P60 in supernatants from mid-log (5h) cultured cells in LB broth. Figure 8E) Secreted levels of P60 in supernatants from mid-log (5h) cultured cells in LB broth expressed as a percentage of protein secreted by the WT strain.

DETAILED DESCRIPTION

General secretory pathway (GSP) mutant *Listeria* bacteria are provided. Aspects of the bacteria include the presence of a general secretory pathway (GSP) mutation, e.g., a SecY and/or SecA mutant. Also provided are methods of making and using the bacteria, e.g., as vectors and vaccines.

Before the present invention is described in greater detail, it is to be understood that aspects of the present disclosure are not limited to the particular embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of embodiments of the present disclosure will be defined only by the appended claims.

Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise,

between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within embodiments of the present disclosure. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges and are also encompassed within embodiments of the present disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in embodiments of the present disclosure.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of embodiments of the present disclosure, representative illustrative methods and materials are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that embodiments of the present disclosure are not entitled to antedate such publication by virtue of prior invention.

Further, the dates of publication provided may be different from the actual publication dates which may need to be independently confirmed.

It is noted that, as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural referents unless the context clearly dictates otherwise. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present invention. Any recited method can be carried out in the order of events recited or in any other order which is logically possible.

In further describing the subject invention, the subject bacteria are reviewed first in greater detail, followed by a review of representative applications in which the subject bacteria and methods find use.

GENERAL SECRETORY PATHWAY (GSP) MUTANT *LISTERIA* BACTERIA

As summarized above, aspects of the invention include general secretory pathway (GSP) mutant *Listeria* bacteria. The subject bacteria may be any *Listeria* species that includes a GSP mutation, e.g., as described below. *Listeria* spp of interest include, but are not limited to: *L. fleischmannii*, *L. grayi*, *L. innocua*, *L. ivanovii*, *L. marthii*, *L. monocytogenes*, *L. rocourtiae*, *L. seeligeri*, *L. weihenstephanensis*, and *L. welshimeri*. Thus, strains of *Listeria* other than *L. monocytogenes* may be used for the generation of bacteria according to the present invention. In certain embodiments, the *Listeria* strain is *L. monocytogenes*.

As the *Listeria* bacteria include a GSP mutation, they are general secretory pathway (GSP) mutants. As such, the bacteria include a mutant member(s) of the general secretory pathway (GSP) (i.e., Secretion system or Sec). Members of the GSP include various GSP pathway proteins, including: transmembrane components, e.g., SecY, SecE, SecG, SecDF, YajC, YidC; Cytoplasmic components, e.g., FtsY and Ffh; ATPases, e.g., SecA, SecA2; Signal peptidases I; e.g., SipX, SipY, SipZ; and Signal peptidases II, e.g., Lsp and LspB. Bacteria according to embodiments of the invention may include one or more mutant GSP proteins, such as two or more, three or more, including four or more GSP mutant proteins, where in some instances the number of mutant GSP proteins is five or less, such as four or less.

In some instances, the GSP mutation confers enhanced protein secretion onto the bacteria (as compared to a suitable control). The enhancement may be manifested in one or more ways. For example, the GSP mutation may increase the amount of a given protein that is secreted by the bacteria, where the magnitude of increase may vary, and in some instances may be 2- fold or greater, such as 5- fold or greater. For example, in some instances the GSP mutation, such as a SecY mutation, provides for an increase in the secretion of p60, where the magnitude of increase may vary, and in some instances may be 2- fold or greater, such as 5- fold or greater. In some instances, the GSP mutation may increase the diversity of proteins that are secreted by the bacteria, where the magnitude of increase may vary, and in some instances may be 2- fold or greater, such as 5- fold or greater. In some instances, the GSP mutation may decrease the toxicity associated with expression/secretion of a given protein that is secreted by the bacteria, where the magnitude of decrease may vary, and in some instances may be 2- fold or greater, such as 5- fold or greater.

As indicated above, the GSP mutation may include one or more mutant GSP proteins. In some instances, the mutant GSP protein is a transmembrane component,

e.g., SecY, SecE, SecG, SecDF, YajC, YidC. In some embodiments, the mutant GSP protein is SecY. Of interest are SecY mutants that result in a conformational change of a translocation pore configuration, e.g., a change in the Sec translocon configuration, which translocon is made up of SecY, SecE and SecG. Conformational changes of interest include changes that result in enhanced protein secretion, e.g., as described above. In some instances, the SecY mutant includes a protein localization (Prl) mutation. Prl mutations are mutations that may be characterized as gain-of-function mutations that expand the repertoire of substrates that can be exported. Prl mutations of interest include PrlA1, and the like.

The SecY mutant may include a variety of different types of mutations in a variety of different locations of the protein. Mutations of interest include insertion, deletion and substitution mutations. In some instances, the mutation is located in a transmembrane helix domain of the SecY protein, such as the tenth transmembrane helix. In some instances, the mutation is a substitution mutation, e.g., a substitution of a single amino acid residue. Of interest are non-conserved substitutions, e.g., a substitution of a hydrophobic (e.g., A, G, I, F, C, L, M, V) for a hydrophilic (e.g., R, E, K, D, H, N, Q, S, T) residue. In some instances, the SecY mutant includes a mutation at G408, such as G408R (where amino acid number is based on wild type *L.monocytogenes* SecY and analogous positions may be readily decided in other *Listeria* SecY proteins).

In some instances, the mutant GSP protein is an ATPase, e.g., SecA, SecA2. SecA mutants of interest include proteins having an AZI mutation (i.e., an azide resistance mutation). The SecA mutant may include a variety of different types of mutations in a variety of different locations of the protein. Mutations of interest include insertion, deletion and substitution mutations. In some instances, the mutation is a substitution mutation, e.g., a substitution of a single amino acid residue. Of interest are conserved substitutions, e.g., a substitution of one hydrophilic residue (e.g., R, E, K, D, H, N, Q, S, T) for another hydrophilic residue. In some instances, the SecA mutant includes a mutation at D599, such as D599N (where amino acid number is based on wild type *L.monocytogenes* SecA and analogous positions may be readily decided in other *Listeria* SecA proteins).

A given bacteria is considered to have a GSP mutation, e.g., as described above, if it includes a mutant GSP protein and/or a mutant nucleic acid coding sequence therefor. For example, SecY mutant bacteria may include a mutation in *lmo2612*, where the mutation may be an insertion, deletion or substitution, and in some instances is an SNP (e.g., as found in a *prlA1* mutation) that encodes a mutant SecY, e.g., as described

above. The mutant nucleic acid coding sequence of such embodiments may be integrated and/or episomally maintained, as desired.

In some instances, the bacteria are SecA2 mutant bacteria, by which is meant that they include a mutant SecA2 protein, which mutant protein is present in addition to the GSP protein mutation, e.g., SecY or SecA mutations, such as described above. The SecA2 mutant protein of such instances is a loss of function mutant. The SecA2 mutant may include a variety of different types of mutations in a variety of different locations of the protein. Mutations of interest include insertion, deletion and substitution mutations. Examples of SecA2 mutants of interest are described in the Experimental section, below.

In some embodiments, the *Listeria* bacteria are attenuated. The term "attenuated," as used herein, describes the diminution in the ability of the subject bacteria to cause disease in an animal as a whole, e.g., as measured by the LD₅₀ of the bacteria, as described below. More specifically, the pathogenic characteristics of the attenuated *Listeria* strain, as viewed from the vantage of the host animal as a whole (as opposed to a cell based perspective) have been lessened compared with wild-type *Listeria*, although the attenuated *Listeria* is capable of growth and maintenance in culture. In certain embodiments, bacteria are considered to be attenuated if, upon the intravenous inoculation of Balb/c mice (as described in the experimental section, below), the lethal dose at which 50% of inoculated animals survive (LD₅₀) is increased above the LD₅₀ of wild-type *Listeria* by at least about 10-fold, such as by at least about 100-fold, including by at least about 1,000 fold, where in certain embodiments the magnitude of increase is at least about 10,000 fold, such as at least about 100,000-fold, as determined using the assay employed in the experimental section below. An attenuated strain of *Listeria* according to the subject invention is thus one which does not kill an animal to which it is administered, or is one which kills the animal only when the number of bacteria administered is vastly greater than the number of wild type non-attenuated bacteria which would be required to kill the same animal.

In certain embodiments, attenuated species according to the subject invention are ones that exhibit a decreased virulence compared to their corresponding wild type strain in the Competitive Index Assay as described in Auerbach et al., "Development of a Competitive Index Assay To Evaluate the Virulence of *Listeria monocytogenes actA* Mutants during Primary and Secondary Infection of Mice," Infection and Immunity, September 2001, p. 5953-5957, Vol. 69, No. 9. In this assay, mice are inoculated with test and reference, e.g., wild-type, strains of bacteria. Following a period of time, e.g., 48 to 60 hours, the inoculated mice are sacrificed and one or more organs, e.g., liver, spleen, are evaluated for bacterial abundance. In these embodiments, a given bacterial

strain is considered to be less virulent if its abundance in the spleen is at least about 50-fold, or more, such as 70-fold or more less than that observed with the corresponding wild-type strain, and/or its abundance in the liver is at least about 10-fold less, or more, such as 20-fold or more less than that observed with the corresponding wild-type strain.

5 In yet other embodiments, bacteria are considered to be less virulent if they show abortive replication in less than about 8 hours, such as less than about 6 hours, including less than about 4 hours, as determined using the assay described in Jones and Portnoy, Intracellular growth of bacteria. (1994b) *Methods Enzymol.* 236:463–467. In yet other
10 embodiments, bacteria are considered to be attenuated or less virulent if, compared to wild-type, they form smaller plaques in the plaque assay employed in U.S. Patent No. 7,794,728 (the disclosure of which is herein incorporated by reference) where cells, such as murine L2 cells, are grown to confluency, e.g., in six-well tissue culture dishes, and then infected with bacteria. Subsequently, DME-agar containing gentamicin is added and plaques are grown for a period of time, e.g., 3 days. Living cells are then visualized by
15 adding an additional DME-agar overlay, e.g., containing neutral red (GIBCO BRL) and incubated overnight. In such an assay, the magnitude in reduction in plaque size observed with the attenuated mutant as compared to the wild-type is, in certain embodiments, 10%, including 15%, such as 25% or more.

Attenuated bacteria of the invention may include one or more different mutations
20 which confer the attenuated phenotype, where mutations of interest include *hly* mutations and/or *lplA* mutations, e.g., as described in U.S. Patent No. 7,794,728 (the disclosure of which is herein incorporated by reference); *actA* and/or *internalin B* (*InlB*) mutations, e.g., as reported in Dung et al., Clin. Cancer Res. (2012) 18:858-868); etc.

A given *Listeria* host cell may include one or more modifications as compared to
25 wildtype, which modifications provide for desirable qualities in the host cell, e.g., attenuation, enhanced immunogenicity, etc. Specific *Listeria* host cells that be assessed in embodiments of the invention include, but are not limited to, those described in PCT Published Application Nos.: WO 2014/106123; WO 2014/074635; WO 2009/143085; WO 2008027560 WO 2008066774; WO 2007117371; WO 2007103225; WO 2005071088;
30 WO 2003102168; WO 2003/092600; WO/2000/009733; and WO 1999/025376; the disclosures of which applications are herein incorporated by reference.

In certain embodiments, bacteria according to the subject invention express a heterologous antigen. The heterologous antigen is, in certain embodiments, one that is capable of providing protection in an animal against challenge by the infectious agent
35 from which the heterologous antigen was derived, or which is capable of affecting tumor growth and metastasis in a manner which is of benefit to a host organism. Heterologous

antigens which may be introduced into a *Listeria* strain of the subject invention by way of DNA encoding the same thus include any antigen which when expressed by *Listeria* serves to elicit a cellular immune response which is of benefit to the host in which the response is induced. Heterologous antigens therefore include those specified by
5 infectious agents, wherein an immune response directed against the antigen serves to prevent or treat disease caused by the agent. Such heterologous antigens include, but are not limited to, viral, bacterial, fungal or parasite surface proteins and any other proteins, glycoproteins, lipoprotein, glycolipids, and the like. Heterologous antigens also include those which provide benefit to a host organism which is at risk for acquiring or
10 which is diagnosed as having a tumor that expresses the heterologous antigen(s). The host organism is may be a mammal, such as a human.

By the term "heterologous antigen," as used herein, is meant a protein or peptide, a glycoprotein or glycopeptide, a lipoprotein or lipopeptide, or any other macromolecule which is not normally expressed in *Listeria*, which substantially corresponds to the same
15 antigen in an infectious agent, a tumor cell or a tumor-related protein. The heterologous antigen is expressed by a strain of *Listeria* according to the subject invention, and is processed and presented to cytotoxic T-cells upon infection of mammalian cells by the strain. The heterologous antigen expressed by *Listeria* species need not precisely match the corresponding unmodified antigen or protein in the tumor cell or infectious agent so
20 long as it results in a T-cell response that recognizes the unmodified antigen or protein which is naturally expressed in the mammal. In other examples, the tumor cell antigen may be a mutant form of that which is naturally expressed in the mammal, and the antigen expressed by the *Listeria* species will conform to that tumor cell mutated antigen. By the term "tumor-related antigen," as used herein, is meant an antigen which affects
25 tumor growth or metastasis in a host organism. The tumor-related antigen may be an antigen expressed by a tumor cell, or it may be an antigen which is expressed by a non-tumor cell, but which when so expressed, promotes the growth or metastasis of tumor cells. The types of tumor antigens and tumor-related antigens which may be introduced into *Listeria* by way of incorporating DNA encoding the same, include any known or
30 heretofore unknown tumor antigen. In other examples, the "tumor-related antigen" has no effect on tumor growth or metastasis, but is used as a component of the *Listeria* vaccine because it is expressed specifically in the tissue (and tumor) from which the tumor is derived. In still other examples, the "tumor-related antigen" has no effect on tumor growth or metastasis, but is used as a component of the *Listeria* vaccine because
35 it is selectively expressed in the tumor cell and not in any other normal tissues.

The heterologous antigen useful in vaccine development may be selected using knowledge available to the skilled artisan, and many antigenic proteins which are expressed by tumor cells or which affect tumor growth or metastasis or which are expressed by infectious agents are currently known. For example, viral antigens which may be considered as useful as heterologous antigens include but are not limited to the nucleoprotein (NP) of influenza virus and the gag protein of HIV. Other heterologous antigens include, but are not limited to, HIV env protein or its component parts gp120 and gp41, HIV nef protein, and the HIV pol proteins, reverse transcriptase and protease. Still other heterologous antigens can be those related to hepatitis C virus (HCV), including but not limited to the E1 and E2 glycoproteins, as well as non-structural (NS) proteins, for example NS3. In addition, other viral antigens such as herpesvirus proteins may be useful. The heterologous antigens need not be limited to being of viral origin. Parasitic antigens, such as, for example, malarial antigens, are included, as are fungal antigens, bacterial antigens and tumor antigens.

As noted herein, a number of proteins expressed by tumor cells are also known and are of interest as heterologous antigens which may be inserted into the vaccine strain of the invention. These include, but are not limited to, the bcr/abl antigen in leukemia, HPVE6 and E7 antigens of the oncogenic virus associated with cervical cancer, the MAGE1 and MZ2-E antigens in or associated with melanoma, and the MVC-1 and HER-2 antigens in or associated with breast cancer. Other coding sequences of interest include, but are not limited to: costimulatory molecules, immunoregulatory molecules, and the like.

In some instances, the heterologous antigen is one that is, or has been modified to be, secreted by a SecA2 mediated pathway. In some instances, the antigen is a protein having a domain that is recognized by SecA2 and secreted by a pathway in which SecA2 is involved. SecA2 recognized domains that may be present in the heterologous antigen include, but are not limited to those described in Renier et al., J. Proteomics (2013) 80:183-195. Such domains also include those found in: PBP 2B; N-acetylmuramidase (NamA); Pheromone transporter (OppA); p60 autolysin; Maltose/maltodextrin ABC transporter; Antigenic lipoprotein (Csa); Conserved lipoprotein; RNA polymerase β' subunit; RNA polymerase β subunit; Pyruvate dehydrogenase E2 subunit; Dnak; GroEL; EF-Tu; Enolase; Phosphomannose isomerase; Ribosomal protein L19; and Ribosomal protein S9. (See e.g., Lenz et al., Proc. Nat'l Acad. Sci. USA (2003) 100: 12432-12437).

Bacteria as described herein may be fabricated using a variety of different protocols. As such, generation of the subject attenuated bacteria may be accomplished

in a number of ways that are well known to those of skill in the art, including deletion mutagenesis, insertion mutagenesis, and mutagenesis which results in the generation of frameshift mutations, mutations which effect premature termination of a protein, or mutation of regulatory sequences which affect gene expression. Mutagenesis can be accomplished using recombinant DNA techniques or using traditional mutagenesis technology using mutagenic chemicals or radiation and subsequent selection of mutants. Representative protocols of different ways to generate bacteria according to the present invention are provided in the Experimental Section, below.

The introduction of DNA encoding a heterologous antigen into a strain of *Listeria* may be accomplished, for example, by the creation of a recombinant *Listeria* in which DNA encoding the heterologous antigen is harbored on a vector, such as a plasmid for example, which plasmid is maintained and expressed in the *Listeria* species, and in whose antigen expression is under the control of prokaryotic promoter/regulatory sequences. Alternatively, DNA encoding the heterologous antigen may be stably integrated into the *Listeria* chromosome by employing, for example, transposon mutagenesis, homologous recombination, or integrase mediated site-specific integration (as described in application serial no. 10/136,860, the disclosure of which is herein incorporated by reference).

Several approaches may be employed to express the heterologous antigen in *Listeria* species as will be understood by one skilled in the art once armed with the present disclosure. In certain embodiments, genes encoding heterologous antigens are designed to either facilitate secretion of the heterologous antigen from the bacterium or to facilitate expression of the heterologous antigen on the *Listeria* cell surface.

In certain embodiments, a fusion protein which includes the desired heterologous antigen and a secreted or cell surface protein of *Listeria* is employed. *Listerial* proteins which are suitable components of such fusion proteins include, but are not limited to, listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase (PI-PLC). A fusion protein may be generated by ligating the genes which encode each of the components of the desired fusion protein, such that both genes are in frame with each other. Thus, expression of the ligated genes results in a protein comprising both the heterologous antigen and the *Listerial* protein. Expression of the ligated genes may be placed under the transcriptional control of a *Listerial* promoter/regulatory sequence such that expression of the gene is effected during growth and replication of the organism. Signal sequences for cell surface expression and/or secretion of the fused protein may also be added to genes encoding heterologous antigens in order to effect cell surface expression and/or secretion of the fused protein. When the heterologous antigen is used alone (i.e.,

in the absence of fused *Listeria* sequences), it may be advantageous to fuse thereto signal sequences for cell surface expression and/or secretion of the heterologous antigen. The procedures for accomplishing this are well known in the art of bacteriology and molecular biology.

5 The DNA encoding the heterologous antigen which is expressed is, in some embodiments, preceded by a suitable promoter to facilitate such expression. The appropriate promoter/regulatory and signal sequences to be used will depend on the type of *Listeria* protein desired in the fusion protein and will be readily apparent to those skilled in the art of *Listeria* molecular biology. For example, preferred *L. monocytogenes* promoter/regulatory and/or signal sequences which may be used to direct expression of a fusion protein include, but are not limited to, sequences derived from the *Listeria hly* gene which encodes LLO, the *Listeria p60 (iap)* gene, and the *Listeria actA* gene which encodes a surface protein necessary for *L. monocytogenes* actin assembly. Other promoter sequences of interest include the *plcA* gene which encodes PI-PLC, the *Listeria mpl* gene, which encodes a metalloprotease, and the *Listeria inlA* gene which encodes internalin, a *Listeria* membrane protein. The heterologous regulatory elements such as promoters derived from phage and promoters or signal sequences derived from other bacterial species may be employed for the expression of a heterologous antigen by the *Listeria* species.

20 In certain embodiments, the attenuated *Listeria* includes a vector. The vector may include DNA encoding a heterologous antigen. In some instances, the vector is a plasmid that is capable of replication in *Listeria*. The vector may encode a heterologous antigen, wherein expression of the antigen is under the control of eukaryotic promoter/regulatory sequences, e.g., is present in an expression cassette. Typical plasmids having suitable promoters that are of interest include, but are not limited to, pCMVbeta comprising the immediate early promoter/enhancer region of human cytomegalovirus, and those which include the SV40 early promoter region or the mouse mammary tumor virus LTR promoter region.

30 As such, in certain embodiments, the subject bacteria include at least one coding sequence for heterologous polypeptide/protein, as described above. In some instances, the coding sequence is one that lacks introns, e.g., is a continuous open reading frame that has a sequence which is the same as a cDNA sequence which may be produced from chromosomal sequence. In some embodiments, this coding sequence is part of an expression cassette, which provides for expression of the coding sequence in the *Listeria* cell for which the vector is designed. The term "expression cassette" as used herein refers to an expression module or expression construct made up of a recombinant

DNA molecule containing at least one desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism, i.e., the *Listeria* cell for which the vector is designed, such as the promoter/regulatory/signal sequences identified above, where the expression
5 cassette may include coding sequences for two or more different polypeptides, or multiple copies of the same coding sequence, as desired. As such, the size of the encoded product may vary greatly, and a broad spectrum of different products may be encoded by the expression cassettes present in the vectors of this embodiment.

As indicated above, the vector may include at least one coding sequence, where
10 in certain embodiments the vectors include two or more coding sequences, where the coding sequences may encode products that act concurrently to provide a desired results. In general, the coding sequence may encode any of a number of different products and may be of a variety of different sizes, where the above discussion merely provides representative coding sequences of interest.

UTILITY

The above-described bacteria find use in a number of different applications. Representative uses of the subject bacteria include, but are not limited to: (a) immunogens for generating antibodies to *Listeria spp.*; (b) adjuvant compositions in
20 immunizing protocols; (c) vectors for introducing macromolecules, e.g., nucleic acids or proteins, into the cytoplasm of target cells; and (d) vaccine compositions, e.g., for eliciting or boosting a cellular immune response in a host. Each of these representative applications is now further described separately below. Uses for attenuated *Listeria spp.* are also described in U.S. Patent Nos. 8,679,476; 8,277,797; 8,192,991; 7,842,289;
25 7,794,728; 7,749,510; 7,488,487; 7,425,449; 6,599,502; 6,504,020; 6,287,556; 6,099,848; 6,004,815; 5,830,702; and 5,643,599; the disclosures of which applications are herein incorporated by reference.

Vaccines

30 The subject bacteria find use as vaccines. The vaccines of the present invention are administered to a vertebrate by contacting the vertebrate with a sub-lethal dose of the attenuated *Listeria* vaccine, where contact typically includes administering the vaccine to the host. In some embodiments, the bacteria are provided in a pharmaceutically acceptable formulation. Administration can be oral, parenteral,
35 intranasal, intramuscular, intradermal, intraperitoneal, intravascular, subcutaneous, direct vaccination of lymph nodes, administration by catheter or any one or more of a variety of

well-known administration routes. In farm animals, for example, the vaccine may be administered orally by incorporation of the vaccine in feed or liquid (such as water). It may be supplied as a lyophilized powder, as a frozen formulation or as a component of a capsule, or any other convenient, pharmaceutically acceptable formulation that
5 preserves the antigenicity of the vaccine. Any one of a number of well-known pharmaceutically acceptable diluents or excipients may be employed in the vaccines of the invention. Suitable diluents include, for example, sterile, distilled water, saline, phosphate buffered solution, and the like. The amount of the diluent may vary widely, as those skilled in the art will recognize. Suitable excipients are also well known to those
10 skilled in the art and may be selected, for example, from A. Wade and P.J. Weller, eds., *Handbook of Pharmaceutical Excipients* (1994) The Pharmaceutical Press: London. The dosage administered may be dependent upon the age, health and weight of the patient, the type of patient, and the existence of concurrent treatment, if any. The vaccines can be employed in dosage forms such as capsules, liquid solutions, suspensions, or elixirs,
15 for oral administration, or sterile liquid for formulations such as solutions or suspensions for parenteral, intranasal intramuscular, or intravascular use. In accordance with the invention, the vaccine may be employed, in combination with a pharmaceutically acceptable diluent, as a vaccine composition, useful in immunizing a patient against infection from a selected organism or virus or with respect to a tumor, etc. Immunizing a
20 patient means providing the patient with at least some degree of therapeutic or prophylactic immunity against selected pathogens, cancerous cells, etc.

The subject vaccines find use in methods for eliciting or boosting a cellular immune response, e.g., a helper T cell or a cytotoxic T-cell response to a selected agent, e.g., pathogenic organism, tumor, etc., in a vertebrate, where such methods include
25 administering an effective amount of the Listeria vaccine. The subject vaccines find use in methods for eliciting in a vertebrate an innate immune response that augments the antigen-specific immune response. Furthermore, the vaccines of the present invention may be used for treatment post-exposure or post diagnosis. In general, the use of vaccines for post-exposure treatment would be recognized by one skilled in the art, for
30 example, in the treatment of rabies and tetanus. The same vaccine of the present invention may be used, for example, both for immunization and to boost immunity after exposure. Alternatively, a different vaccine of the present invention may be used for post-exposure treatment, for example, such as one that is specific for antigens expressed in later stages of exposure. As such, the subject vaccines prepared with the
35 subject vectors find use as both prophylactic and therapeutic vaccines to induce immune responses that are specific for antigens that are relevant to various disease conditions.

The patient may be any human and non-human animal susceptible to infection with the selected organism. The subject vaccines will find particular use with vertebrates such as man, and with domestic animals. Domestic animals include domestic fowl, bovine, porcine, ovine, equine, caprine, Leporidae (such as rabbits), or other animal which may be held in captivity.

The subject vaccines find use in vaccination applications as described in PCT Published Application Nos.: WO 2014/106123; WO 2014/074635; WO 2009/143085; WO 2008027560 WO 2008066774; WO 2007117371; WO 2007103225; WO 2005071088; WO 2003102168; WO 2003/092600; WO/2000/009733; and WO 1999/025376; the disclosures of which applications are herein incorporated by reference.

Generation of Listeria specific antibodies

The subject bacteria find use in the generation of antibodies specific for *Listeria spp.* In these applications, the bacteria are administered to a suitable host according to known techniques, and resultant antibodies are harvested from the immunized host.

Immunization can be carried out in a variety of ways with a number of different animals. Host animals of interest include rabbits, mice, rats, goats and sheep, etc. Any mammal capable of immune response can be employed as the host animal in antibody production. For the most part for commercial production of antibodies, relatively large animals are employed, such as equine, bovine, porcine, canine, ovine, caprine, rodentia, rabbits and hares. A representative antibody production protocol in which the subject attenuated bacteria may be employed includes the antibody generation protocol as described in U.S. Patent No. 4,816,253; the disclosure of which is herein incorporated by reference.

Adjuvant Composition

The subject bacterial strains also find use as immunopotentiating agents, i.e., as adjuvants. In such applications, the subject attenuated bacteria may be administered in conjunction with an immunogen, e.g., a tumor antigen, modified tumor cell, etc., according to methods known in the art where live bacterial strains are employed as adjuvants. See, e.g., Berd et al., Vaccine 2001 Mar 21;19(17-19):2565-70.

In some embodiments, the bacterial strains are employed as adjuvants by chemically coupled to a sensitizing antigen. The sensitizing antigen can be any antigen of interest, where representative antigens of interest include, but are not limited to: viral agents, e.g., Herpes simplex virus; malaria parasite; bacteria, e.g., staphylococcus aureus bacteria, diphtheria toxoid, tetanus toxoid, shistosomula; tumor cells, e.g. CAD₂ mammary adenocarcinoma tumor cells, and hormones such as thyroxine T₄,

triiodothyronine T₃, and cortisol. The coupling of the sensitizing antigen to the immunopotentiating agent can be accomplished by means of various chemical agents having two reactive sites such as, for example, bisdiazobenzidine, glutaraldehyde, diiodoacetate, and diisocyanates, e.g., m-xylenediisocyanate and toluene-2,4-diisocyanate. Use of *Listeria* spp. as adjuvants is further described in U.S. Patent No. 4,816,253; the disclosure of which is herein incorporated by reference.

Delivery Vehicles

The subject bacteria also find use as vectors or delivery vehicles for delivery of macromolecules into target cells, e.g., as described in: PCT publication no. WO 00/09733 (the disclosure of which is herein incorporated by reference); and Dietrich et al., *Nature Biotechnology* (1998) 16: 181-185. A variety of different types of macromolecules may be delivered, including, but not limited to: nucleic acids, polypeptides/proteins, etc., as described in these publications.

15

The following examples are offered by way of illustration and not by way of limitation.

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25

EXPERIMENTAL

I. Materials and Methods

A. Bacterial strains and growth conditions

All *L. monocytogenes* strains used and generated in this study were in the 10403S background and are listed (Table 1).

30

Table 1: *Listeria monocytogenes* strains used in this study.

Strain	Strain#	Description	Source
10403S	-	Wild type (WT) <i>Listeria monocytogenes</i> strain	(1)
$\Delta secA2$	DP-L4342	In frame deletion of <i>lmo0583</i>	(2)
$\Delta pglA\Delta oatA$	DP-L5220	In frame deletion of <i>lmo0415</i> and <i>lmo1291</i>	(3)
Δasp	DP-L4611	In frame deletion of <i>lmo0582</i>	(4)
$\Delta lmo2769$	DP-L6209	In frame deletion of <i>lmo2769</i>	This study
$\Delta lmo2769\Delta secA2$	DP-L6210	In frame deletion of <i>lmo2769</i> in a $\Delta secA2$ background	This study
$\Delta lmo2769\Delta secA2$ +pPL2-2767-2769	DP-L6211	$\Delta lmo2769\Delta secA2$ strain complemented with pPL2 <i>lmo2767-2769</i> construct	This study
R57+pPL2-2767-2769	DP-L6212	R57 strain complemented with pPL2 <i>lmo2767-2769</i> construct	This study
<i>lmo2768::Tn</i>	DP-L6155	<i>HimarI</i> Transposon insert in <i>lmo2768</i>	(5)
$\Delta secA2$: <i>lmo2768::Tn</i>	DP-L6213	<i>HimarI</i> Transposon insert in <i>lmo2768</i> in $\Delta secA2$ strain	This study
$\Delta lmo1721$	DP-L6214	In frame deletion of <i>lmo1721</i>	This study
$\Delta lmo1721\Delta secA2$	DP-L6215	In frame deletion of <i>lmo1721</i> in a $\Delta secA2$ background	This study
$\Delta lmo2769\Delta lmo1721$	DP-L6216	In frame deletion of <i>lmo1721</i> in a $\Delta lmo2769$ background	This study
$\Delta lmo2769\Delta lmo1721\Delta secA2$	DP-L6217	In frame deletion of <i>lmo1721</i> in a $\Delta lmo2769\Delta secA2$ background	This study
$\Delta secA2$: <i>lmo2637::Tn</i>	DP-L6218	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in $\Delta secA2$ strain	This study
$\Delta secA2$ <i>prlA1</i>	DP-L6219	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in $\Delta secA2$ strain with <i>secY</i> (G408R)	This study
WT: <i>lmo2637::Tn</i>	DP-L6220	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in 10403S (WT) strain	Lab collection
<i>prlA1</i>	DP-L6221	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in WT strain with <i>secY</i> (G408R)	This study
$\Delta lmo2769\Delta secA2$: <i>lmo2637::Tn</i>	DP-L6222	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in $\Delta lmo2769\Delta secA2$ strain with WT <i>secY</i>	This study
$\Delta lmo2769\Delta secA2$ <i>prlA1</i>	DP-L6223	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in $\Delta lmo2769\Delta secA2$ strain with <i>secY</i> (G408R)	This study
$\Delta lmo1721\Delta secA2$ <i>prlA1</i>	DP-L6224	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in $\Delta lmo1721\Delta secA2$ strain with <i>secY</i> (G408R)	This study
$\Delta lmo2769\Delta lmo1721\Delta secA2$: <i>Tn</i>	DP-L6225	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in $\Delta lmo2769\Delta lmo1721\Delta secA2$ strain with WT <i>secY</i>	This study
$\Delta lmo2769\Delta lmo1721\Delta secA2$ <i>prlA1</i>	DP-L6226	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in $\Delta lmo2769\Delta lmo1721\Delta secA2$ strain with <i>secY</i> (G408R)	This study
R57 <i>prlA1</i>	DP-L6227	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in strain R57 with the <i>secY</i> (G408R)	This study
R57: <i>lmo2637::Tn</i>	DP-L6228	<i>HimarI</i> Transposon insert in <i>lmo2637</i> in strain R57 with WT <i>secY</i>	This study

The $\Delta secA2$ previously characterized (Lenz et al., "SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis," Proc. Nat'l Acad. Sci USA (2003) 100:12432–12437; Lenz & Portnoy "Identification of a second *Listeria* secA gene associated with protein secretion and the rough phenotype," Mol. Microbiol (2002) 45:1043–1056) served as a parent strain for generating motility revertants. Unless otherwise stated all *L. monocytogenes* strains were grown in brain heart infusion medium (BHI, Difco, Detroit, MI). All *E. coli* strains used for generating in-frame deletion and complement constructs were grown in Luria-Bertani (LB, Difco, Detroit, MI) medium.

B. Swarming motility

A single colony of Δ secA2 was used to generate a single motility revertant by stab-inoculating semi-solid LB 0.4% agar at 30°C for five days. Revertant strains were grown overnight in BHI broth at 30°C without shaking, 1 μ L of culture was then
 5 incorporated into a semi-solid LB agar and swarming area was evaluated using ImageJ (available at the website produced by placing "http://" before rsweb.nih.gov/ij/") following incubation at 30°C for 48 h.

C. L2 plaque assay

10 Plaque assays using murine L2 fibroblasts were performed as previously described (Sun et al., "Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread," *Infect. Immun.* (1990) 58:3770-3778). Briefly, overnight 30°C static cultures of *L. monocytogenes* were allowed to infect monolayers of L2 cells for 1h. Cells were washed and overlaid with 0.7% agarose in
 15 DMEM (Gibco/Invitrogen, CA) containing 10 μ g/mL gentamycin (GM). After 3 days at 37°C, plaques were overlaid with 2 mL of 0.7% agarose in DMEM with GM and 0.3% neutral red (Sigma-Aldrich). Monolayers were stained overnight and plaque size was evaluated using ImageJ.

D. DNA isolation and sequencing

20 Genomic DNA was isolated from stationary phase cultures of *L. monocytogenes* using the MasterPure DNA purification kit (Epicentre). DNA was then fragmented using Covaris S22 (Covaris Inc.). Libraries were constructed using Apollo 324 (IntegenX Inc.), PCR amplified, and multiplexed at the Vincent J. Coates Genomics Sequencing
 25 Laboratory at UC Berkeley. The resulting libraries were sequenced using single-end reads with a HiSeq 2000 Illumina platform. Sequence data were aligned to the *L. monocytogenes* 10403S reference genome CP002002 using Bowtie 2 (Langmead & Salzberg, "Fast gapped-read alignment with Bowtie 2," *Nat. Methods* (2012) 9:357–359) and SNPs were identified using SAM tools (Li et al., "The sequence alignment/map
 30 format and SAMtools," *Bioinformatics* (2009) 25:2078–2079). Approximately 93% of all reads aligned to the reference genome, resulting in >50X coverage.

E. In-frame deletion and complementation constructs

In-frame deletion mutants of *Imo2769* and *Imo1721* were constructed by splice
 35 overlap extension and introduced by allelic exchange using pKSV7 using JD17-JD20 and JD30-JD33 primers, respectively (Table 2) as previously described (Camilli et al.

"Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis," (1993) *Mol. Microbiol* 8:143–157).

Table 2. Deletion and Complement Primer Sequences

Primer	5'-3' Sequence	Description
JD17	ATTAGTCGACCTCGGAGTTTGGTGTCTTCTGG (SEQ ID NO:01)	<i>lmo2769</i> deletion primer A
JD18	ATTACTGCAGAAACGATGCGGACTCAAACG (SEQ ID NO:02)	<i>lmo2769</i> deletion primer D
JD19	CTCCCGTCTGTTTTAAATCTCGTATTTAGTTAAGTTCCG AATTTTCAT (SEQ ID NO:03)	<i>lmo2769</i> deletion primer B
JD20	ATGAAAATTCGGAACCTTAATAACGAGATTTAAAC AGACGGGAG (SEQ ID NO:04)	<i>lmo2769</i> deletion primer C
JD30	ATTAGTCGAC ACAGATGTAGCGGCTCGTGG (SEQ ID NO:05)	<i>lmo1721</i> deletion primer A
JD31	GATTCCTTTTTCTTAATTTCTTCGACTTCTTCTTTCTA CTAGACAT (SEQ ID NO:06)	<i>lmo1721</i> deletion primer B
JD32	ATGTCTAGTAGAAAAGAAGTCAAGAAAATTAAGA AAAAGGAATC (SEQ ID NO:07)	<i>lmo1721</i> deletion primer C
JD33	ATTACTGCAGCGCCGTCCATTGTTCCATAG (SEQ ID NO:08)	<i>lmo1721</i> deletion primer D
JD46	ATTAGTCGACCTGGATGTGGCGTAAGGG (SEQ ID NO:09)	<i>lmo2769-lmo2767</i> F' primer
JD47	ATTAGGATCCATAACTTTGTCCCGATTGTCC (SEQ ID NO:10)	<i>lmo2769-lmo2767</i> R' primer

5

The pPL2 integration vector was used to complement $\Delta lmo2769$ mutants and R57 revertant strain with the *lmo2769-2767* operon and all the endogenous regulatory sequences using JD46-JD47 primers (Table 2) as previously described (Lauer et al., "Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors," *J. Bacteriol.* (2002) 184:4177–4186).

10

F. Transductions

Transductions of the *lmo2768::Tn* and *lmo2637::Tn* donor strains were generated using phage U153 and erythromycin as a selection marker, as previously described (Hodgson DA, "Generalized transduction of serotype 1/2 and serotype 4b strains of *Listeria monocytogenes*," *Mol. Microbiol.* (2000) 35:312–323). Strain R57 containing the *prlA1* was used as the recipient strain for *lmo2637::Tn* and subsequent recipient strains of *secY* mutation were confirmed by Sanger sequencing (Elim Biopharmaceuticals, Hayward, CA).

15

G. *In vivo* mouse infections

20

CD1 (CrI:CD1(ICR)) mice (Charles River Laboratories) were injected intravenously with 1×10^5 CFU of wild-type or mutant *L. monocytogenes* strains. Spleens

and livers were harvested after 48 h and bacterial burdens were evaluated as previously described (Witte et al., "Cyclic di-AMP is critical for *Listeria monocytogenes* growth, cell wall homeostasis, and establishment of infection," mBio (2013) 4:e00282–00213).

5

H. Disk diffusion assay

A total of 3×10^8 stationary phase *L. monocytogenes* cells were plated on BHI agar plates. Sterile Whatman paper discs (7 mm in diameter) containing 1 mg of chicken egg white lysozyme (Sigma), 10 µg of vancomycin or 120 µg of penicillin in 10 µL volume were added to plates. The area of growth inhibition around each disc was measured using ImageJ following 24 hr incubation at 37°C.

I. Microscopy

Phase contrast microscopy was performed using stationary BHI culture at 37°C. Fluorescence microscopy was conducted using Olympus IX81 TIRF microscope on mid-log cells stained with SYTO9 green fluorescent stain (Invitrogen).

J. Western blotting

Secreted proteins from mid-log LB culture supernatants were precipitated with 10% trichloro-acetic acid (TCA), as previously described (Zemansky et al., "Development of a mariner-based transposon and identification of *Listeria monocytogenes* determinants, including the peptidyl-prolyl isomerase PrsA2, that contribute to its hemolytic phenotype," J. Bacteriol. (2009) 191:3950–3964) and solubilized in NuPAGE LDS buffer (Invitrogen) containing 5% β –mercaptoethanol at a volume adjusted to OD₆₀₀ for each strain. Samples were fractionated by SDS-PAGE, transferred to a PVDF membrane for immunoblotting with polyclonal anti-P60 antibodies and quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

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II. Results:

A. Isolation and initial characterization of $\Delta secA2$ suppressor mutants

The inability of bacteria to properly septate during cell division can negatively influence swarming efficiency in semisolid media (Blackman et al., "The role of autolysins during vegetative growth of *Bacillus subtilis* 168," *Micobiology* (1998) 144:73-82; Halbedel et al., "DivIVA affects secretion of virulence-related autolysins in *Listeria monocytogenes*," *Mol. Microbiol.* (2012) 83:821–839; Rashid et al., "Bacillus subtilis mutant deficient in the major autolytic amidase and glucosaminidase is impaired in motility," *FEMS Microbiol. Lett.* (1993) 112:135–140). Indeed, the filamentous nature of the *L. monocytogenes secA2* mutant led to a 92% reduction in swarming motility compared to the wild type (WT) 10403S strain (Figure 1). Seventy spontaneous, independent $\Delta secA2$ suppressor mutants were generated by identifying swarming bacteria that appeared after five days incubation at 30°C in semisolid LB agar. These suppressor mutants displayed a wide range of swarming phenotypes (11 to 112%) when compared to the WT strain (Figure 1). Motility revertants were further characterized based on their colony and microscopic appearance (Table 3).

Table 3. Colony and microscopic morphology of $\Delta secA2$ revertants.

Strain	Colony	Chaining [#]	Lysozyme ^{\$}	Strain	Colony	Chaining [#]	Lysozyme ^{\$}
R1	smooth	+	1.79 (± 0.10)	R33	rough	+++	1.71 (± 0.00)
R2	rough	++++	1.64 (± 0.10)	R34	smooth	++ [€]	2.67 (± 0.20)
R3.1	smooth	+	1.71 (± 0.00)	R35	smooth	++	2.67 (± 0.60)
R3.2	rough	++++	1.71 (± 0.20)	R36	smooth	+	1.64 (± 0.10)
R4	smooth	+	1.64 (± 0.10)	R37	smooth	++	1.71 (± 0.00)
R5	smooth	++	1.71 (± 0.00)	R38	smooth	++	1.57 (± 0.00)
R6	rough	+++	1.57 (± 0.00)	R39	rough	++++	2.00 (± 0.20)
R7	smooth	+	2.36 (± 0.10)	R40	rough	+++ [€]	1.79 (± 0.10)
R8	rough	+++	2.50 (± 0.10)	R41	smooth	+	1.79 (± 0.10)
R9	rough	+++	2.17 (± 0.30)	R42	smooth	+	2.14 (± 0.20)
R10.1	smooth	++	1.71 (± 0.00)	R43	rough	+++	1.57 (± 0.20)
R10.2	rough	+++	1.86 (± 0.00)	R44	smooth	+	2.21 (± 0.30)
R11	smooth	++ [€]	1.86 (± 0.00)	R45	smooth	+	2.29 (± 0.20)
R12	smooth	++	2.29 (± 0.00)	R46	smooth	+	2.50 (± 0.10)
R13	rough	+++	1.64 (± 0.10)	R47.1	smooth	+	2.57 (± 0.00)
R14	rough	+++	1.93 (± 0.10)	R47.2	rough	+++	2.64 (± 0.10)
R15	rough	+++	1.86 (± 0.00)	R48	smooth	+	1.71 (± 0.00)
R16	smooth	+	1.71 (± 0.00)	R49	smooth	++	1.71 (± 0.20)
R17	smooth	++ [€]	1.71 (± 0.00)	R50	smooth	++	2.21 (± 0.10)
R18.1	smooth	+ [€]	1.71 (± 0.00)	R51	rough	+	1.71 (± 0.00)
R18.2	rough	++++	1.64 (± 0.10)	R52	smooth	+	1.64 (± 0.10)
R19	rough	++++	1.79 (± 0.10)	R53	smooth	+	2.29 (± 0.20)
R20	rough	+++	1.79 (± 0.10)	R54	smooth	++	2.57 (± 0.20)
R21	smooth	++	1.93 (± 0.10)	R55	rough	+++	1.86 (± 0.40)
R22	rough	+++	1.79 (± 0.10)	R56	smooth	++	2.14 (± 0.00)
R23	smooth	+	2.10 (± 0.40)	R57	smooth	+	2.87 (± 0.40)
R24	smooth	+ [€]	2.14 (± 0.20)	R58	smooth	+	1.86 (± 0.20)
R25	rough	++++	1.64 (± 0.10)	R59	smooth	+	1.79 (± 0.10)
R26	smooth	+ [€]	2.93 (± 0.30)	R60.1	smooth	++	2.49 (± 0.20)
R27	rough	+++	1.64 (± 0.10)	R60.2	rough	+++	1.71 (± 0.00)
R28	smooth	++	1.71 (± 0.00)	R61	smooth	+	2.29 (± 0.00)
R29	smooth	+	1.71 (± 0.20)	R62	smooth	++	2.50 (± 0.10)
R30	smooth	+	3.36 (± 0.10)	R63.1	smooth	++	2.21 (± 0.10)
R31	rough	+++	1.79 (± 0.10)	R63.2	rough	+++	2.21 (± 0.10)
R32	smooth	++ [€]	1.64 (± 0.10)	R64	smooth	+ [€]	3.00 (± 0.20)

Microscopy was conducted on stationary phase cells grown in BHI at 37°C. Chaining phenotype was scored from + to +++++, where WT = + and $\Delta secA2$ =++++ according to the following algorithm:

5

- + = single cells and chains of up to 3 cells/field x40 magnification
- ++ = single cells and chains of up to 4 cells/field x40 magnification
- +++ = chains of up to 6 cells/field x40 magnification
- ++++ = chains of >6 cells/field x40 magnification

10

€ Pleomorphic cells

\$ Disk diffusion susceptibility to 1mg of lysozyme expressed as ratio of WT, where a ratio >1 indicates increased 10 susceptibility. Values in parenthesis represent standard deviations.

The majority of suppressor mutants (64%) reverted from a rough to a smooth colony morphology. Rough colony morphology correlated with chaining, with less chaining observed for isolates forming smooth colonies. Whereas WT (42-44) and *secA2* mutants of *L. monocytogenes* are lysozyme resistant, all 70 suppressor mutants were susceptible to lysozyme (Table 3).

A critical aspect of *L. monocytogenes* pathogenesis is the capacity to spread from cell-to-cell and form plaques in monolayers of tissue culture cells, which correlates well with mouse virulence (Roche et al., "Assessment of the virulence of *Listeria monocytogenes*: agreement between a plaque-forming assay with HT-29 cells and infection of immunocompetent mice," Int. J. Food Microbiol. (2001) 68:33-44). The *secA2* mutant forms plaques that are approximately 30% of WT (Figure 2 and (Lenz et al., "SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis," Proc. Nat'l Acad. Sci. USA (2003) 100:12432–12437)). The Δ *secA2* swarming suppressor mutants varied greatly in their ability to form plaques, ranging from 0% to 87% of WT (Figure 2). There was no significant correlation between chaining phenotype and plaque size, suggesting that chaining alone does not directly influence cell-to-cell spread as measured by the plaque assay.

B. Whole-genome sequencing of five Δ *secA2* suppressor mutants

To identify mutations responsible for the observed phenotypes, five strains were selected for further analysis (Table 4).

Table 4: Strains selected for sequencing and their phenotypes.

Strain	Colony	Chaining [€]	Motility [*]	Plaque [¢]
R9	Rough	+++	29% ($\pm$ 3)	41% ($\pm$ 9)
R23	Smooth	+	55% ($\pm$ 13)	0%
R35	Smooth	++	102% ($\pm$ 26)	0%
R57	Smooth	+	112% ($\pm$ 15)	87% ($\pm$ 13)
R60.1	Smooth	++	87% ($\pm$ 23)	21% ($\pm$ 6)
Δ <i>secA2</i> [#]	Rough	++++	9% ($\pm$ 2)	29% ($\pm$ 9)

[#] Parent Δ *secA2* was included as a reference strain

[€] Chaining scores interpretation by phase microscopy

+ = single cells and chains of up to 3 cells/field x40 magnification

++ = single cells and chains of up to 4 cells/field x40 magnification

+++ = chains of up to 6 cells/field x40 magnification

++++ = chains of >6 cells/field x40 magnification

^{*} Expressed as an average percentage (standard deviation) of the swarming area normalized to WT strain on 0.4% LB after 48h at 30°C

[¢] Expressed as an average percentage (standard deviation) of plaque area of WT strain

Whole-genome sequencing revealed a number of single-nucleotide polymorphisms (SNP) that differed from the WT strain and confirmed that each lacked the *secA2* gene (Table 5).

Table 5: Single-nucleotide polymorphisms (SNP) of strains sequenced.

Strain	Position ^a	Reference ^a	Alteration	Change	Lmo no.	Gene name	Encoded protein or function
R9	1104029	C	T	A141V	<i>lmo1087</i>		Ribitol-5-phosphate 2-dehydrogenase
	1921584	T	C	V195 silent	<i>lmo1892</i>	<i>pbpA</i>	Penicillin-binding protein 1
	2812912	A	G	48 bp upstream of <i>lmo2769</i> transcription start site	<i>lmo2769</i>		Antibiotic transport system ATP-binding protein
R23	714441	A	G	E28G	<i>lmo0699</i>	<i>fliM</i>	Flagellar motor switch
	2734020	G	C	G122 silent	<i>lmo2694</i>		Lysine decarboxylase
	2811145	G	T	T169 stop codon	<i>lmo2768</i>		Membrane protein (permease)
R35	728791	G	A	A340T	<i>lmo0714</i>	<i>fliG</i>	Flagellar motor switch protein G
	736353	A	G	E176G	<i>lmo0723</i>		Methyl-accepting chemotaxis protein
	1102328	C	T	P499S	<i>lmo1085</i>		Similar to teichoic acid biosynthesis protein B
	1191185	T	C	I486T	<i>lmo1208</i>	<i>cbiP</i>	Cobayric acid synthase
	1264242	CTTTTT TTT	CTTTTT T	58 bp upstream of <i>lmo1281</i> transcription start site	<i>lmo1281</i>		Hydroxybenzoyl coenzyme A thioesterase
	1343106	G	A	R232H	<i>lmo1360</i>	<i>folD</i>	Methylenetetrahydrofolate dehydrogenase/cyclohydrolase
	1390074	GAAAA AAAA	GAAAAAA A	I309S, premature stop at codon 319	<i>lmo1403</i>	<i>mutS</i>	DNA mismatch repair protein MutS
	1932076	A	G	S247A	<i>lmo1901</i>	<i>panC</i>	Pantothenate synthetases
	2137030	A	G	L219W	<i>lmo2100</i>		Similar to transcriptional regulator (GntR family)
	2323057	G	A	R264C	<i>lmo2278</i>	<i>lysA</i>	L-Alanoyl-D-glutamate peptidase
	2447109	CAAAA AAA	CAAAAAA AA	A25C, premature stop at codon 49	<i>lmo2421</i>		Similar to two-component sensor histidine kinase
	2468643	CTTTTT TTT	CTTTTT T	A46Q, premature stop at codon 62	<i>lmo2444</i>		Similar to glycosidase
	2812179	ATTTTT TT	ATTTTT TT	I229N, premature stop at codon 236	<i>lmo2769</i>		Antibiotic transport system ATP-binding protein
R57	1738627	TAA	TAAA	L780F, premature stop at codon 791	<i>lmo1721</i>	<i>lacR</i>	Sigma-54 interaction domain-containing protein
	2658496	C	T	G408R	<i>lmo2612</i>	<i>secY</i>	Protein translocase

Strain	Position ^a	Reference ^a	Alteration	Change	Lmo no.	Gene name	Encoded protein or function
	2812179	ATTTT TT	ATTTT TT	I229N, premature stop at codon 236	<i>lmo2769</i>		subunit SecY Antibiotic transport system ATP-binding protein
R60.1	311857	CAAAA AA	CAAAAA A	G109R, premature stop at codon 279	<i>lmo0290</i>	<i>wall</i>	Similar to <i>B. subtilis</i> YycI protein
	2542503	C	T	D559N	<i>lmo2510</i>	<i>secA</i>	Protein translocase subunit SecA
	2568428	T	CA	V211G, premature stop at codon 223	<i>lmo2537</i>	<i>mnaA</i>	UDP-N- acetylglucosamine 2- epimerase

Most revertants encoded three to four SNPs while revertant R35 was an outlier with 13 SNPs, one of which mapped to *lmo1403*, a gene encoding the DNA mismatch repair protein MutS. We hypothesize that inactivation of *mutS* led to a hypermutation phenotype (Mérino et al., "A hypermutator phenotype attenuates the virulence of *Listeria monocytogenes* in a mouse model," *Mol. Microbiol.* (2002) 44:877–887.).

Two revertants, R23 and R35, contained SNPs in genes encoding flagella motor switch proteins FliM and FliG, respectively (Table 5). Both of these proteins function in controlling the direction of flagella rotation (Dyer et al., "A molecular mechanism of bacterial flagellar motor switching," *J. Mol. Biol.* (2009) 388:71-84; Kihara et al., "Deletion analysis of the flagellar switch protein FliG of *Salmonella*," *J. Bacteriol.* (2000) 182:3022-3028; Mariconda et al., "A mechanical role for the chemotaxis system in swarming motility," *Mol. Microbiol.* (2006) 60:1590–1602; Toker et al., "Deletion analysis of the FliM flagellar switch protein of *Salmonella typhimurium*," *J. Bacteriol.* (1996) 178:7069-7079.) and single point mutations in FliM have been shown to restore swarming motility in a chemotaxis deficient mutant of *Salmonella enterica* (Mariconda et al., *id.*). We hypothesize that SNPs in these genes led to enhanced swarming motility of the corresponding revertants by direct manipulation of the flagella motor. However, neither of these suppressor mutants formed plaques in tissue culture cells and therefore were not subjected to further analysis.

All suppressor mutants were significantly more susceptible to lysozyme than WT or the parent *secA2* mutant strain (Figure 3B; Table 3). Susceptibility to cell wall-acting antibiotics was less prominent but still evident for R23, R35 and R60.1 (Supplementary Figure 6A-B). Additionally, four out of five suppressors had mutations in the *lmo2769* operon, previously shown to be required for lysozyme resistance (Burke et al., "*Listeria monocytogenes* is resistant to lysozyme by the regulation, not acquisition, of cell wall modifying enzymes," *J. Bacteriol.* (2014) JB.02053-02014). Lastly, mutant R60.1

contained a mutation in *wall*, a regulator of the WalRK two-component system required for autolysin regulation and lysozyme resistance in *L. monocytogenes* (Burke et al., *id.*).

Most notably, mutants R57 and R60.1 contained SNPs in genes encoding two essential components of the canonical Sec pathway, SecY and SecA, respectively.

- 5 These mutations resulted in amino acid substitutions leading to altered protein function and the SNP in *secY* is analyzed in detail below. The identification of mutations in *secY* and *secA* shows a link between SecA2 and the general Sec-pathway in *L. monocytogenes*.

- 10 Improved swarming motility and lack of chaining did not improve cell-to-cell spread in most of the mutants, with the exception of R57. This mutant showed a complete loss of chaining (Figure 3A) and restored cell-to-cell spread to 87% of the WT strain (Figure 1). Based on the increased plaque size, we hypothesized that this strain would show increased virulence *in vivo*. However, upon intravenous infection of mice, the R57 mutant was just as attenuated as the $\Delta secA2$ parental strain (Figure 3C-D). The
- 15 lack of even a partial rescue of virulence was somewhat surprising and suggested that either the *secA2* mutation cannot be overcome *in vivo*, or that the combination of SNPs resulted in virulence suppression. Because of these prominent phenotypes and a possible link with the SecYEG translocon, R57 was chosen for further analysis.

20 **C. Mutations in the *lmo2769* operon resulted in lysozyme susceptibility and increased swarming motility but did not contribute to increased plaque formation.**

- Four out of five sequenced revertants had SNPs in *lmo2769* (Table 5). The first gene of this uncharacterized operon, *lmo2769*, encodes an ATP-binding protein, followed by an ABC transporter permease and a membrane protein encoded by *lmo2768*
- 25 and *lmo2767*, respectively. The function of the ABC transporter is unknown; however, it has been implicated in lysozyme sensitivity (Burke et al., J Bacteriol. (2014)196:3756-67). It seemed probable that mutations in this operon, in addition to contributing to lysozyme susceptibility, also enhanced swarming motility of $\Delta secA2$ revertants. Indeed the swarming motility of $\Delta lmo2769\Delta secA2$ mutant was twice that of the parent $\Delta secA2$
- 30 strain (Figure 4A). The $\Delta lmo2769$ mutants were also significantly more susceptible to lysozyme than WT or $\Delta secA2$ (Figure 4B, Figure 6 and (Burke et al, *id.*)). There was no change in susceptibility to other cell wall acting agents tested (Figure 6), however *lmo2768::Tn* has been shown to be more susceptible to CAMPs and cefuroxime (Burke et al, *id.*). The increase in swarming motility and lysozyme susceptibility was diminished
- 35 by complementing either the $\Delta lmo2769\Delta secA2$ double mutant or R57 with the *lmo2769* operon (Figure 4A-B).

Lysozyme susceptibility caused by the disruption of the *Imo2769* operon had negligible impact on virulence (Figure 4C and Figures 7A-B). Plaque size of the $\Delta secA2\Delta Imo2769$ double mutant increased 10% from that of $\Delta secA2$, however complementation of R57 with the *Imo2769-Imo2767* operon had no effect on plaque formation of the revertant (Figure 4C). We concluded that disruption of *Imo2769* led to a cell wall defect that increased swarming motility of the mutant, but was not responsible for the increased plaque size in the originally isolated R57 suppressor strain.

Deletion of *Imo1721*, a gene harboring the second SNP identified in R57, had a suppressive effect on the swarming motility of $\Delta secA2$ and the double $\Delta secA2\Delta Imo2769$ mutants (Figure 8D) and had no effect on lysozyme susceptibility (Figure 6F). This gene encodes the transcriptional regulator LacR, which has been suggested to suppress virulence in *L. monocytogenes* in response to cellobiose (Milenbachs et al., "Deregulation of *Listeria monocytogenes* virulence gene expression by two distinct and semi-independent pathways," *Micobiology* (2004) 150:321–333; Stoll & Goebel, "The major PEP-phosphotransferase systems (PTSs) for glucose, mannose and cellobiose of *Listeria monocytogenes*, and their significance for extra- and intracellular growth," *Micobiology* (2010) 156:1069-1083) in addition to regulating the phosphotransferase system (Dalet et al., "Characterization of a unique $\sigma 54$ -dependent PTS operon of the lactose family in *Listeria monocytogenes*," *Biochimie* (2003) 85:633–638). Plaque formation was unaffected by in-frame deletion of $\Delta Imo1721$ alone or in combination with a *secA2* deletion (Figure 4D), but had a suppressive effect on the $\Delta Imo1721\Delta Imo2769$ double mutant, suggesting that the combination of the two mutations negatively influence virulence when *SecA2* is undisturbed. Collectively these observations suggest that the SNP in *Imo1721* did not contribute to the revertant phenotype.

D. The *secY* mutation significantly improved virulence and swarming motility of $\Delta secA2$.

The third SNP identified in the R57 revertant was in *Imo2612*, encoding *SecY*, which resulted in amino-acid substitution G408R. The G408 residue corresponded to V411 when aligned to the *E. coli* *SecY* and mapped to the last transmembrane segment of the protein. Mutations in this region of *E. coli* *SecY*, such as L407R and I408N, have been described as protein localization (*prl*) mutations (Osborne et al., "PrIA suppressor mutations cluster in regions corresponding to three distinct topological domains," *EMBO J.* (1993) 12:3391-3398; Smith et al., "Modeling the effects of *prl* mutations on the *Escherichia coli* *SecY* complex," *J. Bacteriol.* (2005) 187:6454-6465; Lycklama a Nijeholt et al., "Immobilization of the plug domain inside the *SecY* channel allows unrestricted

protein translocation," J. Biol. Chem. (2010) 285:23747–23754.). We refer to the *L. monocytogenes* *secY* (G408R) allele as *prlA1* in the remainder of this manuscript.

As in other bacteria, *secY* is essential in *L. monocytogenes* (Hossain et al., "Identification of putative drug targets of *Listeria monocytogenes* F2365 by subtractive genomics approach," J. Bio. Sci. Biotech. (2013) 2:63-71.) and is transcribed with other essential genes in a 28-gene operon. Attempts to introduce the *prlA1* mutation into a WT background by allelic exchange at the native *secY* locus were unsuccessful. Therefore, we used phage transduction utilizing a *Himar1* transposon insertion in a neighboring gene (*lmo2637*) as an antibiotic marker to transduce the *prlA1* allele from the R57 revertant and the WT *secY* into various strains. Disruption of *lmo2637* had a minor effect on swarming motility (Figure 8A) but no effect on virulence (Figures 8C and 7C-D).

Introduction of the *prlA1* mutation into the $\Delta secA2$ strain or the $\Delta secA2 \Delta lmo2769$ double mutant resulted in enhanced motility (Figure 5A) and restored the smooth colony morphology of these mutants. In addition, motility of R57 diminished significantly (88%) upon replacement of *prlA1* with the WT allele (Figure 5A), which was coupled with a change in colony morphology from smooth to rough. This suggested that the *prlA1* mutation significantly contributed to the R57 motility and cell morphology phenotypes.

Surprisingly the *prlA1* mutation alone was sufficient to completely restore cell-to-cell spread of the $\Delta secA2$ and the $\Delta lmo2769 \Delta secA2$ double mutants (Figure 5B). Exchanging the *prlA1* mutation for the WT allele in R57 decreased plaque formation from 87% to 39%, the level observed for the $\Delta lmo2769 \Delta secA2$ double mutant (Figure 8C).

To investigate whether destabilization of the SecY channel in response to the *prlA1* mutation was sufficient to restore protein secretion in $\Delta secA2$ mutants, the abundance of P60 in the supernatants of mutant cultures was evaluated as a proxy for other SecA2-dependent proteins. Notably, P60 levels were completely restored to WT levels in the *secA2* constructs expressing the *prlA1* mutation (Figure 5C). Restoration of P60 was entirely *PrlA1*- dependent as exchanging the *prlA1* for the WT allele in R57 background diminished the secreted P60 levels to those observed for the $\Delta secA2$ parent strain (Figure 8B). Further, to address the possibility of differing secretion levels between the WT and $\Delta secA2 prlA1$ strains, we undertook a timed experiment in which secretion levels of P60 were assessed. The introduction of the *prlA1* mutation restored the levels of P60 secretion in the $\Delta secA2$ strain, while not affecting the secretion of SecA-dependent listeriolysin O (LLO) (Fig. 5D). Interestingly, P60 levels of R57 mutant were only 80% of the WT strain (Figure 5C) and were not directly linked to $\Delta lmo1721$ (Figure 5D) or $\Delta lmo2769$ (Figure 5C). Collectively these observations show that a *prlA1* mutation alone was sufficient to overcome the motility, cell morphology and cell-to-cell spread

phenotypes associated with $\Delta secA2$, by restoring secretion of the autolysin P60 and likely other SecA2-dependent proteins to WT levels.

Lastly, the effect of the *prlA1* allele on virulence *in vivo* was assessed. Somewhat surprisingly, a *prlA1* mutation alone in a WT background did not affect virulence (Figure 7C). However, virulence of both *secA2* and the $\Delta mo2769\Delta secA2$ double mutant were partially restored (Figure 5E-F and Figures 7E-F). Whereas the *secA2* mutant and the R57 suppressor were 1,000-fold less virulent in the spleen and 10,000-fold less virulent in the liver (Figure 3C-D and Figure 5E-F), the *prlA1* mutation partially rescued virulence by approximately, 100-fold in the spleen and 20-fold in the liver (Figures 5E-F). Notably though, it was still considerably less virulent than WT (20-fold in the spleen and 1000-fold in the liver).

III. Discussion

Much of what is known about the general secretory pathway (Sec) in bacteria originates from seminal genetic studies that led to the identification and characterization of the Sec components including the SecYEG channel and SecA ATPase (Beckwith J, "The Sec-dependent pathway," Res. Microbiol. (2013) 164:497-504; Kusters & Driessen, "SecA, a remarkable nanomachine," Cell. Mol. Life Sci. (2011) 68:2053-2066; Tam et al., "Investigating the SecY plug movement at the SecYEG translocation channel," EMBO J. (2005) 24:3380-3388; Flower AM, "The SecY translocation complex: convergence of genetics and structure, Trends Microbiol. (2007) 15:203-210; Osborne & Silhavy, "PrfA suppressor mutations cluster in regions corresponding to three distinct topological domains," EMBO J. (1993) 12:3391-3398; Smith et al., "Modeling the effects of prf mutations on the Escherichia coli SecY complex," J. Bacteriol. (2005) 187:6454-6465; Duong & Wickner, "The PrfA and PrfG phenotypes are caused by a loosened association among the translocase SecYEG subunits," EMBO J. (1999) 18:3263-3270). While the *secA* is essential in all bacteria, a subset of Gram-positive bacteria harbor an additional, nonessential homologous gene called *secA2* (Feltcher & Braunstein, "Emerging themes in SecA2-mediated protein export," Nature Rev. Microbiol. (2012) 10:779-789; Lenz et al., "SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis," Proc. Nat'l Acad. Sci USA (2003) 100:12432-12437; Rigel & Braunstein, "A new twist on an old pathway – accessory secretion systems," Mol. Microbiol. (2008) 69:291-302.; Bensing et al., "Selective transport by SecA2: An expanding family of customized motor proteins," Biochim. Biophys. Acta (2014) 1843:1674-1686; Schneewind & Missiakas, "Protein secretion and surface display in Gram-positive bacteria," Phil. Trans. R Soc. B (2012)

367:1123-1139; Nguyen-Mau et al., "Secretion genes as determinants of *Bacillus anthracis* chain length," J. Bacteriol (2012) 194:3841-3850). Both pathogenic and nonpathogenic species of *Listeria* encode SecA2 (Mishra et al., "Genetic organisation and molecular characterization of secA2 locus in *Listeria* species," Gene (2011) 489:76-85), which facilitates the secretion of two major autolysins contributing to septation, swarming motility, plaque formation in tissue culture cells and virulence in mice (Lenz et al., "SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis," Proc. Nat'l Acad. Sci USA (2003)100:12432-12437; Lenz & Portnoy, "Identification of a second *Listeria* secA gene associated with protein secretion and the rough phenotype," Mol. Microbiol. (2002) 45:1043-1056; Machata et al., "Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in *Listeria monocytogenes*," J. Bacteriol. (2005) 187:8385-8394; Mishra et al., "Genetic organisation and molecular characterization of secA2 locus in *Listeria* species," Gene (2011) 489:76-85). It remains unclear, however, why certain bacteria encode secA2 while others do not, and why secA2 is dispensable for cell viability.

In this study, we took advantage of the swarming motility defect to isolate spontaneous mutants capable of swarming motility (Figure 1). One mutant was phenotypically very similar to WT and was characterized in depth, with its phenotype being genetically traced directly to a single amino acid mutation in SecY. Similar mutations in *E. coli* secY, called *prlA* alleles are gain-of-function mutations that expand the repertoire of substrates that can be exported (Flower, *id.*; Osborne & Silhavy, *id.*; Smith et al., *id.*; van der Wolk et al., "PrIA4 prevents the rejection of signal sequence defective preproteins by stabilizing the SecA-SecY interaction during the initiation of translocation," EMBO J(1998) 17:3631-3639). Our findings indicate that the secY mutant identified in this study, here named *prlA1*, rescued the *L. monocytogenes* secA2 mutant by allowing for secretion of proteins that are normally SecA2-dependent. Recent work in *Mycobacterium smegmatis* reported that increased SecY levels suppressed the severe phenotypes of a secA2 mutant (Ligon et al., "Suppressor analysis reveals a role for SecY in the SecA2-dependent protein export pathway of Mycobacteria," J. Bacteriol. (2013) 195:4456-4465), suggesting that SecA2-dependent protein secretion occurs through interaction with the canonical SecY. Thus, in both *L. monocytogenes* and *M. smegmatis*, the canonical secY is very likely part of the translocation pore for SecA2 substrates unlike other pathogenic bacteria including *Bacillus anthracis* and some *Streptococcus* and *Staphylococcus* species, all of which have SecA2 and SecY2 (Feltcher & Braunstein, "Emerging themes in SecA2-mediated protein export," Nature Rev. Microbiol. (2012) 10:779-789; Bensing et al., "Selective transport by SecA2: An

expanding family of customized motor proteins," *Biochim. Biophys. Acta* (2014) 1843:1674-1686; Schneewind & Missiakas, "Protein secretion and surface display in Gram-positive bacteria," *Phil. Trans. R Soc. B* (2012) 367:1123-1139; Nguyen-Mau S, "Secretion genes as determinants of *Bacillus anthracis* chain length," *J. Bacteriol.* (2012) 194:3841-3850.).

Wild-type *L. monocytogenes* is highly lysozyme resistant (Rae et al., "Mutations of the *Listeria monocytogenes* peptidoglycan N-deacetylase and O-acetylase result in enhanced lysozyme sensitivity, bacteriolysis, and hyperinduction of innate immune pathways," *Infect. Immun.* (2011) 79:3596-3606; Burke et al., "*Listeria monocytogenes* is resistant to lysozyme by the regulation, not acquisition, of cell wall modifying enzymes," *J. Bacteriol.* (2014) JB.02053-02014; Boneca et al., "A critical role for peptidoglycan N-deacetylation in *Listeria* evasion from the host innate immune system," *Proc. Nat'l Acad. Sci. USA* (2007) 104:997-1002). One curious finding reported in this study was that all 70 suppressors were lysozyme-sensitive and in four of the five sequenced suppressor mutants, lysozyme sensitivity was caused by mutations in an uncharacterized ABC transporter encoded by the *lmo2769-lmo2767* operon. Lysozyme susceptibility of the fifth mutant was likely due to a mutation in *wall*, which was previously shown to be required for lysozyme resistance (Burke et al., *id.*). The first gene in the *lmo2769-lmo2767* operon, encodes an ATP-binding protein similar to a poorly characterized *B. subtilis* protein (YtrB) whose expression has been observed following exposure to cell wall acting agents (Dyer et al., "A molecular mechanism of bacterial flagellar motor switching," *J. Mol. Biol.* (2009) 388:71-84). Mutation of either *lmo2769* or *lmo2768* conferred lysozyme susceptibility, suggesting that these mutations altered the cell wall and may have led to decreased chaining and increased swarming. Although the exact function of this operon requires further characterization, these data suggest that the *lmo2769* operon is involved in maintaining cell wall homeostasis (Collins et al., "The ABC transporter AnrAB contributes to the innate resistance of *Listeria monocytogenes* to nisin, bacitracin, and various β -lactam antibiotics," *Antimicrob. Agents Chemother.* (2010) 54:4416-4423; Cuthbertson et al., "ABC transporters involved in export of cell surface glycoconjugates," *Microbiol Mol. Biol. Rev.* (2010) 74:341-362). The high frequency of mutations detected in this operon, associated with increased swarming, likely provided a growth advantage in semisolid media that increased the probability of acquiring less frequent mutations, for example the *prlA1*.

The *prlA1* allele characterized in this study resulted in a glycine substitution for arginine (G408R) in the tenth transmembrane helix of SecY. Variations in this region of SecY in *E. coli*, such as L407R (PrIA301) and I408N (PrIA4), as well as PrIA-like

mutation in the accessory SecY2 of *Streptococcus gordonii* (Bensing & Sullam, "Transport of preproteins by the accessory Sec system requires a specific domain adjacent to the signal peptide," J. Bacteriol (2010) 192:4223–4232), promote translocation of proteins with defective signal peptides by destabilizing the pore ring within the SecYEG channel (Osborne & Silhavy, *id.*; Smith et al., *id.*; Lycklama a Nijeholt et al., "Immobilization of the plug domain inside the SecY channel allows unrestricted protein translocation," J. Biol. Chem. (2010) 285:23747–23754). It is likely that the substitution of a hydrophobic glycine for a positively charged hydrophilic arginine residue would also induce a conformational change to the pore ring. The results indicate that the rescue in protein secretion is a result of a more efficient interaction between SecA and SecY induced by the conformational change of the pore ring, similar to that observed in a PrlA4 variant of *E. coli* (van der Wolk et al., *id.*; de Keyzer et al., "The F286Y mutation of PrlA4 tempers the signal sequence suppressor phenotype by reducing the SecA binding affinity," FEBS Lett. (2002) 510:17–21).

Protein secretion is an energetically costly process and in the absence of SecA2, another ATPase, such as SecA, is required to provide energy for translocation of P60 in the *L. monocytogenes* $\Delta secA2prlA1$ mutant. Since SecA is dimeric during protein translocation (Kusters et al., "Quaternary structure of SecA in solution and bound to SecYEG probed at the single molecule level," Structure (2011) 19:430-439) it is possible that SecA2 and SecA form a heterodimer, which interacts with the SecYEG, thereby modulating substrate specificity of the channel. The interaction between SecA2 and the canonical SecA ATPase has previously been shown to occur in *Streptococcus* (Zhou et al., "Canonical SecA associates with an accessory secretory protein complex involved in biogenesis of a Streptococcal serine-rich repeat glycoprotein," J. Bacteriol. (2011) 193:6560-6566) and implied in *Mycobacteria* (Rigel et al., "The accessory SecA2 system of Mycobacteria requires ATP binding and the canonical SecA1," J. Biol. Chem/ (2009) 284:9927-9936) and more recently in *Listeria* (Halbedel et al., "A systematic proteomic analysis of Listeria monocytogenes house-keeping protein secretion systems," Mol. Cell Proteomics (2014) mcp.M114.041327). In the absence of a functional SecA2, SecA may drive SecA2-dependent secretion, although at a much lower rate, potentially due to a lower affinity of SecA alone for the specific substrates. This view is supported by the observation that the export of P60 and other SecA2-dependent proteins is not completely abolished in the *secA2* mutant (Lenz et al., *id.*; Lenz & Portnoy, *id.*).

Although not yet characterized, we also identified a suppressor mutation in *secA*, providing further support for a possible interaction between SecA2 and SecA in *L. monocytogenes*. This mutation resulted in an amino-acid substitution D599N

corresponding to D649 in the second nucleotide-binding domain of SecA in *E.coli*. Amino acid alterations in this region of SecA, in particular residues 631 to 653, are known as *azi* as these confer azide resistance resulting from an increased ATPase activity (Das et al., "The variable subdomain of Escherichia coli SecA functions to regulate SecA ATPase activity and ADP release," J. Bacteriol. (2012) 194:2205-2213; Schmidt et al., "Nucleotide binding activity of SecA homodimer is conformationally regulated by temperature and altered by *prlD* and *azi* mutations," J. Biol. Chem. (2000) 275:15440-15448) in addition to having an increased affinity for SecY (Schmidt et al., *id.*). A SecA homodimer with enhanced affinity for SecY either through *prlA1* or *azi* mutations may suffice in overcoming the requirement for SecA2 in a *secA2* mutant. The enhanced interaction of SecA with SecY would indirectly increase the rate of secretion of SecA2-dependent proteins independent of altering affinity of the ATPase for these substrates.

Another piece of evidence supporting the interaction between the two ATPases comes from a recent proteomics study, which showed that P60 secretion is indistinguishable between WT *L. monocytogenes* EGD-e and $\Delta secA2$ when grown in a defined medium at 37°C, but was diminished in the mutant at 20°C (Renier et al., "Exoproteomic analysis of the SecA2-dependent secretion in *Listeria monocytogenes* EGD-e," J. Proteomics (2013) 80:183–195), thus suggesting that SecA2-dependent secretion is able to engage an alternative pathway under different environmental conditions. One ATPase or dimer combination may be favored over the other depending on the energetic state of the cell. For example, the affinity of SecA2 for ATP in *Mycobacteria* is higher than that of SecA (Hou et al., "ATPase activity of *Mycobacterium tuberculosis* SecA1 and SecA2 proteins and its importance for SecA2 function in macrophages," J. Bacteriol. (2008) 190:4880-4887). In order not to compete for substrate, SecA2 is bound to ADP in a dormant state until it is required (D'Lima et al., "ADP-dependent conformational changes distinguish *Mycobacterium tuberculosis* SecA2 from SecA1," J. Biol. Chem. (2014) 289:2307-2317). Considering there is no conservation in the signal peptides of proteins being secreted through the SecA2-dependent pathway in *L. monocytogenes*, it remains unclear how proteins are targeted to the SecA2 (Lenz et al., *id.*; Renier et al., *id.*) and how that can be overcome in certain conditions.

It is curious why *Listeria* species require SecA2 for normal septation, while most other bacteria use other SecA2-independent mechanisms. One possibility is that *secA2* mutants have an advantage under certain conditions. Indeed, *secA2* mutants arise spontaneously in the lab as rough colonies that emanate from a smooth colony and spread on solid media (Lenz & Portnoy, *id.*; Monk et al., "Morphotypic conversion in

Listeria monocytogenes biofilm formation: biological significance of rough colony
 isolates," Appl. Environ. Microbiol. (2004) 70:6686-6694; Gutekunst et al., "A
 filamentous-like mutant of Listeria monocytogenes with reduced expression of a 60-
 kilodalton extracellular protein invades and grows in 3T6 and Caco-2 cells," Can. J.
 5 Microbiol. (1992) 38:843-851). At ambient temperatures, these mutants readily form
 distinct filamentous biofilms, which are thicker than those formed by the WT strain (24,
 69). The spontaneous and reversible morphologic conversion to a rough phenotype may
 provide a potential advantage to *L. monocytogenes* in its saprophytic phase by
 enhancing the ability to colonize abiotic surfaces. This may also be one of the reasons
 10 why the nonpathogenic *Listeria* species have retained SecA2, however, this potential
 advantage outside the host comes at a significant cost to its pathogenic phase, as *L.*
monocytogenes *secA2* mutants are approximately 1,000-fold attenuated for virulence in
 mice (Figure 3C-D and (Lenz et al., *id.*; Lenz & Portnoy, *id.*; Machata et al.,
 "Simultaneous deficiency of both MurA and p60 proteins generates a rough phenotype in
 15 *Listeria monocytogenes*," J. Bacteriol. (2005) 187:8385-8394), although rough mutants
 can persist in the gut (Zachar & Savage, "Microbial interference and colonization of the
 murine gastrointestinal tract by *Listeria monocytogenes*," Infect. Immun. (1979) 23:168-
 174) and the gallbladder (Hardy et al., "Extracellular replication of *Listeria*
monocytogenes in the murine gall bladder," Science (2004) 303:851-853). Some of this
 20 attenuation has been traced to the lower levels of secreted autolysins primarily P60 and
 to a lesser extend NamA (Lenz et al., *id.*). However, even though the *prlA1* mutation
 described in the study restored WT levels of secreted P60 and cell-to-cell spread, it only
 resulted in a 100-fold rescue in virulence *in vivo* of the attenuated *secA2* mutant. The
 suppressor mutant was still less virulent than the WT strain suggesting that a functional
 25 SecA2 is still required for full expression of *L. monocytogenes* virulence. SecA2-
 dependent secretion may have evolved in *L. monocytogenes* to provide a mechanism for
 switching between the parasitic and the saprophytic phase thus providing an advantage
 in its ability to thrive in the environment as well as inside a host.

30 VI. Summary

The bulk of bacterial protein secretion occurs through the conserved SecY
 translocation channel that is powered by SecA-dependent ATP hydrolysis. Many Gram-
 positive bacteria, including the human pathogen *Listeria monocytogenes*, possess an
 additional nonessential specialized ATPase, SecA2. SecA2-dependent secretion is
 35 required for normal cell morphology and virulence in *L. monocytogenes*; however, the
 mechanism of export via this pathway is poorly understood. *L. monocytogenes* *secA2*

mutants form rough colonies, have septation defects, are impaired for swarming motility, and form small plaques in tissue culture cells. In this study, 70 spontaneous mutants were isolated that restored swarming motility to *L. monocytogenes secA2* mutants. Most of the mutants had smooth colony morphology and septated normally, but all were

5 lysozyme sensitive. Five representative mutants were subjected to whole-genome sequencing. Four of the five had mutations in proteins encoded by the *lmo2769* operon that conferred lysozyme sensitivity and increased swarming but did not rescue virulence defects. A point mutation in *secY* was identified that conferred smooth colony morphology to *secA2* mutants, restored wild-type plaque formation, and increased

10 virulence in mice. This *secY* mutation resembled a *pri* suppressor known to expand the repertoire of proteins secreted through the SecY translocation complex. Accordingly, the $\Delta secA2 priA1$ mutant showed wild-type secretion levels of P60, an established SecA2-dependent secreted autolysin. Although the *pri* mutation largely suppressed almost all of the measurable SecA2-dependent traits, the $\Delta secA2 priA1$ mutant was still less virulent in

15 vivo than the wild-type strain, suggesting that SecA2 function was still required for pathogenesis.

Notwithstanding the appended clauses, the disclosure set forth herein is also defined by the following clauses:

1. A *Listeria* bacterium comprising a general secretory pathway (GSP)

20 mutation and a heterologous nucleic acid.

2. The *Listeria* bacterium according to Clause 1, wherein the GSP mutation results in enhanced protein secretion.
3. The *Listeria* bacterium according to Clauses 1 or 2, wherein the GSP pathway mutation comprises a mutant GSP protein.

25 4. The *Listeria* bacterium according to Clause 3, wherein the mutant GSP protein is a mutant of SecY or SecA.

5. The *Listeria* bacterium according to Clause 4, wherein the mutant GSP protein is a SecY mutant.
6. The *Listeria* bacterium according to Clause 5, where the SecY mutant

30 results in a conformational change of a translocation pore configuration.

7. The *Listeria* bacterium according to Clauses 4 or 5, wherein the SecY mutant comprises a protein localization (PRL) mutation.
8. The *Listeria* bacterium according to any of Clauses 5 to 7, wherein the Sec Y mutant comprises a mutation in the SecY tenth transmembrane helix.

9. The *Listeria* bacterium according to Clause 8, wherein the mutation comprises a substitution mutation.
10. The *Listeria* bacterium according to Clause 9, wherein the mutation comprises a substitution of a hydrophobic residue for a hydrophilic residue.
- 5 11. The *Listeria* bacterium according to Clause 10, wherein the mutation comprises a mutation at G408.
12. The *Listeria* bacterium according to Clause 11, wherein the mutation comprises a G408R mutation.
- 10 13. The *Listeria* bacterium according to Clause 4, wherein the mutant GSP protein is a SecA mutant.
14. The *Listeria* bacterium according to Clause 13, wherein the SecA mutant comprises an AZI mutation.
15. The *Listeria* bacterium according to Clauses 13 or 14, wherein the SecA mutant comprises a substitution mutation.
- 15 16. The *Listeria* bacterium according to Clause 13, wherein the substitution mutation comprises a conserved mutation.
17. The *Listeria* bacterium according to Clause 16, wherein the conserved mutation comprises a substitution of polar residues.
18. The *Listeria* bacterium according to Clause 17, wherein the mutation
20 comprises a mutation at D599.
19. The *Listeria* bacterium according to Clause 18, wherein the mutation comprises a D599N mutation.
20. The *Listeria* bacterium according to any of the preceding clauses, wherein the heterologous nucleic acid is integrated.
- 25 21. The *Listeria* bacterium according to Clause 20, wherein the heterologous nucleic acid encodes at least one product.
22. The *Listeria* bacterium according to Clause 21, wherein the at least one product is an antigen.
23. The *Listeria* bacterium according to Clause 22, wherein the antigen is
30 secreted by a SecA2 mediated pathway.
24. The *Listeria* bacterium according to any of the preceding clauses, wherein the bacterium is attenuated.
25. The *Listeria* bacterium according to any of the preceding clauses, wherein the bacterium comprises a SecA2 mutant.
- 35 26. The *Listeria* bacterium according to any of the preceding clauses, wherein the *Listeria* bacterium is *Listeria monocytogenes*.

27. A vaccine comprising a *Listeria* bacterium according to any of Clauses 1 to 26.

28. A method of eliciting or boosting a cellular immune response in a subject, said method comprising:

5 administering to said subject an effective amount of a vaccine according to Clause 27.

29. A method of delivering a nucleic acid or polypeptide into a cell, said method comprising:

10 introducing into said cell a *Listeria* bacterium according to any of Clauses 1 to 26, wherein said *Listeria* bacterium comprises nucleotide coding sequence for the nucleic acid or protein.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein.

35 Rather, the scope and spirit of present invention is embodied by the appended claims.

WHAT IS CLAIMED IS:

1. A *Listeria* bacterium comprising a general secretory pathway (GSP) mutation and a heterologous nucleic acid.
- 5 2. The *Listeria* bacterium according to Claim 1, wherein the GSP mutation results in enhanced protein secretion.
3. The *Listeria* bacterium according to Claims 1 or 2, wherein the GSP
10 pathway mutation comprises a mutant GSP protein.
4. The *Listeria* bacterium according to Claim 3, wherein the mutant GSP protein is a mutant of SecY or SecA.
- 15 5. The *Listeria* bacterium according to Claim 4, wherein the mutant GSP protein is a SecY mutant.
6. The *Listeria* bacterium according to Claim 5, where the SecY mutant results in a conformational change of a translocation pore configuration.
20
7. The *Listeria* bacterium according to Claims 4 or 5, wherein the SecY mutant comprises a protein localization (PRL) mutation.
8. The *Listeria* bacterium according to any of Claims 5 to 7, wherein
25 the Sec Y mutant comprises a mutation in the SecY tenth transmembrane helix.
9. The *Listeria* bacterium according to Claim 8, wherein the mutation comprises a substitution mutation.
- 30 10. The *Listeria* bacterium according to Claim 9, wherein the mutation comprises a substitution of a hydrophobic residue for a hydrophilic residue.

11. The *Listeria* bacterium according to Claim 10, wherein the mutation comprises a mutation at G408.

12. The *Listeria* bacterium according to Claim 4, wherein the mutant
5 GSP protein is a SecA mutant.

13. The *Listeria* bacterium according to Claim 12, wherein the SecA mutant comprises an AZI mutation.

10 14. The *Listeria* bacterium according to any of the preceding claims, wherein the heterologous nucleic acid is integrated.

15 15. The *Listeria* bacterium according to any of the preceding claims, wherein the *Listeria* bacterium is *Listeria monocytogenes*.

16. A vaccine comprising a *Listeria* bacterium according to any of Claims 1 to 15.

17. A method of eliciting or boosting a cellular immune response in a
20 subject, said method comprising:

administering to said subject an effective amount of a vaccine according to Claim 16.

18. A method of delivering a nucleic acid or polypeptide into a cell, said
25 method comprising:

introducing into said cell a *Listeria* bacterium according to any of Claims 1 to 15, wherein said *Listeria* bacterium comprises nucleotide coding sequence for the nucleic acid or protein.

30



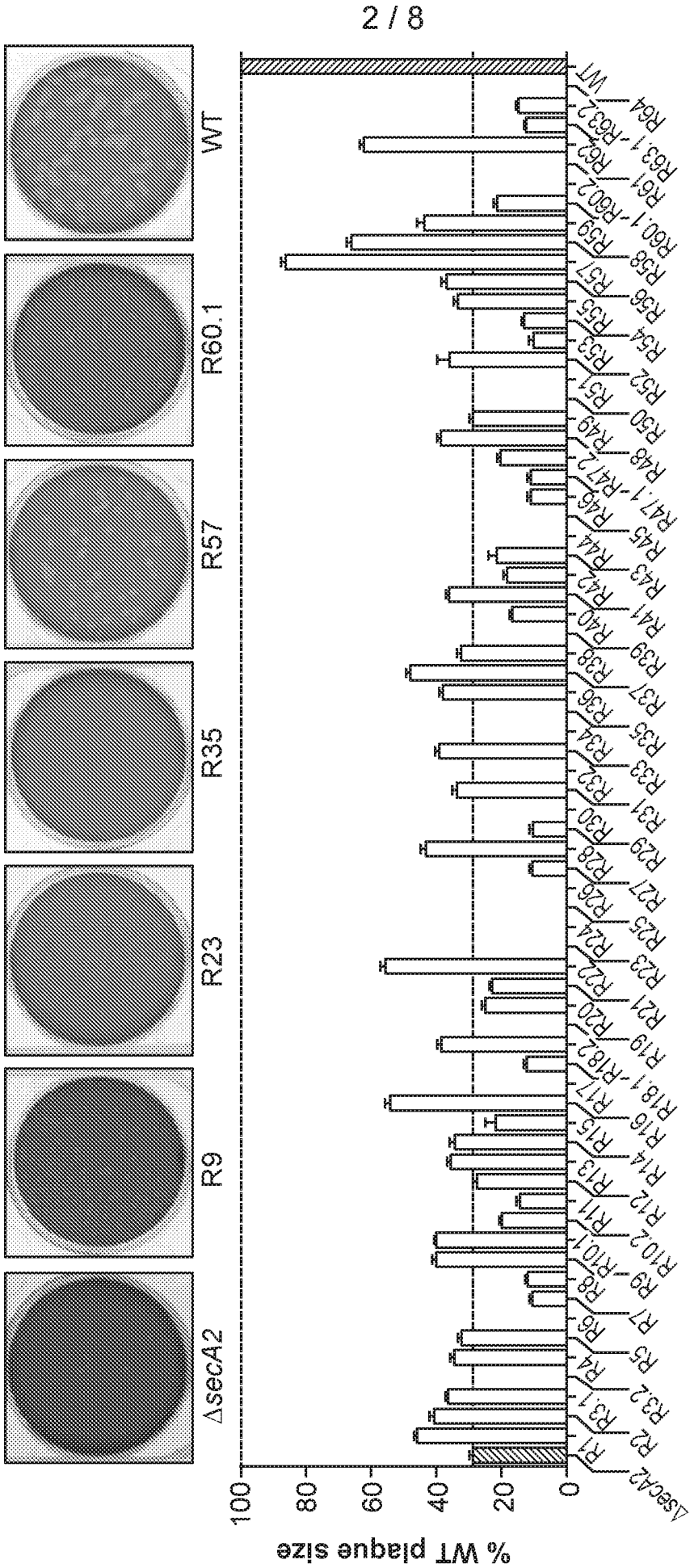


FIG. 2

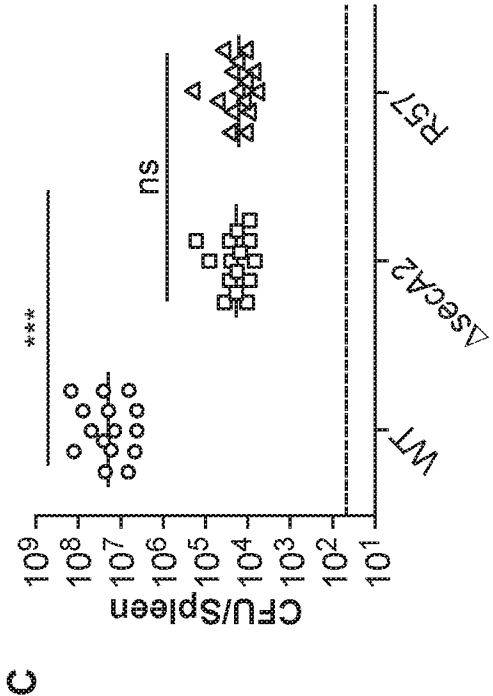
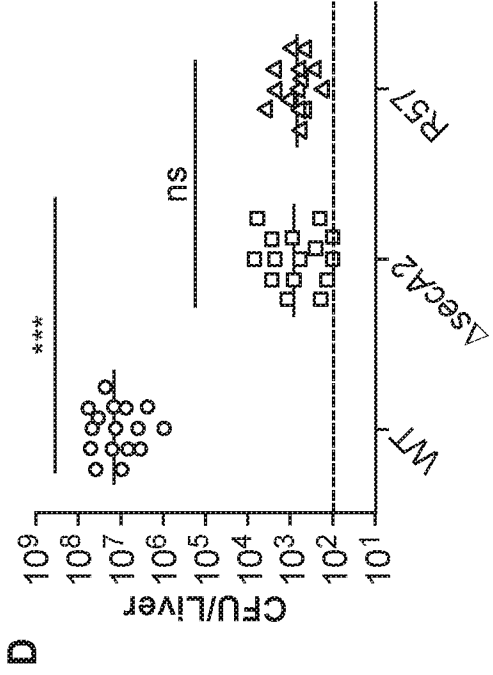
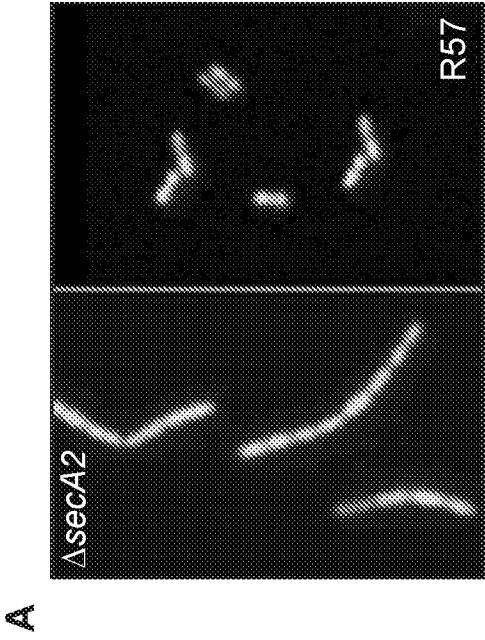
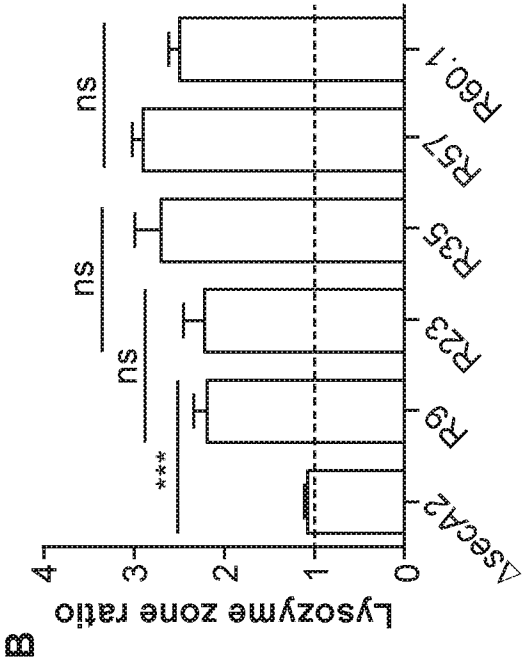


FIG. 3

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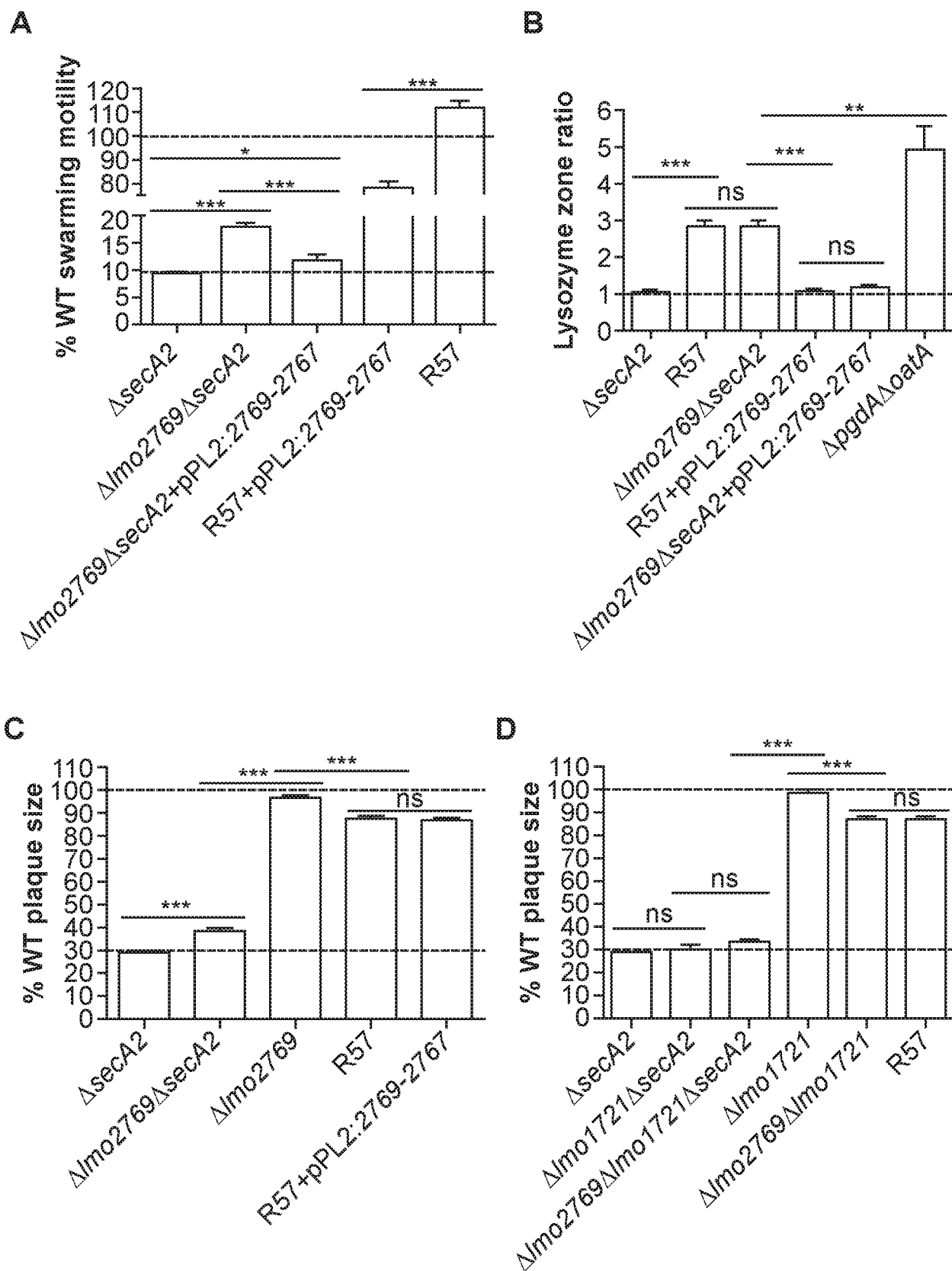


FIG. 4

SUBSTITUTE SHEET (RULE 26)

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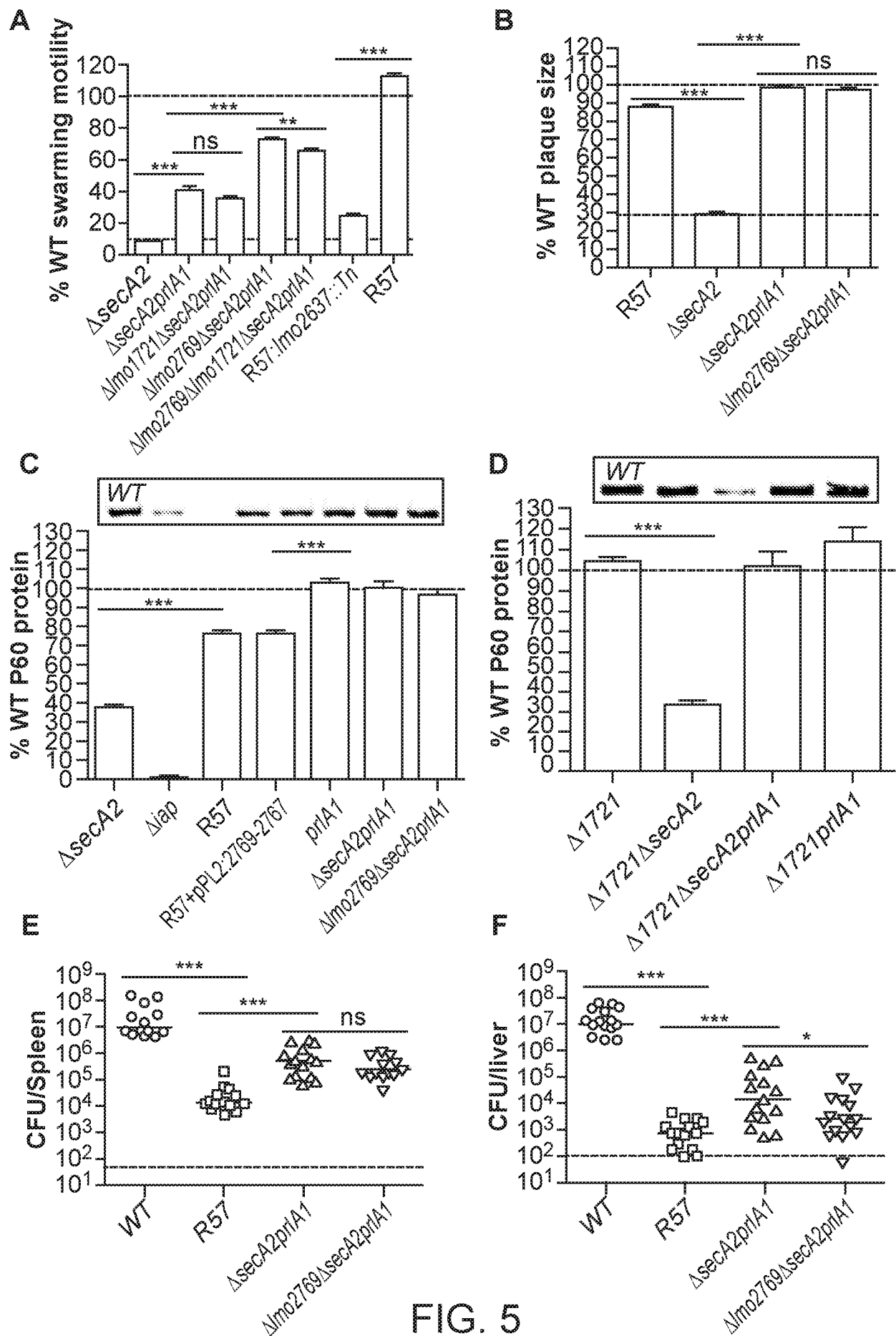


FIG. 5

SUBSTITUTE SHEET (RULE 26)

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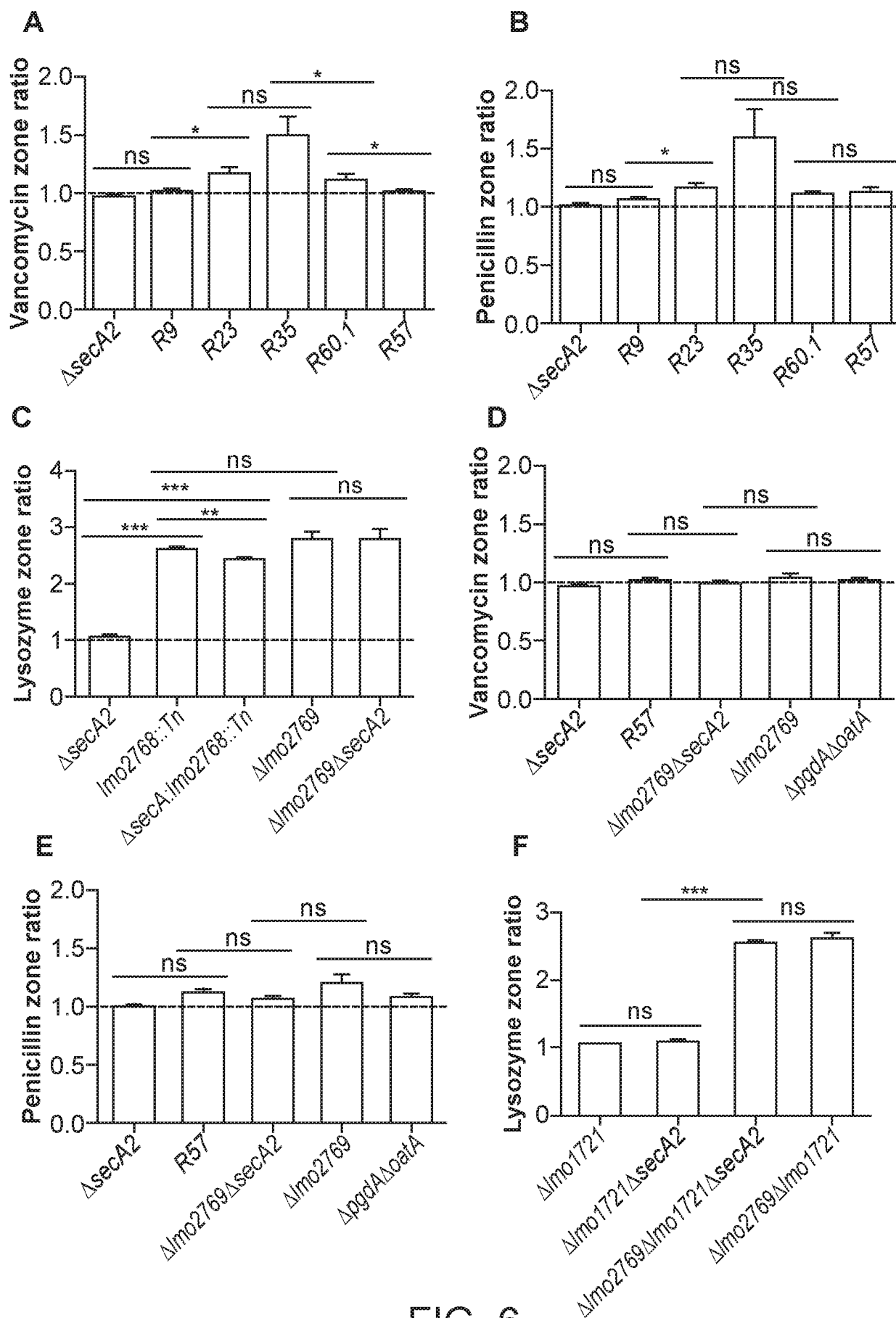


FIG. 6

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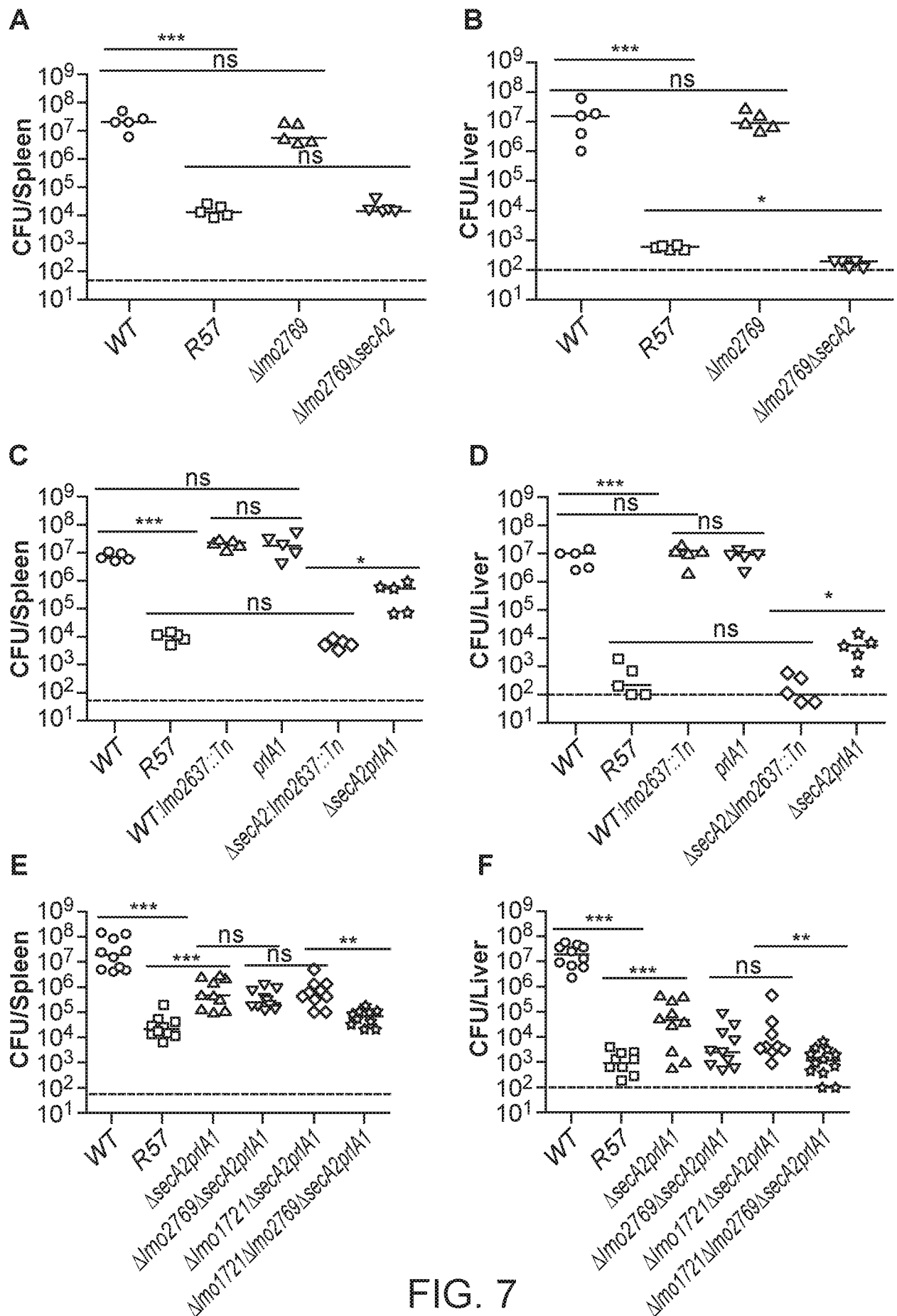


FIG. 7

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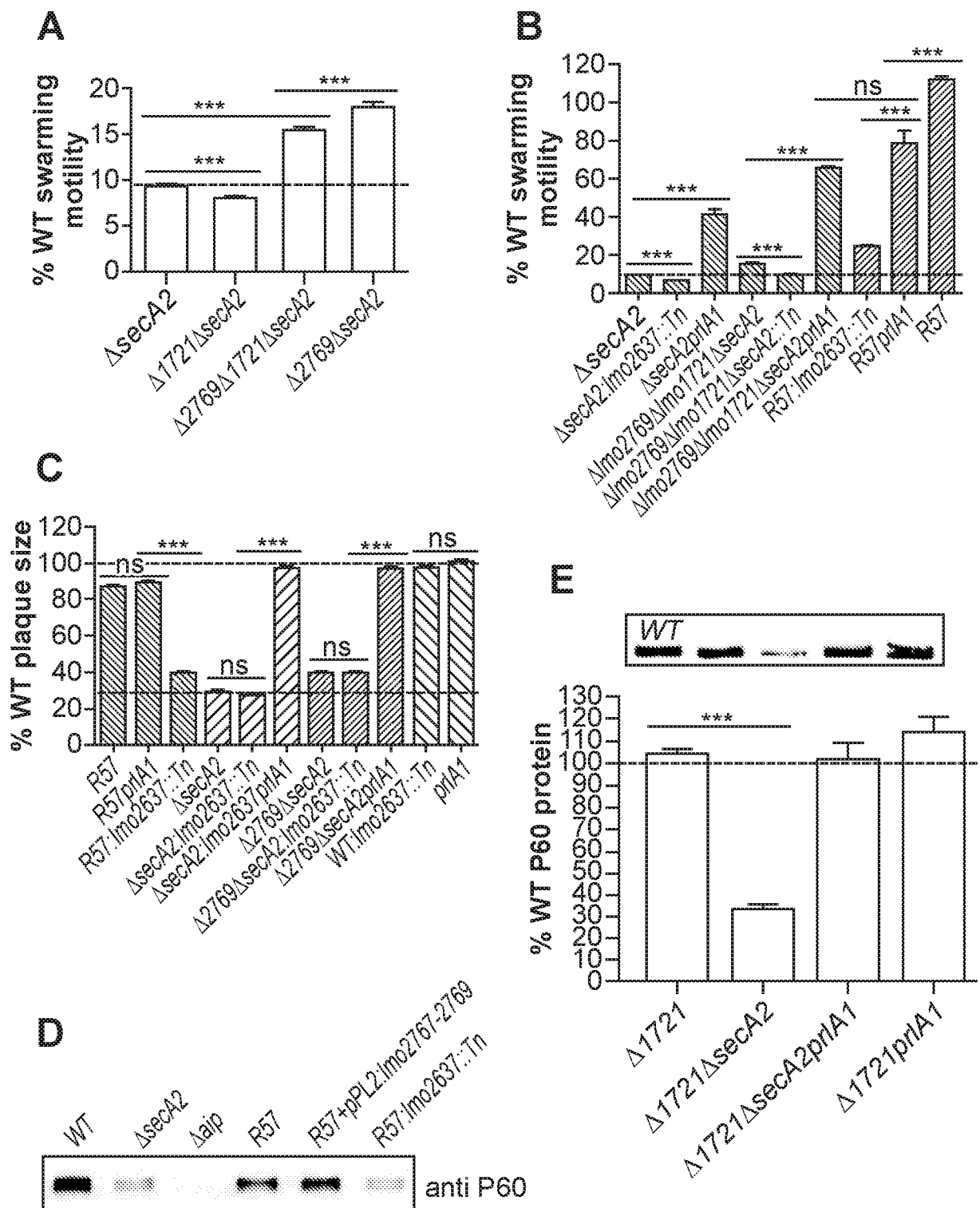


FIG. 8

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/59007

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A61K 39/02; A61K 48/00; C12N 1/20; C12N 15/74 (2016.01) CPC - A61K 2039/522; A61K 2039/523; A61K 48/00; C12N 15/74 According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC(8) - A61K 39/02; A61K 48/00; C12N 1/20; C12N 15/74 (2016.01) CPC - A61K 2039/522; A61K 2039/523; A61K 48/00; C12N 15/74; USPC - 424/235.1; 424/93.2; 435/252.3; 435/471 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched IPC(8) - A61K 39/02; A61K 48/00; C12N 1/20; C12N 15/74 (2016.01) - see keyword below CPC - A61K 2039/522; A61K 2039/523; A61K 48/00; C12N 15/74; USPC - 424/235.1; 424/93.2; 435/252.3; 435/471 Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PubWEST(USPT,PGPB,EPAB,JPAB); PatBase; Medline, Google; Listeria, monocytogenes, general secretory pathway, GSP, mutation, mutant, SecA, SecY, vaccine, DNA, heterologous, nucleic acid, PrA1, PrI, Imo2612, avirulent, attenuated, reduced, decrease, virulence, secrete, SecE, SecD, YajC, YidC, polynucleotide, enhance, increase, therapy, treat				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
Y	HALBEDEL et al., A Systematic Proteomic Analysis of Listeria monocytogenes House-keeping Protein Secretion Systems. Mol Cell Proteomics. 2014 Nov, Vol. 13(11), p. 3063-81. Epub 2014 Jul 23. Abstract; pg 3063, col 2, lower para; pg 3064, col 1, col 2, lower para, and Table 1; pg 3065, col 1, last para; pg 3069, col 1, last para; pg 3070, col 2, lower para; pg 3071, Fig 2D; pg 3072, col 1, lower para, and col 2; pg 3074, col 2; and pg 3075, col 2, and Fig 5	1-6		
Y	US 2011/0287055 A1 (LAUER et al.) 24 November 2011 (24.11.2011), para [0002], [0010], [0059], [0140], [0167], [0168], [0178], and [0250]	1-6		
A	NCBI_NC_00321_SecA_SecY, Listeria monocytogenes strain EGD, complete genome. 08 November 2011 [online]. [Retrieved on 2016.01.05]. Retrieved from the Internet: <URL: http://www.ncbi.nlm.nih.gov/nuccore/16802048?sat=8&satkey=2081954 > pg 1, Definition; pg 475, the last gene complement(2587300..2589825) SecA; and pg 496, the third gene complement (2695476..2696771), SecY	1-6		
A	BONDAR et al., Dynamics of SecY Translocons with Translocation-Defective Mutations. Structure. 2010, Vol. 18(7), p. 847-85. Entire documentation especially pg 848, Fig 1; pg 851, Fig 3; and pg 852, col 2	1-6		
A	DESVAUX et al., The protein secretion systems in Listeria: inside out bacterial virulence. FEMS Microbiol Rev. 2006, Vol. 30(5), p. 774-805. Entire documentation, especially Abstract; pg 776, Table 1; pg 777, col 2, last para; and pg 779, Fig 2	1-6		
Y,P	DURACK et al., A prl Mutation in SecY Suppresses Secretion and Virulence Defects of Listeria monocytogenes secA2 Mutants. J Bacteriol. 2015 Mar, Vol. 197(5), p. 932-42. Epub 2014 Dec 22. Entire documentation, especially, Abstract; pg 932, col 1, para 1; pg 935, Table 2; pg 936, col 2, last para; and pg 939, col 2, top and last para	1-6		
☐ Further documents are listed in the continuation of Box C.				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; border: none;"> * Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; vertical-align: top; border: none;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family </td> </tr> </table>			* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family			
Date of the actual completion of the international search 08 January 2016		Date of mailing of the international search report 04 MAR 2016		
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774		

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 15/59007

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 7-11, 14-18
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+: Claims 1-6, 12-13, directed to a *Listeria* bacterium comprising a general secretory pathway (GSP) mutation and a heterologous nucleic acid. The GSP pathway mutation will be searched to the extent that the GSP pathway mutation comprises a mutant GSP protein, wherein the mutant GSP protein is a mutant of SecY. It is believed that claims 1-6 encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass the GSP pathway mutation comprises a mutant GSP protein, wherein the mutant GSP protein is a mutant of SecY. Additional GSP pathway mutation will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected GSP pathway mutation. Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be wherein the mutant GSP protein is a mutant of SecA [claims: (1-4), 12-13]. *****Continued in the extra sheet*****

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-6

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Continuation of:
Box No III (unity of invention is lacking)

The inventions listed as Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Feature

Among Groups I+, (a mutant of) SecY is structurally and functionally different from (a mutant of) SecA.

Common Technical Features

The inventions of Groups I+ share the technical feature of a *Listeria* bacterium comprising a general secretory pathway (GSP) mutation and a heterologous nucleic acid (claim 1),

- wherein the GSP mutation results in enhanced protein secretion (claim 2); and
- wherein the GSP pathway mutation comprises a mutant GSP protein (claim 3).

However, these shared technical features do not represent a contribution over prior art as being obvious over an article entitled 'A Systematic Proteomic Analysis of *Listeria monocytogenes* House-keeping Protein Secretion Systems' by HALBEDEL et al. (hereinafter 'Halbedel'; Mol Cell Proteomics. 2014 Nov, Vol. 13(11), p. 3063-81. Epub 2014 Jul 23), in view of US 2011/0287055 A1 to LAUER et al. (hereinafter 'Lauer') as follows:

Halbedel discloses a *Listeria* bacterium comprising a general secretory pathway (GSP) mutation (pg 3065, col 1, last para - 'protein secretion systems of *L. monocytogenes*. Mutant strains lacking...expressing the SecA ATPase, the YidC membrane insertases SpoIIJ and YqjG', wherein '*L. monocytogenes* is a *Listeria* bacterium', each of 'SecA' and 'YidC membrane insertases SpoIIJ and YqjG' is 'a general secretory pathway (GSP)' protein; Abstract - '*Listeria monocytogenes*...Most of its virulence factors are secretory proteins... *L. monocytogenes* encodes... protein secretion pathways. ...Sec-dependent transport, the YidC membrane insertases SpoIIJ and YqjG'; pg 3069, col 1, last para - 'Deletion of *L. monocytogenes* Secretion Genes... secretion mutants were constructed ...a mutant allowing for conditional expression of the secA (lmo2510) gene encoding the housekeeping secretion ATPase SecA (strain LMS104)... constructed mutants in the spoIIJ (lmo2854, LMS100) and yqjG (lmo1379, LMS6) genes'; pg 3063, col 2, lower para to pg 3064, col 1 - 'the general secretion (Sec) system...composed of the protein conducting SecYEG channel and the auxiliary SecDF and YajC proteins. Protein secretion is driven by the SecA ATPase'; Specification: pg 6, ln 36 to pg 7, ln 1 - 'the mutant GSP protein is a transmembrane component, e.g., SecY, SecE, SecG, SecDF, YajC, YidC').

Halbedel further discloses wherein the general secretory pathway (GSP) secretory proteins are bacterial virulence factors (Abstract - '*Listeria monocytogenes* ... Most of its virulence factors are secretory proteins either released to the medium or attached to the bacterial surface'), and further wherein the mutation of a certain the general secretory pathway (GSP) protein reduces the virulence of the *Listeria* (pg 3070, col 2, lower para - 'the greater wax moth *Galleria mellonella* was used as an infection model for a better discrimination of possible attenuation effects in the three mutant strains. ... 80% of the larvae infected with the Del.SpoIIJ mutant were still viable 7 days post infection (Fig. 2D). This demonstrates that SpoIIJ ... has an impact on pathogenicity of *L. monocytogenes*', wherein 'SpoIIJ' is 'a general secretory pathway (GSP)' protein 'YidC'; pg 3065, col 1, last para - 'protein secretion systems of *L. monocytogenes*. Mutant strains lacking...expressing the SecA ATPase, the YidC membrane insertases SpoIIJ'; pg 3071, Fig 2D).

Halbedel does not specially teach wherein the *Listeria* bacterium comprising a heterologous nucleic acid. Lauer discloses a method of using a mutant *Listeria* strain with reduced virulence comprising a heterologous nucleic acid for expressing a therapeutic heterologous polypeptide (para [0002] - 'recombinant *Listeria* useful for expression of polypeptides, including heterologous polypeptides...recombinant bacteria comprising mutations in PrfA which are useful in vaccine compositions'; para [0010] - 'recombinant *Listeria* bacterium is attenuated ... an actA mutation ...recombinant PrfA* *Listeria* bacterium ... is attenuated for proliferation'; para [0059] - 'polynucleotides encoding the heterologous polypeptide(s) are recombinant'; para [0168] - 'vaccine compositions ... treating conditions in a host'), as a vaccine composition (para [0140] - 'making a vaccine composition comprising... *Listeria* nucleic acid is modified so that the proliferation of the *Listeria* population is attenuated'; para [0167] - 'recombinant *Listeria*...a vaccine comprising a recombinant bacterium (e.g. a recombinant *Listeria* bacterium) comprises introducing a recombinant nucleic acid molecule into the bacterium, wherein the recombinant nucleic acid molecule encodes an antigen'; para [0002] - 'recombinant *Listeria* useful for expression of polypeptides, including heterologous polypeptides... comprising mutations in PrfA which are useful in vaccine compositions'; para [0250] - 'enhanced immune potency of KBMA PrfA* based vaccines ...over-expression of PrfA-dependent virulence genes can increase cytotoxicity, resulting in decreased virulence of wild-type strains'; para [0178] - 'Vaccines can be evaluated for their ability to provide either a prophylactic or therapeutic effect against a particular disease').

It would have been obvious to one of ordinary skill in the art at the time the invention was made to combine the teachings of Halbedel and Lauer, to obtain a *Listeria* bacterium comprising a general secretory pathway (GSP) mutation, as taught by Halbedel, and further wherein the *Listeria* bacterium comprising a heterologous nucleic acid, based on the combination of Lauer and Halbedel, in order to combine the technologies and methods available in the art for facilitating generating vaccine compositions by using *Listeria* mutant strains with reduced or attenuated virulence for facilitating delivering heterologous nucleic acids for treating different disorders with an expected success and without undue experimentation.

*****Continued in the next extra sheet*****

Continuation of:

The previous extra sheet - Box No III (unity of invention is lacking)

Halbedel further discloses wherein the GSP mutation results in enhanced protein secretion (pg 3072, col 2 - 'Identification of SecA-dependent Extracellular Proteins by Proteomics-Extracellular proteins isolated from SecA-expressing and SecA-depleted LMS104 cells ... In total, 562 spots were detected on these gels. Proteins spots were considered to be significantly accumulated ...upon SecA depletion when their spot volume was at least two times greater ... A total of 166 spots with a significantly increased level were found', wherein 'SecA-dependent Extracellular Proteins ...A total of 166 spots with a significantly increased level were found' is an indication of 'the GSP mutation results in enhanced protein secretion'; pg 3075, col 2 - 'a single mutation in the secA gene is sufficient to restore p60 and MurA secretion'; pg 3075, Fig 5; pg 3064, col 1 - 'Protein secretion is driven by the SecA ATPase, which energizes translocation of secreted and trans-membrane proteins through the membrane-embedded SecYEG pore...secretion of some listerial proteins requires the presence of the accessory SecA2 ATPase ... includes the autolysins p60 ...MurA'); and -- wherein the GSP pathway mutation comprises a mutant GSP protein (pg 3069, col 1, last para - 'Deletion of L. monocytogenes Secretion Genes... secretion mutants were constructed ...a mutant allowing for conditional expression of the secA (lmo2510) gene encoding the housekeeping secretion ATPase SecA (strain LMS104).... constructed mutants in the spoIIJ (lmo2854, LMS100) and yqjG (lmo1379, LMS6) genes'; Abstract - 'Listeria monocytogenes... L. monocytogenes encodes... protein secretion pathways. ... Sec-dependent transport, the YidC membrane insertases SpoIIJ and YqjG'; pg 3063, col 2, lower para to pg 3064, col 1 - 'the general secretion (Sec) system... composed of the protein conducting SecYEG channel and the auxiliary SecDF and YajC proteins. Protein secretion is driven by the SecA ATPase'; Specification: pg 6, ln 36 to pg 7, ln 1 - 'the mutant GSP protein is a transmembrane component, e.g., SecY, SecE, SecG, SecDF, YajC, YidC').

Without a shared special technical feature, the inventions lack unity with one another.

Groups I+ therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Continuation of item 4: Claims 7-11, 14-18 are not drafted in accordance with the second and third sentences of Rule 6.4 (a). These claims are improper multiple dependent claims.