

FACULTATIVELY ATTENUATED BACTERIAL SPECIES AND METHODS OF PREPARATION AND USE THEREOF

CROSS REFERENCES TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 61/723,234, filed November 6, 2012, which is hereby incorporated in its entirety including all tables, figures, and claims.

BACKGROUND OF THE INVENTION

[0002] The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

[0003] Pathogenic organisms are, by definition, capable of causing disease in an infected host. For clinical use of such organisms, attenuated vaccine strains are often created which exhibit reduced or eliminated virulence, but which still retain sufficient viability to stimulate a desired immune response against the pathogen or heterologous antigen(s) of interest. Attenuated vector platforms have been demonstrated to elicit protective responses specific for encoded heterologous antigens in a number of experimental models, including infectious and malignant diseases.

[0004] Although most attenuated vaccine vectors are viral, bacterial vaccine vector platforms have been developed for both prophylactic and therapeutic applications. Attenuated strains of many otherwise pathogenic bacteria are now available and the ease of manipulation for generating recombinant strains provides a means for using bacteria as efficacious delivery vehicles for a number of foreign proteins such as antigens associated with infectious diseases and cancer. Live attenuated bacterial vaccine strains have been developed from, inter alia, *Listeria*, *Escherichia*, *Salmonella*, *Shigella*, *Lactobacillus*, and *Yersinia* species.

[0005] While such vaccine strains may exhibit reduced virulence, their safety, particularly in immune compromised individuals, remains a concern. One example of a

strategy to further reduce the risk of bacterial vaccines is the so-called Killed But Metabolically Active (“KBMA”) approach. KBMA vaccine strains are constructed by abrogating the capacity for nucleotide excision repair through deletion of DNA repair genes such as *uvrA* and *uvrB*. The gene deletion renders the bacteria exquisitely sensitive to photochemical inactivation through the combined treatment of psoralens and UVA. Because of their inability to repair the psoralen-induced DNA cross-links formed, KBMA bacterial strains are unable to replicate and are thus functionally noninfectious. This characteristic provides an improved safety profile in comparison to live attenuated strains. The very limited number of cross-links, however, preserves their metabolic activity, including antigen expression, and thus their immune potential. Manufacturing of KBMA strains exhibiting consistent properties, however, may be difficult, as titration of the number of cross-links is dependent on a number of difficult to control variables.

[0006] There remains a need in the art to provide attenuated bacterial vaccine strains with advantageous safety profiles for use treatment or prevention of diseases having a risk-benefit profile not appropriate for live attenuated vaccines.

BRIEF SUMMARY OF THE INVENTION

[0007] The present invention provides facultatively attenuated bacterial species and methods of preparation and use thereof. The term “facultatively attenuated” as used herein refers to a bacterium which comprises a set of defined recombinant modifications which have substantially no effect on the ability of the bacterium to grow by multiplication when the bacterium is outside of its host organism, but which result in deletion of one or more genes essential for multiplication of the bacterium when the bacterium is introduced into its host organism, for example within host cells of a vaccinee recipient. These recombinant modifications take advantage of regulatory sequences which preferentially induce expression of genes within the mammalian host.

[0008] As described hereinafter, the bacterium is most preferably an intracellular pathogen, such as *Listeria monocytogenes*.

[0009] In a first aspect of the invention, the invention relates to methods of configuring a bacterium to delete one or more genes in the bacterial genome which are essential for multiplication of the bacterium, the deletion occurring preferentially when the bacterium is introduced into a host. These methods comprise:

- (i) recombinantly introducing into the bacterium a nucleic acid encoding a recombinase heterologous to the bacterium, wherein the nucleic acid encoding the recombinase is operably connected to regulatory sequences which preferentially induce expression of the recombinase in a mammalian host cell, and
- (ii) recombinantly introducing in the bacterial genome a first attachment site for the recombinase upstream from the gene(s) essential for multiplication of the bacterium, and a second attachment site for the recombinase downstream from the gene(s) essential for multiplication of the bacterium.

[0010] The recombinase, when specifically induced and expressed in the mammalian host cell, catalyzes a site specific recombination event which deletes the gene(s) essential for multiplication of the bacterium flanked by the first and second attachment sites. Following this site specific recombination event the bacterium is deficient for multiplication.

[0011] The phrase “substantially no effect on the ability of the bacterium to grow by multiplication” as used herein refers to a bacterium in which colony formation is at least 75% of that of a bacterium which is otherwise identical, but which lacks the defined recombinant modifications described above. For convenience, such a bacterium which lacks the recombinantly introduced recombinase sequence and the recombinantly introduced first and second attachment sites are referred to herein as a “wild type” bacterium. In preferred embodiments, colony formation is at least 80% of that of a wild type bacterium, more preferably at least 90% of a wild type bacterium, and most preferably at least 95% of a wild type bacterium. Colony formation is determined in an in vitro colony formation assay, and is expressed as colony-forming units (CFU).

[0012] As used herein with regard to a gene (or genes), the term “essential for multiplication” refers to a gene that, when deleted, results in a bacterium in which colony formation is reduced to 1% of wild type or less, and/or in which the growth of the bacterium (measured by CFU) in host cells contained in the organism is reduced by at least 1000-fold relative to wild type. Preferably, colony formation is reduced to 0.01% of wild type or less. A bacterium in which such a gene (or genes) has been deleted is referred to herein as being “deficient for multiplication.”

[0013] In the context of the present invention, deletion of a gene (or genes) essential for multiplication occurs preferentially when the bacterium is introduced into a host organism.

As used herein, a deletion event occurs “preferentially in a host” if introduction of the bacterium into the host organism converts a bacterium in which colony formation is at least 75% of that of a wild type bacterium into a bacterium in which colony formation is reduced to 1% of wild type or less. Preferably, colony formation is reduced to 0.01% of wild type or less.

[0014] The term “regulatory sequences which preferentially induce expression of the recombinase in the host cell” refer to regulatory sequences which induce expression of a gene under control thereof by at least 10-fold upon introduction of the bacterium into a host organism. By way of example only, expression of genes under the *actA* promoter of *Listeria* is dependent upon a regulatory factor known as PrfA for transcriptional activation. Relative to broth-grown *Listeria*, gene expression under *actA*/PrfA regulation is induced approximately 200-fold when *Listeria* is present in host cells. Thus, in certain embodiments the regulatory sequences comprise a *Listeria monocytogenes* promoter which is PrfA-dependent. PrfA-dependent promoters may be selected from the group consisting of the *inlA* promoter, the *inlB* promoter, the *inlC* promoter, the *hpt* promoter, the *hly* promoter, the *plcA* promoter, the *mpl* promoter, and the *actA* promoter. Similar systems to induce gene expression in host organisms for other bacterial species are described hereinafter. In preferred embodiments, such preferentially induced expression increases at least 50-fold, more preferably at least 100-fold, and still more preferably at least 1000-fold, upon introduction of the bacterium into a host organism.

[0015] The term “host organism” as used herein refer to an organism in which the bacterium of interest is able to multiply in the absence of deletion of a gene (or genes) essential for multiplication as described herein. In certain embodiments, a host organism is a mammalian species, most preferably a human. Also, in certain embodiments, the bacterium is an intracellular pathogen, and the regulatory sequences preferentially induce expression of the recombinase when the bacterium is in a mammalian host cell. Preferred bacterial genuses are selected from the group consisting of *Listeria*, *Neisseria*, *Mycobacterium*, *Francisella*, *Bacillus*, *Salmonella*, *Shigella*, *Yersinia*, *Brucella*, *Legionella*, *Rickettsia*, and *Chlamydia*. This list is not meant to be limiting. Most preferably, the bacterium is a facultative intracellular bacterium such as *Listeria*, *Salmonella*, *Shigella*, *Francisella*, *Mycobacterium*, *Legionella*, *Burkholderia* and *Brucella*. In certain exemplary embodiments described hereinafter, the bacterium is *Listeria monocytogenes*, including modified such as *Listeria*

monocytogenes $\Delta ActA/\Delta InlB$ (a *L. monocytogenes* in which the native *ActA* and *InlB* genes have been deleted or rendered functionally deleted by mutation).

[0016] As noted above, a recombinase sequence which is recombinantly introduced into a bacterium is heterologous to the bacterium. As used herein, this term refers to a recombinase which is not a normal constituent of the bacterial genome. In various embodiments, the recombinase may be selected from the group consisting of ϕ C31 integrase, R4 integrase, TP901 integrase, ϕ BT1 integrase, BxB1 integrase, PSA integrase, Cre recombinase, Flp recombinase, XerC recombinase, λ integrase, HK022 integrase, P22 integrase, HP1 integrase, L5 integrase, $\gamma\delta$ recombinase, Tn3 recombinase, gin recombinase, RV integrase, SPBc integrase, TG1 integrase, ϕ C1 integrase, MR11 integrase, ϕ 370 integrase, ϕ K38 integrase, W β integrase, and BL3 integrase. Suitable recombinase attachment sites can include recombinantly introduced *attB* and *attP* sites.

[0017] In certain embodiments, the gene(s) essential for multiplication of the bacterium comprise at least one gene involved in DNA replication. Such genes may be selected from the group consisting of *ori*, *dnaA*, *dnaN*, *gyrA*, *gyrB*, *polC*, *dnaE*, *ftsK*, *ftsZ*, *ligA*, *dnaG*, *parC*, *parE*, *holB*, *dnaX*, *SMC*, and *ftsY*.

[0018] As described hereinafter, a plurality of genes essential for multiplication of the bacterium are often grouped together as a single operon. In this case, the first attachment site may be preferentially recombinantly introduced upstream of a portion of the operon, and the second attachment site recombinantly introduced downstream of a portion of the operon, such that the site specific recombination event deletes a plurality of such genes in a single event. Preferably, the first attachment site is upstream of the operon, and the second attachment site is downstream of the operon. The first and second attachment sites can flank a nucleic acid sequence about 20kb in length or less, about 10kb in length or less, and about 6kb in length. The term "about" in this context refers to +/- 10% of a given length.

[0019] In certain embodiments, the bacterium is utilized as an expression platform for expressing one or more genes which are heterologous to the bacterium, for example for purposes of generating an immune response to the heterologous proteins expressed from those genes. In these embodiments, the bacterium can comprise within the bacterial genome an exogenous nucleic acid sequence encoding a heterologous polypeptide(s), wherein the exogenous nucleic acid sequence is operably connected to regulatory sequences which

preferentially induce expression of the heterologous polypeptide when the bacterium is in a mammalian host.

[0020] In a related aspect, the invention relates to methods of deleting one or more genes in a bacterial genome which are essential for multiplication of a bacterium. These methods comprise introducing the facultatively attenuated bacterium described herein to a host organism host under conditions wherein the recombinase is expressed by the bacterium, and wherein the expressed recombinase deletes the one or more genes essential for multiplication of the bacterium by the site specific recombination event.

[0021] In yet another aspect, the present invention relates to a facultatively attenuated bacterium, or population thereof such as a bacterial culture, as described herein. Such a bacterium comprises:

- (i) a nucleic acid encoding a recombinase heterologous to the bacterium, wherein the nucleic acid encoding the recombinase is operably connected to regulatory sequences which preferentially induce expression of the recombinase in the host, and
- (ii) a first attachment site for the recombinase upstream from the gene(s) essential for multiplication of the bacterium, and a second attachment site for the recombinase downstream from the gene(s) essential for multiplication of the bacterium, wherein the first and second attachment sites are operably linked such that the recombinase, when expressed in the mammalian host, catalyzes a site specific recombination event which deletes the gene(s) essential for multiplication of the bacterium flanked by the first and second attachment sites.

As noted above, in certain embodiments the bacterium is an intracellular pathogen, and is most preferably a facultative intracellular bacterium, and the regulatory sequences preferentially induce expression of the recombinase when the bacterium is in a mammalian host cell. A suitable bacterium is of a genus selected from the group consisting of *Listeria*, *Neisseria*, *Mycobacterium*, *Francisella*, *Bacillus*, *Salmonella*, *Shigella*, *Yersinia*, *Brucella*, *Legionella*, *Rickettsia*, and *Chlamydia*. This list is not meant to be limiting. Most preferably, the bacterium is a facultative intracellular bacterium such as *Listeria*, *Salmonella*, *Shigella*, *Francisella*, *Mycobacterium*, *Legionella*, *Burkholderia*, and *Brucella*. In certain exemplary embodiments described hereinafter, the bacterium is *Listeria monocytogenes*, including modified such as *Listeria monocytogenes* $\Delta ActA/\Delta InlB$. In certain embodiments, the gene(s)

essential for multiplication of the bacterium comprise at least one gene involved in DNA replication. Such genes may be selected from the group consisting of *ori*, *dnaA*, *dnaN*, *gyrA*, *gyrB*, *polC*, *dnaE*, *ftsK*, *ftsZ*, *ligA*, *dnaG*, *parC*, *parE*, *holB*, *dnaX*, *SMC*, and *ftsY*.

[0022] As also noted above, a recombinase sequence which is recombinantly introduced into a bacterium is heterologous to the bacterium. As used herein, this term refers to a recombinase which is not a normal constituent of the bacterial genome. In various embodiments, the recombinase may be selected from the group consisting of ϕ C31 integrase, R4 integrase, TP901 integrase, ϕ BT1 integrase, BxB1 integrase, PSA integrase, Cre recombinase, Flp recombinase, XerC recombinase, λ integrase, HK022 integrase, P22 integrase, HP1 integrase, L5 integrase, $\gamma\delta$ recombinase, Tn3 recombinase, gin recombinase, RV integrase, SPBc integrase, TG1 integrase, ϕ C1 integrase, MR11 integrase, ϕ 370 integrase, ϕ K38 integrase, W β integrase, and BL3 integrase. Suitable recombinase attachment sites can include recombinantly introduced *attB* and *attP* sites.

[0023] As described hereinafter, a plurality of genes essential for multiplication of the bacterium are often grouped together as a single operon. In this case, the first attachment site may be preferentially recombinantly introduced upstream of a portion of the operon, and the second attachment site recombinantly introduced downstream of a portion of the operon, such that the site specific recombination event deletes a plurality of such genes in a single event. Preferably, the first attachment site is upstream of the operon, and the second attachment site is downstream of the operon. The first and second attachment sites can flank a nucleic acid sequence about 20kb in length or less, about 10kb in length or less, about 6kb in length, or of any length that is sufficient to inactivate multiplication of the bacterium in the host cell. The term "about" in this context refers to +/- 10% of a given length.

[0024] The bacterium of the present invention may be utilized as an expression platform for expressing one or more genes which are heterologous to the bacterium, for example for purposes of generating an immune response to the heterologous proteins expressed from those genes. In these embodiments, the bacterium can comprise within the bacterial genome an exogenous nucleic acid sequence encoding a heterologous polypeptide, wherein the exogenous nucleic acid sequence is operably connected to regulatory sequences which preferentially induce expression of the heterologous polypeptide when the bacterium is in a mammalian host.

BRIEF DESCRIPTION OF THE FIGURES

[0025] Figure 1. Schematic representation of Lm-RHD. (A) In broth culture or fermentation, Lm-RHD do not express the recombinase, thus allowing growth and manufacturing of vaccines. (B) After vaccination, Lm-RHD enter dendritic cells where they simultaneously express the recombinase that results in bacterial death and the vaccine antigen which results in specific immunogenicity.

[0026] Figure 2. Overview of regions deleted in Lm-RHD strains

[0027] Figure 3. Deletion 1. (A) Lox recombinase sites flanking gyrase region (yaaA, recI, gyrB, gyrA); (B) Growth curve in broth culture; (C) Intracellular growth curve in DC2.4 cells.

[0028] Figure 4. Deletion 2. (A) Lox recombinase sites flanking lmo2587, origin, dnaA, dnaN, and lmo0003; (B) Growth curve in BHI Broth; (C) Intracellular growth curve in DC2.4 cells.

[0029] Figure 5. Deletion 3. (A) Lox recombinase sites flanking lmo2582-origin-gyrA region; (B) In vitro growth curve in BHI; (C) Intracellular growth curve in DC2.4 cells.

[0030] Figure 6. Deletion 4. (A) Frt recombinase sites flanking the gyrase region (yaaA, recI, gyrB, gyrA); (B) In vitro growth curve in BHI; (C) Intracellular growth curve in DC2.4 cells.

[0031] Figure 7. Comparison of intracellular expression of various antigens from live attenuated (Lm11) and Lm-RHD (BH3618) platforms. A-D: Intracellular Western blot of four pairs of strains in the Lm11 or BH3618 strain background. (A) Lane 1: live Lm strain Lm11; Lane 2: Lm RHD strain BH3618; platform strains without added antigen. (B) Lane 3: live Lm strain BH3943; lane 4: Lm-RHD strain BH3953; Antigen: ActAN100-Ny-ESO-1-MAGE A3 fusion protein (<). (C) Lane 5: Live Lm strain BH3947; lane 6: Lm-RHD strain BH3957; Antigen: ActAN100-HBV Polymerase-HBxAg fusion protein (*). (D) Lane 9: Live Lm strain BH3951; lane 10: Lm-RHD strain BH3961; Antigen: ActAN100-Plasmodium falciparum CSP fusion protein (◄).

[0032] Figure 8. Accelerated clearance and safety of Lm-RHD strains in immune-competent and immune-deficient mice.

[0033] Figure 9. Immunogenicity of Lm-RIID strains. Open circles: unstimulated; gray: stimulated with peptide noted above.

[0034] Figure 10. Protective immunity to heterologous challenge. (A) Protection from WT-Lm challenge with live attenuated, KBMA, and Lm-RIID strains. (B) Protection from a heterologous challenge with WT vaccinia virus with live-attenuated, KBMA, and Lm-RIID strains.

[0035] Figure 11. Therapeutic efficacy of Lm-RIID vaccines in a CT-26 tumor model.

DETAILED DESCRIPTION OF THE INVENTION

[0036] The present invention relates to compositions and methods for preparing and using facultatively attenuated bacterial species. The present invention can provide attenuated bacterial vaccine strains with advantageous safety profiles for use treatment or prevention of diseases having a risk-benefit profile not appropriate for live attenuated vaccines.

[0037] While described hereinafter in detail with regard to *Listeria monocytogenes*, the skilled artisan will understand that the methods and compositions described herein are generally applicable to bacterial species, and in particular to facultative intracellular bacterial species.

[001] *Listeria monocytogenes* (Lm) is a facultative intracellular bacterium characterized by its ability to induce a profound innate immune response that leads to robust and highly functional CD4 and CD8 T cell immunity specific for vaccine-encoded Ags. Lm is a food-borne bacterium with increased pathogenicity among immune compromised individuals, including patients with cancer or other viral-induced immune deficiencies, pregnant women, the elderly and infants.

[002] Live-attenuated recombinant Lm vaccine platforms engineered to encode a designated antigen(s) relevant to a selected targeted pathogenic agent or malignancy have formed the basis for several human clinical trials. In particular, genetically defined live-attenuated Lm $\Delta actA \Delta inlB$, which is deleted of two virulence genes and is attenuated >3 logs in the mouse listeriosis model, retains its immunologic potency and has been shown to induce robust CD4 and CD8 T cell immunity in both mouse models of human disease as

well as in humans, and has been shown to be safe and well-tolerated in clinical settings among patients with various solid tumor malignancies.

[003] To prime a desired CD8 T cell response, Lm-based vaccines must retain the ability to escape from the vacuole of infected dendritic cells (DCs) in a process mediated by expression of a pore-forming cytolysin known as listeriolysin O (LLO), and desired antigens are engineered to be expressed and secreted from bacteria in the cytoplasm, where they are subsequently processed and presented on MHC class I molecules. Thus, inactivated Lm vaccines, such as those inactivated by heat (Heat-Killed Lm; HKLM) and are not metabolically active and cannot induce a desired CD8 T cell response that can effectively protect against challenge with a pathogen containing the vaccine immunogen or conferring efficacy in tumor-bearing animals. This dichotomy is well-known in the field and represents a challenge to vaccinologists developing Lm vaccine platforms that retain immunologic potency that is comparable to live-attenuated Lm vaccine platforms, yet have the safety of HKLM.

[004] Facultatively attenuated Lm, referred to herein as Recombinase-Induced Intracellular Death (Lm-RIID), has been developed to address the need for safe and immunologically potent Lm-based vaccine platforms. Lm-RIID vaccines concurrently express the vaccine antigen of interest *and* induce the deletion of genes essential for bacterial viability after the Lm vaccine strain escapes into the cytosol of the host cell. Lm-RIID vaccines grow in broth culture with the same properties as live-attenuated Lm vaccines such as Lm $\Delta actA \Delta inlB$ based vaccines, but commit suicide once inside host cells. This is achieved by flanking essential target Lm genes with recombinase (e.g., loxP) sites, and driving expression of a sequence-specific recombinase (e.g., Cre recombinase) as well as vaccine antigen expression, from promoters that are specifically induced in the host organism, in this case in the host cytoplasm. This results in a self-limiting infection even when administered intravenously into a host animal. As described hereinafter, Lm-RIID vaccines can be derived from previously described live-attenuated Lm vaccine strains, for example Lm $\Delta actA \Delta inlB$. Expression of the ActA protein is induced >200-fold with infected host mammalian cells, compared to broth culture. However, the PrfA-dependent *actA* promoter is *NOT* substantially induced in broth culture. Lm-RIID vaccines can be manufactured with the same methods used for growth of live-attenuated Lm vaccines, such as the Lm $\Delta actA \Delta inlB$ strain.

[0038] It is to be understood that the invention is not limited in its application to the details of construction and to the arrangements of the components set forth in the following description or illustrated in the drawings. The invention is capable of embodiments in addition to those described and of being practiced and carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein, as well as the abstract, are for the purpose of description and should not be regarded as limiting.

[0039] As such, those skilled in the art will appreciate that the conception upon which this disclosure is based may readily be utilized as a basis for the designing of other structures, methods and systems for carrying out the several purposes of the present invention. It is important, therefore, that the claims be regarded as including such equivalent constructions insofar as they do not depart from the spirit and scope of the present invention.

[0040] 1. Definitions

[0041] Abbreviations used to indicate a mutation in a gene, or a mutation in a bacterium comprising the gene, are as follows. By way of example, the abbreviation "*L. monocytogenes* $\Delta actA$ " means that part, or all, of the *actA* gene was deleted. The delta symbol (Δ) means deletion. An abbreviation including a superscripted minus sign (*Listeria* ActA⁻) means that the *actA* gene was mutated, *e.g.*, by way of a deletion, point mutation, or frameshift mutation, but not limited to these types of mutations.

[0042] "Administration" as it applies to a human, mammal, mammalian subject, animal, veterinary subject, placebo subject, research subject, experimental subject, cell, tissue, organ, or biological fluid, refers without limitation to contact of an exogenous ligand, reagent, placebo, small molecule, pharmaceutical agent, therapeutic agent, diagnostic agent, or composition to the subject, cell, tissue, organ, or biological fluid, and the like.

"Administration" can refer, *e.g.*, to therapeutic, pharmacokinetic, diagnostic, research, placebo, and experimental methods. Treatment of a cell encompasses contact of a reagent to the cell, as well as contact of a reagent to a fluid, where the fluid is in contact with the cell. "Administration" also encompasses in vitro and ex vivo treatments, *e.g.*, of a cell, by a reagent, diagnostic, binding composition, or by another cell.

[0043] An "agonist," as it relates to a ligand and receptor, comprises a molecule, combination of molecules, a complex, or a combination of reagents, that stimulates the receptor. For example, an agonist of granulocyte-macrophage colony stimulating factor

(GM-CSF) can encompass GM-CSF, a mutein or derivative of GM-CSF, a peptide mimetic of GM-CSF, a small molecule that mimics the biological function of GM-CSF, or an antibody that stimulates GM-CSF receptor.

[0044] An “antagonist,” as it relates to a ligand and receptor, comprises a molecule, combination of molecules, or a complex, that inhibits, counteracts, downregulates, and/or desensitizes the receptor. “Antagonist” encompasses any reagent that inhibits a constitutive activity of the receptor. A constitutive activity is one that is manifest in the absence of a ligand/receptor interaction. “Antagonist” also encompasses any reagent that inhibits or prevents a stimulated (or regulated) activity of a receptor. By way of example, an antagonist of GM-CSF receptor includes, without implying any limitation, an antibody that binds to the ligand (GM-CSF) and prevents it from binding to the receptor, or an antibody that binds to the receptor and prevents the ligand from binding to the receptor, or where the antibody locks the receptor in an inactive conformation.

[0045] As used herein, an “analog” or “derivative” with reference to a peptide, polypeptide or protein refers to another peptide, polypeptide or protein that possesses a similar or identical function as the original peptide, polypeptide or protein, but does not necessarily comprise a similar or identical amino acid sequence or structure of the original peptide, polypeptide or protein. An analog preferably satisfies at least one of the following: (a) a proteinaceous agent having an amino acid sequence that is at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% identical to the original amino acid sequence (b) a proteinaceous agent encoded by a nucleotide sequence that hybridizes under stringent conditions to a nucleotide sequence encoding the original amino acid sequence; and (c) a proteinaceous agent encoded by a nucleotide sequence that is at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 99% identical to the nucleotide sequence encoding the original amino acid sequence.

[0046] “Antigen presenting cells” (APCs) are cells of the immune system used for presenting antigen to T cells. APCs include dendritic cells, monocytes, macrophages, marginal zone Kupffer cells, microglia, Langerhans cells, T cells, and B cells. Dendritic cells occur in at least two lineages. The first lineage encompasses pre-DC1, myeloid DC1,

and mature DC1. The second lineage encompasses CD34⁺CD45RA⁻ early progenitor multipotent cells, CD34⁺CD45RA⁺ cells, CD34⁺CD45RA⁺CD4⁺ IL-3R α ⁺ pro-DC2 cells, CD4⁺CD11c⁻ plasmacytoid pre-DC2 cells, lymphoid human DC2 plasmacytoid-derived DC2s, and mature DC2s.

[0047] “Attenuation” and “attenuated” encompasses a bacterium, virus, parasite, infectious organism, prion, tumor cell, gene in the infectious organism, and the like, that is modified to reduce toxicity to a host. The host can be a human or animal host, or an organ, tissue, or cell. The bacterium, to give a non-limiting example, can be attenuated to reduce binding to a host cell, to reduce spread from one host cell to another host cell, to reduce extracellular growth, or to reduce intracellular growth in a host cell. Attenuation can be assessed by measuring, e.g., an indicum or indicia of toxicity, the LD₅₀, the rate of clearance from an organ, or the competitive index (see, e.g., Auerbuch, *et al.* (2001) *Infect. Immunity* 69:5953-5957). Generally, an attenuation results an increase in the LD₅₀ and/or an increase in the rate of clearance by at least 25%; more generally by at least 50%; most generally by at least 100% (2-fold); normally by at least 5-fold; more normally by at least 10-fold; most normally by at least 50-fold; often by at least 100-fold; more often by at least 500-fold; and most often by at least 1000-fold; usually by at least 5000-fold; more usually by at least 10,000-fold; and most usually by at least 50,000-fold; and most often by at least 100,000-fold.

[0048] “Attenuated gene” encompasses a gene that mediates toxicity, pathology, or virulence, to a host, growth within the host, or survival within the host, where the gene is mutated in a way that mitigates, reduces, or eliminates the toxicity, pathology, or virulence. The reduction or elimination can be assessed by comparing the virulence or toxicity mediated by the mutated gene with that mediated by the non-mutated (or parent) gene. “Mutated gene” encompasses deletions, point mutations, and frameshift mutations in regulatory regions of the gene, coding regions of the gene, non-coding regions of the gene, or any combination thereof.

[0049] “Conservatively modified variants” applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, a conservatively modified variant refers to nucleic acids encoding identical amino acid sequences, or amino acid sequences that have one or more conservative substitutions. An example of a conservative substitution is the exchange of an amino acid in one of the following groups for another

amino acid of the same group (U.S. Pat. No. 5,767,063 issued to Lee, *et al.*; Kyte and Doolittle (1982) J. Mol. Biol. 157:105-132).

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

(2) Neutral hydrophilic: Cys, Ser, Thr;

(3) Acidic: Asp, Glu;

(4) Basic: Asn, Gln, His, Lys, Arg;

(5) Residues that influence chain orientation: Gly, Pro;

(6) Aromatic: Trp, Tyr, Phe; and

(7) Small amino acids: Gly, Ala, Ser.

[0050] “Effective amount” encompasses, without limitation, an amount that can ameliorate, reverse, mitigate, prevent, or diagnose a symptom or sign of a medical condition or disorder. Unless dictated otherwise, explicitly or by context, an “effective amount” is not limited to a minimal amount sufficient to ameliorate a condition.

[0051] An “extracellular fluid” encompasses, e.g., serum, plasma, blood, interstitial fluid, cerebrospinal fluid, secreted fluids, lymph, bile, sweat, fecal matter, and urine. An “extracellular fluid” can comprise a colloid or a suspension, e.g., whole blood or coagulated blood.

[0052] The term “fragments” in the context of polypeptides include a peptide or polypeptide comprising an amino acid sequence of at least 5 contiguous amino acid residues, at least 10 contiguous amino acid residues, at least 15 contiguous amino acid residues, at least 20 contiguous amino acid residues, at least 25 contiguous amino acid residues, at least 40 contiguous amino acid residues, at least 50 contiguous amino acid residues, at least 60 contiguous amino acid residues, at least 70 contiguous amino acid residues, at least 80 contiguous amino acid residues, at least 90 contiguous amino acid residues, at least 100 contiguous amino acid residues, at least 125 contiguous amino acid residues, at least 150 contiguous amino acid residues, at least 175 contiguous amino acid residues, at least 200 contiguous amino acid residues, or at least 250 contiguous amino acid residues of the amino acid sequence of a larger polypeptide.

[0053] “Gene” refers to a nucleic acid sequence encoding an oligopeptide or polypeptide. The oligopeptide or polypeptide can be biologically active, antigenically active, biologically inactive, or antigenically inactive, and the like. The term gene encompasses, e.g., the sum of the open reading frames (ORFs) encoding a specific oligopeptide or polypeptide; the sum of the ORFs plus the nucleic acids encoding introns; the sum of the ORFs and the operably linked promoter(s); the sum of the ORFs and the operably linked promoter(s) and any introns; the sum of the ORFs and the operably linked promoter(s), intron(s), and promoter(s), and other regulatory elements, such as enhancer(s). In certain embodiments, “gene” encompasses any sequences required in cis for regulating expression of the gene. The term gene can also refer to a nucleic acid that encodes a peptide encompassing an antigen or an antigenically active fragment of a peptide, oligopeptide, polypeptide, or protein. The term gene does not necessarily imply that the encoded peptide or protein has any biological activity, or even that the peptide or protein is antigenically active. A nucleic acid sequence encoding a non-expressable sequence is generally considered a pseudogene. The term gene also encompasses nucleic acid sequences encoding a ribonucleic acid such as rRNA, tRNA, or a ribozyme.

[0054] “Growth” of a bacterium such as *Listeria* encompasses, without limitation, functions of bacterial physiology and genes relating to colonization, replication, increase in protein content, and/or increase in lipid content. Unless specified otherwise explicitly or by context, growth of a *Listeria* encompasses growth of the bacterium outside a host cell, and also growth inside a host cell. Growth related genes include, without implying any limitation, those that mediate energy production (e.g., glycolysis, Krebs cycle, cytochromes), anabolism and/or catabolism of amino acids, sugars, lipids, minerals, purines, and pyrimidines, nutrient transport, transcription, translation, and/or replication. In some embodiments, “growth” of a *Listeria* bacterium refers to intracellular growth of the *Listeria* bacterium, that is, growth inside a host cell such as a mammalian cell. While intracellular growth of a *Listeria* bacterium can be measured by light microscopy or colony forming unit (CFU) assays, growth is not to be limited by any technique of measurement. Biochemical parameters such as the quantity of a *Listerial* antigen, *Listerial* nucleic acid sequence, or lipid specific to the *Listeria* bacterium, can be used to assess growth. In some embodiments, a gene that mediates growth is one that specifically mediates intracellular growth. In some embodiments, a gene that specifically mediates intracellular growth encompasses, but is not limited to, a gene where inactivation of the gene reduces the rate of intracellular growth but

does not detectably, substantially, or appreciably, reduce the rate of extracellular growth (e.g., growth in broth), or a gene where inactivation of the gene reduces the rate of intracellular growth to a greater extent than it reduces the rate of extracellular growth. To provide a non-limiting example, in some embodiments, a gene where inactivation reduces the rate of intracellular growth to a greater extent than extracellular growth encompasses the situation where inactivation reduces intracellular growth to less than 50% the normal or maximal value, but reduces extracellular growth to only 1-5%, 5-10%, or 10-15% the maximal value. The invention, in certain aspects, encompasses a *Listeria* attenuated in intracellular growth but not attenuated in extracellular growth, a *Listeria* not attenuated in intracellular growth and not attenuated in extracellular growth, as well as a *Listeria* not attenuated in intracellular growth but attenuated in extracellular growth.

[0055] A composition that is “labeled” is detectable, either directly or indirectly, by spectroscopic, photochemical, biochemical, immunochemical, isotopic, or chemical methods. For example, useful labels include ^{32}P , ^{33}P , ^{35}S , ^{14}C , ^3H , ^{125}I , stable isotopes, epitope tags, fluorescent dyes, electron-dense reagents, substrates, or enzymes, e.g., as used in enzyme-linked immunoassays, or fluorettes (see, e.g., Rozinov and Nolan (1998) Chem. Biol. 5:713-728).

[0056] “Ligand” refers to a small molecule, peptide, polypeptide, or membrane associated or membrane-bound molecule, that is an agonist or antagonist of a receptor. “Ligand” also encompasses a binding agent that is not an agonist or antagonist, and has no agonist or antagonist properties. By convention, where a ligand is membrane-bound on a first cell, the receptor usually occurs on a second cell. The second cell may have the same identity (the same name), or it may have a different identity (a different name), as the first cell. A ligand or receptor may be entirely intracellular, that is, it may reside in the cytosol, nucleus, or in some other intracellular compartment. The ligand or receptor may change its location, e.g., from an intracellular compartment to the outer face of the plasma membrane. The complex of a ligand and receptor is termed a “ligand receptor complex.” Where a ligand and receptor are involved in a signaling pathway, the ligand occurs at an upstream position and the receptor occurs at a downstream position of the signaling pathway.

[0057] “Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single stranded, double-stranded form, or multi-stranded form. Non-limiting examples of a nucleic acid are a, e.g., cDNA, mRNA, oligonucleotide, and

polynucleotide. A particular nucleic acid sequence can also implicitly encompass “allelic variants” and “splice variants.”

[0058] “Operably linked” in the context of a promoter and a nucleic acid encoding a mRNA means that the promoter can be used to initiate transcription of that nucleic acid.

[0059] The terms “percent sequence identity” and “% sequence identity” refer to the percentage of sequence similarity found by a comparison or alignment of two or more amino acid or nucleic acid sequences. Percent identity can be determined by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. An algorithm for calculating percent identity is the Smith-Waterman homology search algorithm (see, e.g., Kann and Goldstein (2002) *Proteins* 48:367-376; Arslan, et al. (2001) *Bioinformatics* 17:327-337).

[0060] By “purified” and “isolated” is meant, when referring to a polypeptide, that the polypeptide is present in the substantial absence of the other biological macromolecules with which it is associated in nature. The term “purified” as used herein means that an identified polypeptide often accounts for at least 50%, more often accounts for at least 60%, typically accounts for at least 70%, more typically accounts for at least 75%, most typically accounts for at least 80%, usually accounts for at least 85%, more usually accounts for at least 90%, most usually accounts for at least 95%, and conventionally accounts for at least 98% by weight, or greater, of the polypeptides present. The weights of water, buffers, salts, detergents, reductants, protease inhibitors, stabilizers (including an added protein such as albumin), and excipients, and molecules having a molecular weight of less than 1000, are generally not used in the determination of polypeptide purity. See, e.g., discussion of purity in U.S. Pat. No. 6,090,611 issued to Covacci, *et al.*

[0061] “Peptide” refers to a short sequence of amino acids, where the amino acids are connected to each other by peptide bonds. A peptide may occur free or bound to another moiety, such as a macromolecule, lipid, oligo- or polysaccharide, and/or a polypeptide. Where a peptide is incorporated into a polypeptide chain, the term “peptide” may still be used to refer specifically to the short sequence of amino acids. A “peptide” may be connected to another moiety by way of a peptide bond or some other type of linkage. A peptide is at least two amino acids in length and generally less than about 25 amino acids in

length, where the maximal length is a function of custom or context. The terms “peptide” and “oligopeptide” may be used interchangeably.

[0062] “Protein” generally refers to the sequence of amino acids comprising a polypeptide chain. Protein may also refer to a three dimensional structure of the polypeptide. “Denatured protein” refers to a partially denatured polypeptide, having some residual three dimensional structure or, alternatively, to an essentially random three dimensional structure, i.e., totally denatured. The invention encompasses reagents of, and methods using, polypeptide variants, e.g., involving glycosylation, phosphorylation, sulfation, disulfide bond formation, deamidation, isomerization, cleavage points in signal or leader sequence processing, covalent and non-covalently bound cofactors, oxidized variants, and the like. The formation of disulfide linked proteins is described (see, e.g., Woycechowsky and Raines (2000) *Curr. Opin. Chem. Biol.* 4:533-539; Creighton, *et al.* (1995) *Trends Biotechnol.* 13:18-23).

[0063] “Recombinant” when used with reference, e.g., to a nucleic acid, cell, animal, virus, plasmid, vector, or the like, indicates modification by the introduction of an exogenous, non-native nucleic acid, alteration of a native nucleic acid, or by derivation in whole or in part from a recombinant nucleic acid, cell, virus, plasmid, or vector. Recombinant protein refers to a protein derived, e.g., from a recombinant nucleic acid, virus, plasmid, vector, or the like. “Recombinant bacterium” encompasses a bacterium where the genome is engineered by recombinant methods, e.g., by way of a mutation, deletion, insertion, and/or a rearrangement. “Recombinant bacterium” also encompasses a bacterium modified to include a recombinant extra-genomic nucleic acid, e.g., a plasmid or a second chromosome, or a bacterium where an existing extra-genomic nucleic acid is altered.

[0064] “Sample” refers to a sample from a human, animal, placebo, or research sample, e.g., a cell, tissue, organ, fluid, gas, aerosol, slurry, colloid, or coagulated material. The “sample” may be tested *in vivo*, e.g., without removal from the human or animal, or it may be tested *in vitro*. The sample may be tested after processing, e.g., by histological methods. “Sample” also refers, e.g., to a cell comprising a fluid or tissue sample or a cell separated from a fluid or tissue sample. “Sample” may also refer to a cell, tissue, organ, or fluid that is freshly taken from a human or animal, or to a cell, tissue, organ, or fluid that is processed or stored.

[0065] A “selectable marker” encompasses a nucleic acid that allows one to select for or against a cell that contains the selectable marker. Examples of selectable markers include, without limitation, e.g.: (1) A nucleic acid encoding a product providing resistance to an otherwise toxic compound (e.g., an antibiotic), or encoding susceptibility to an otherwise harmless compound (e.g., sucrose); (2) A nucleic acid encoding a product that is otherwise lacking in the recipient cell (e.g., tRNA genes, auxotrophic markers); (3) A nucleic acid encoding a product that suppresses an activity of a gene product; (4) A nucleic acid that encodes a product that can be readily identified (e.g., phenotypic markers such as beta-galactosidase, green fluorescent protein (GFP), cell surface proteins, an epitope tag, a FLAG tag); (5) A nucleic acid that can be identified by hybridization techniques, for example, PCR or molecular beacons.

[0066] “Specifically” or “selectively” binds, when referring to a ligand/receptor, nucleic acid/complementary nucleic acid, antibody/antigen, or other binding pair (e.g., a cytokine to a cytokine receptor) indicates a binding reaction which is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated conditions, a specified ligand binds to a particular receptor and does not bind in a significant amount to other proteins present in the sample. Specific binding can also mean, e.g., that the binding compound, nucleic acid ligand, antibody, or binding composition derived from the antigen-binding site of an antibody, of the contemplated method binds to its target with an affinity that is often at least 25% greater, more often at least 50% greater, most often at least 100% (2-fold) greater, normally at least ten times greater, more normally at least 20-times greater, and most normally at least 100-times greater than the affinity with any other binding compound.

[0067] In a typical embodiment an antibody will have an affinity that is greater than about 10^9 liters/mol, as determined, e.g., by Scatchard analysis (Munsen, *et al.* (1980) *Analyt. Biochem.* 107:220-239). It is recognized by the skilled artisan that some binding compounds can specifically bind to more than one target, e.g., an antibody specifically binds to its antigen, to lectins by way of the antibody’s oligosaccharide, and/or to an Fc receptor by way of the antibody’s Fc region.

[0068] “Spread” of a bacterium encompasses “cell to cell spread,” that is, transmission of the bacterium from a first host cell to a second host cell, as mediated, for example, by a vesicle. Functions relating to spread include, but are not limited to, e.g., formation of an

actin tail, formation of a pseudopod-like extension, and formation of a double-membraned vacuole.

[0069] The term “subject” as used herein refers to a human or non-human organism. Thus, the methods and compositions described herein are applicable to both human and veterinary disease. In certain embodiments, subjects are “patients,” i.e., living humans that are receiving medical care for a disease or condition. This includes persons with no defined illness who are being investigated for signs of pathology.

[0070] The “target site” of a recombinase is the nucleic acid sequence or region that is recognized, bound, and/or acted upon by the recombinase (see, e.g., U.S. Pat. No. 6,379,943 issued to Graham, et al.; Smith and Thorpe (2002) *Mol. Microbiol.* 44:299-307; Groth and Calos (2004) *J. Mol. Biol.* 335:667-678; Nunes-Duby, et al. (1998) *Nucleic Acids Res.* 26:391-406).

[0071] “Therapeutically effective amount” is defined as an amount of a reagent or pharmaceutical composition that is sufficient to induce a desired immune response specific for encoded heterologous antigens, show a patient benefit, i.e., to cause a decrease, prevention, or amelioration of the symptoms of the condition being treated. When the agent or pharmaceutical composition comprises a diagnostic agent, a “diagnostically effective amount” is defined as an amount that is sufficient to produce a signal, image, or other diagnostic parameter. Effective amounts of the pharmaceutical formulation will vary according to factors such as the degree of susceptibility of the individual, the age, gender, and weight of the individual, and idiosyncratic responses of the individual (see, e.g., U.S. Pat. No. 5,888,530 issued to Netti, *et al.*).

[0072] “Treatment” or “treating” (with respect to a condition or a disease) is an approach for obtaining beneficial or desired results including and preferably clinical results. For purposes of this invention, beneficial or desired results with respect to a disease include, but are not limited to, one or more of the following: improving a condition associated with a disease, curing a disease, lessening severity of a disease, delaying progression of a disease, alleviating one or more symptoms associated with a disease, increasing the quality of life of one suffering from a disease, and/or prolonging survival. Likewise, for purposes of this invention, beneficial or desired results with respect to a condition include, but are not limited to, one or more of the following: improving a condition, curing a condition, lessening

severity of a condition, delaying progression of a condition, alleviating one or more symptoms associated with a condition, increasing the quality of life of one suffering from a condition, and/or prolonging survival.

[0073] “Vaccine” encompasses preventative vaccines. Vaccine also encompasses therapeutic vaccines, e.g., a vaccine administered to a mammal that comprises a condition or disorder associated with the antigen or epitope provided by the vaccine. A number of bacterial species have been developed for use as vaccines and can be used in the present invention, including, but not limited to, *Shigella flexneri*, *Escherichia coli*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella typhimurium*, *Salmonella typhi* or *mycobacterium species*. This list is not meant to be limiting. See, e.g., WO04/006837; WO07/103225; and WO07/117371, each of which is hereby incorporated by reference in its entirety, including all tables, figures, and claims. The bacterial vector used in the vaccine composition may be a facultative, intracellular bacterial vector. The bacterium may be used to deliver a polypeptide described herein to antigen-presenting cells in the host organism. As described herein, *L. monocytogenes* provides a preferred vaccine platform for expression of the antigens of the present invention.

[0074] 2. Targeting essential genes for facultative deletion

[0075] Bacteria engineered for Recombinase-Induced Intracellular Death (RIID) are programmed to “commit suicide” by linking expression of a recombinantly introduced recombinase gene to a promoter that is facultatively expressed when the bacterium is in a host organism. By way of example below, expression of the recombinase can be made facultative in *Listeria* using a PrfA-dependent promoter which may be selected from the *inlA* promoter, the *inlB* promoter, the *inlC* promoter, the *hpt* promoter, the *hly* promoter, the *plcA* promoter, the *mpl* promoter, and the *actA* promoter. PrfA is a transcription factor activated intracellularly which induces expression of linked genes in appropriately engineered vaccine strains.

[0076] The sequence of *L. monocytogenes* PrfA is as follows (SEQ ID NO: 1):

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MNAQAEEFKK YLETNGIKPK QFHKKELIFN QWDPQEYCIF LYDGITKLTS      50
ISENGTIMNL QYYKGAFVIM SGFIDTETSV GYYNLEWISE QATAYVIKIN 100
ELKELLSKNL THFFYVFQTL QKQVSYSIAK FNDFSINGKL GSICGQLIL 150
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TYVYGKETPD GIKITLDNLT MQELGYSSGI AHSSAVSRII SKLKQEKVIV 200
YKNSCFYVQN LDYLKRYAPK LDEWFYLACP ATWGKLN 237

[0077] As noted above, in the following examples expression of the *actA* gene is responsive to PrfA, and the *actA* promoter is a PrfA responsive regulatory element. The *actA* promoter is a suitable promoter for facultative high-level expression of recombinase genes, as ActA is the most abundantly expressed *Listeria* protein and its expression is induced >200-fold within infected cells as compared to *in vitro* culture conditions.

[0078] Other regulatory systems which may be used in a manner similar to that of *Listeria* PrfA include the following:

genus	species	regulator
<i>Mycobacterium</i>	<i>tuberculosis</i>	PhoP, ArsR
<i>Francisella</i>	<i>tularensis</i>	MigR
<i>Salmonella</i>	<i>enterica</i>	SlyA, SsrB
<i>Shigella</i>	<i>flexneri</i>	VirB
<i>Burkholderia</i>	<i>cenocepacia</i>	ShvR
<i>Brucella</i>	<i>melitensis</i>	BlxR
<i>Legionella</i>	<i>pneumophila</i>	PmrA
<i>Yersinia</i>	<i>pestis, enterocolitica</i>	RovA
<i>Bacillus</i>	<i>anthracis</i>	AtxA
<i>Staphylococcus</i>	<i>aureus</i>	SarA

[0079] In the following examples, expression of Cre recombinase is linked to the *actA* promoter for expression in *Listeria*. In alternatives, recombinases such as ϕ C31 integrase, R4 integrase, TP901 integrase, ϕ BT1 integrase, BxB1 integrase, PSA integrase, Cre recombinase, Flp recombinase, XerC recombinase, λ integrase, HK022 integrase, P22 integrase, HP1 integrase, L5 integrase, $\gamma\delta$ recombinase, Tn3 recombinase, gin recombinase, RV integrase, SPBc integrase, TG1 integrase, ϕ C1 integrase, MR11 integrase, ϕ 370 integrase, ϕ K38 integrase, W β integrase, and BL3 integrase may find use in the present invention in a manner similar to that shown below for Cre recombinase .

[0080] Upon expression, Cre excises a bacterial gene required for viability that has been targeted for deletion by the recombinant introduction of flanking recombinase binding sites (e.g., loxP). Advantageously, mutant recombinase sites such as lox66 and lox71 mutant lox P sites may be utilized. Unlike native loxP, lox66 and lox71 sites which are joined following

excision of the targeted gene(s) cannot be used subsequently as a template for Cre-mediated recombination, thus driving the equilibrium of excision to completion.

[0081] Targeting deletion genes essential for DNA replication (e.g., *gyrA*, *gyrB*) results in bacterial cell death in infected cells, as compared to its isogenic parent *Listeria* strain. However, because the *actA* promoter is not induced during fermentation, growth of RIID Lm in bacterial growth media is indistinguishable from the parent strain. Expression of Lm-encoded antigens can also be induced in the infected APC using a PrfA-dependent promoter such as *ActA*, where synthesized antigens are secreted from the listerial bacterium into the cytosol through linkage with a bacterial signal peptide/chaperone and then processed and presented via the MHC class I pathway. Although RIID Lm strain is programmed for death post-infection of APCs, the vaccine still elicits potent CD8 T cell responses that are comparable to vaccination with an isogenic live-attenuated Lm vaccine strain.

[0082] The following is a non-limiting list of exemplary genes involved in DNA replication which may be targeted for excision in the RIID approach.

- ori (origin of replication)
- dnaA* (replication initiation)
- dnaN* (DNA polymerase III, beta subunit)
- gyrA* (DNA gyrase, subunit A)
- gyrB* (DNA gyrase, subunit B)
- polC* (DNA polymerase III, alpha subunit)
- dnaE* (DNA polymerase III, alpha subunit)
- ftsK* (DNA translocase, chromosome separation)
- ftsZ* (tubulin-like, septation)
- ligA* (DNA ligase)
- dnaG* (DNA primase)
- parC* (topo IV subunit)
- parE* (topo IV subunit)
- holB* (DNA polymerase III, delta subunit)
- dnaX* (DNA polymerase III, gamma and tau subunits)
- SMC (chromosome segregation)

[0083] Preferred but non-limiting examples of genes to target for deletion include those that are involved in replication and bacterial cell division such as *polC*, *dnaE*, *ftsK*, *ftsZ*,

ligA, *dnaG*, *parC*, *parE*, *holB*, *dnaX*, *SMC*, and *fisY*. There are additional gene targets that are essential for the multiplication of *Listeria* that may be targeted for intracellular excision as an alternative, or in addition to, genes involved in DNA replication. Since Lm RIID strains have utility as a vaccine platform, in which Ag expression *de novo* is required for vaccine potency (Brockstedt et. al., 2005), one must consider the impact of the excised gene(s) on antigen expression/secretion to select gene targets. In a preferred embodiment, genes encoding proteins involved in RNA transcription and protein synthesis should be avoided because of a potential decrease of immunologic potency, which may not be desired for particular uses. Other preferred non-limiting examples include targeting bacterial genes affecting virulence, such as *hly* and *dacA*. It will be clear to the skilled artisan that any gene targeted for deletion in the cytosol of the infected host cell can be accomplished by the methods described herein, accordingly: First, the inter-genic regions upstream and downstream of the essential gene(s) to be deleted is identified; second PCR primers that include the desired *loxP* variants are designed; third, the corresponding allelic exchange vectors to insert the *lox* sites in the non-essential/inter-genic space are constructed; and, fourth, the *lox* sites are sequentially inserted into the *Listeria* vaccine strain by allelic exchange. Alternatively, it will be apparent to the skilled artisan that if the gene to be targeted for deletion is small, than both *lox* sites can be added with a single allelic exchange step. In still a further embodiment, the *PactA*-Cre cassette for induction of Cre recombinase expression in the cytosol of the infected cell can be introduced at alternate locations that are either amenable to site-specific integration (e.g. *comK* using pPL1) or any chromosomal location that is amenable to insertion using allelic exchange methodology. In yet another embodiment, PrfA-dependent promoters other than *actA* can be used for the cytosolic induced expression of Cre recombinase; *inlC* is a non-limiting example of an alternative promoter.

[0084] As an alternative to the Cre/*lox* strategy discussed above, genes essential for the multiplication of *Listeria* that can also be targeted for intracellular excision with FLP recombinase and *frt* sites. First, the inter-genic regions upstream and downstream of the essential gene(s) to be deleted is identified; second PCR primers that include the desired *frt* sites are designed; third, the corresponding allelic exchange vectors to insert the *frt* sites in the non-essential/inter-genic space are constructed; and, fourth, the *frt* sites are sequentially inserted into the *Listeria* vaccine strain by allelic exchange. Alternatively, it will be apparent to the skilled artisan that if the gene to be targeted for deletion is small, than both *frt* sites

can be added with a single allelic exchange step. In still a further embodiment, the *PactA*-FLP cassette for induction of FLP recombinase expression in the cytosol of the infected cell can be introduced at alternate locations that are either amenable to site-specific integration (e.g. *comK* using pPL1) or any chromosomal location that is amenable to insertion using allelic exchange methodology.

4. Antigenic constructs

[0085] Target antigens

[0086] A preferred feature of the RIID bacteria described herein when used as a vaccine platform is the ability to initiate both the innate immune response as well as an antigen-specific T cell response against the recombinantly expressed antigen(s). For example, *L. monocytogenes* expressing the antigen(s) described herein can induce Type 1 interferon (IFN- α/β) and a cascade of co-regulated chemokine and cytokine protein which shape the nature of the vaccine-induced immune response. In response to this immune stimulation, NK cells and antigen presenting cells (APCs) are recruited to the liver following intravenous vaccination routes, or, alternatively to the vaccination site following other routes of vaccination, for example, by intramuscular, subcutaneous, or intradermal immunization routes. In certain embodiments, the vaccine platform of the present invention induces an increase at 24 hours following delivery of the vaccine platform to the subject in the serum concentration of one or more, and preferably all, cytokines and chemokines selected from the group consisting of IL-12p70, IFN- γ , IL-6, TNF α , and MCP-1; and induces a CD4+ and/or CD8+ antigen-specific T cell response against one or more antigens expressed by the vaccine platform. In other embodiments, the vaccine platform of the present invention also induces the maturation of resident immature liver NK cells as demonstrated by the upregulation of activation markers such as DX5, CD11b, and CD43 in a mouse model system, or by NK cell-mediated cytolytic activity measured using ^{51}Cr -labeled YAC-1 cells that were used as target cells.

[0087] The ability of *L. monocytogenes* to serve as a vaccine vector has been reviewed in Wesikrich, *et al.*, *Immunol. Rev.* 158:159-169 (1997). A number of desirable features of the natural biology of *L. monocytogenes* make it an attractive platform for application to a therapeutic vaccine. The central rationale is that the intracellular lifecycle of *L. monocytogenes* enables effective stimulation of CD4+ and CD8+ T cell immunity. Multiple

pathogen associated molecular pattern (PAMP) receptors including TLRs (TLR2, TLR5, TLR9) nucleotide-binding oligomerization domains (NOD), and Stimulator of Interferon Genes (STING) are triggered in response to interaction with *L. monocytogenes* macromolecules upon infection, resulting in the pan-activation of innate immune effectors and release of Th-1 polarizing cytokines, exerting a profound impact on the development of a CD4+ and CD8+ T cell response against the expressed antigens.

[0088] Strains of *L. monocytogenes* have recently been developed as effective intracellular delivery vehicles of heterologous proteins providing delivery of antigens to the immune system to induce an immune response to clinical conditions that do not permit injection of the disease-causing agent, such as cancer and HIV. See, e.g., U.S. Pat. No. 6,051,237; Gunn *et al.*, *J. Immunol.*, 167:6471-6479 (2001); Liao, *et al.*, *Cancer Research*, 62: 2287-2293 (2002); U.S. Pat. No. 6,099,848; WO 99/25376; WO 96/14087; and U.S. Pat. No. 5,830,702), each of which is hereby incorporated by reference in its entirety, including all tables, figures, and claims. A recombinant *L. monocytogenes* vaccine expressing an lymphocytic choriomeningitis virus (LCMV) antigen has also been shown to induce protective cell-mediated immunity to the antigen (Shen *et al.*, *Proc. Natl. Acad. Sci. USA*, 92: 3987-3991 (1995)).

[0089] In certain embodiments, the *L. monocytogenes* used in the vaccine compositions of the present invention is RIID strain which further comprises an attenuating mutation in *actA* and/or *inlB*, and preferably a deletion of all or a portion of *actA* and *inlB* (referred to herein as "*Lm* Δ*actA*/Δ*inlB*"), and contains recombinant DNA encoding for the expression of the one or more antigen(s) of interest. The antigen(s) are preferably under the control of bacterial expression sequences and are stably integrated into the *L. monocytogenes* genome.

[0090] The invention also contemplates a *Listeria* attenuated in at least one regulatory factor, e.g., a promoter or a transcription factor. The following concerns promoters. *ActA* expression is regulated by two different promoters (Vazwuez-Boland, *et al.* (1992) *Infect. Immun.* 60:219-230). Together, *InlA* and *InlB* expression is regulated by five promoters (Lingnau, *et al.* (1995) *Infect. Immun.* 63:3896-3903). The transcription factor *prfA* is required for transcription of a number of *L. monocytogenes* genes, e.g., *hly*, *plcA*, *ActA*, *mpl*, *prfA*, and *iap*. *PrfA*'s regulatory properties are mediated by, e.g., the *PrfA*-dependent promoter (*PinlC*) and the *PrfA*-box. The present invention, in certain embodiments, provides a nucleic acid encoding inactivated, mutated, or deleted in at least one of *ActA*

promoter, *inlB* promoter, *PrfA*, *PinlC*, *PrfA* box, and the like (see, e.g., Lalic Mullthaler, et al. (2001) Mol. Microbiol. 42:111-120; Shetron-Rama, et al. (2003) Mol. Microbiol. 48:1537-1551; Luo, et al. (2004) Mol. Microbiol. 52:39-52). *PrfA* can be made constitutively active by a Gly145Ser mutation, Gly155Ser mutation, or Glu771Lys mutation (see, e.g., Mueller and Freitag (2005) Infect. Immun. 73:1917-1926; Wong and Freitag (2004) J. Bacteriol. 186:6265-6276; Ripio, et al. (1997) J. Bacteriol. 179:1533-1540).

[0091] Examples of target antigens that may find use in the invention are listed in the following table. The target antigen may also be a fragment or fusion polypeptide comprising an immunologically active portion of the antigens listed in the table. This list is not meant to be limiting.

Table 1. Antigens.

Antigen	Reference
Tumor antigens	
Mesothelin	GenBank Acc. No. NM_005823; U40434; NM_013404; BC003512 (see also, e.g., Hassan, <i>et al.</i> (2004) Clin. Cancer Res. 10:3937-3942; Muminova, <i>et al.</i> (2004) BMC Cancer 4:19; Iacobuzio-Donahue, <i>et al.</i> (2003) Cancer Res. 63:8614-8622).
Wilms' tumor-1 associated protein (WT-1), including isoform A; isoform B; isoform C; isoform D.	WT-1 isoform A (GenBank Acc. Nos. <u>NM_000378</u> ; NP_000369). WT-1 isoform B (GenBank Acc. Nos. <u>NM_024424</u> ; NP_077742). WT-1 isoform C (GenBank Acc. Nos. <u>NM_024425</u> ; NP_077743). WT-1 isoform D (GenBank Acc. Nos. <u>NM_024426</u> ; NP_077744).
Stratum corneum chymotryptic enzyme (SCCE), and variants thereof.	GenBank Acc. No. NM_005046; NM_139277; AF332583. See also, e.g., Bondurant, <i>et al.</i> (2005) Clin. Cancer Res. 11:3446-3454; Santin, <i>et al.</i> (2004) Gynecol. Oncol. 94:283-288; Shigemasa, <i>et al.</i> (2001) Int. J. Gynecol. Cancer 11:454-461; Sepehr, <i>et al.</i> (2001) Oncogene 20:7368-7374.
MHC class I chain-related protein A (MICA); MHC class I	See, e.g., Groh, <i>et al.</i> (2005) Proc. Natl. Acad. Sci. USA 102:6461-6466; GenBank Acc. Nos. NM_000247; BC_016929; AY750850; NM_005931.

Table 1. Antigens.

Antigen	Reference
chain-related protein A (MICB).	
Gastrin and peptides derived from gastrin; gastrin/CCK-2 receptor (also known as CCK-B).	Harris, <i>et al.</i> (2004) Cancer Res. 64:5624-5631; Gilliam, <i>et al.</i> (2004) Eur. J. Surg. Oncol. 30:536-543; Laheru and Jaffee (2005) Nature Reviews Cancer 5:459-467.
Glypican-3 (an antigen of, e.g., hepatocellular carcinoma and melanoma).	GenBank Acc. No. <u>NM_004484</u> , Nakatsura, <i>et al.</i> (2003) Biochem. Biophys. Res. Commun. 306:16-25; Capurro, <i>et al.</i> (2003) Gastroenterol. 125:89-97; Nakatsura, <i>et al.</i> (2004) Clin. Cancer Res. 10:6612-6621).
Coactosin-like protein.	Nakatsura, <i>et al.</i> (2002) Eur. J. Immunol. 32:826-836; Laheru and Jaffee (2005) Nature Reviews Cancer 5:459-467.
Prostate stem cell antigen (PSCA).	GenBank Acc. No. AF043498; AR026974; AR302232 (see also, e.g., Argani, <i>et al.</i> (2001) Cancer Res. 61:4320-4324; Christiansen, <i>et al.</i> (2003) Prostate 55:9-19; Fuessel, <i>et al.</i> (2003) 23:221-228).
Prostate acid phosphatase (PAP); prostate-specific antigen (PSA); PSM; PSMA.	Small, <i>et al.</i> (2000) J. Clin. Oncol. 18:3894-3903; Altwein and Luboldt (1999) Urol. Int. 63:62-71; Chan, <i>et al.</i> (1999) Prostate 41:99-109; Ito, <i>et al.</i> (2005) Cancer 103:242-250; Schmittgen, <i>et al.</i> (2003) Int. J. Cancer 107:323-329; Millon, <i>et al.</i> (1999) Eur. Urol. 36:278-285.
Six-transmembrane epithelial antigen of prostate (STEAP).	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. NM_018234; NM_001008410; NM_182915; NM_024636; NM_012449; BC011802.
Prostate carcinoma tumor antigen-1 (PCTA-1).	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. L78132.
Prostate tumor-inducing gene-1	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442).

Table 1. Antigens.

Antigen	Reference
(PTI-1).	
Prostate-specific gene with homology to G protein-coupled receptor.	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442).
Prostase (an antrogon regulated serine protease).	See, e.g., Machlenkin, <i>et al.</i> (2005) Cancer Res. 65:6435-6442; GenBank Acc. No. BC096178; BC096176; BC096175.
Proteinase 3.	GenBank Acc. No. X55668.
Cancer-testis antigens, e.g., NY-ESO-1; SCP-1; SSX-1; SSX-2; SSX-4; GAGE, CT7; CT8; CT10; MAGE-1; MAGE-2; MAGE-3; MAGE-4; MAGE-6; LAGE-1.	GenBank Acc. No. NM_001327 (NY-ESO-1) (see also, e.g., Li, <i>et al.</i> (2005) Clin. Cancer Res. 11:1809-1814; Chen, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. U S A. 101(25):9363-9368; Kubuschok, <i>et al.</i> (2004) Int. J. Cancer. 109:568-575; Scanlan, <i>et al.</i> (2004) Cancer Immun. 4:1; Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047; Scanlan, <i>et al.</i> (2000) Cancer Lett. 150:155-164; Dalerba, <i>et al.</i> (2001) Int. J. Cancer 93:85-90; Ries, <i>et al.</i> (2005) Int. J. Oncol. 26:817-824.
MAGE-A1, MAGE-A2; MAGE-A3; MAGE-A4; MAGE-A6; MAGE-A9; MAGE-A10; MAGE-A12; GAGE-3/6; NT-SAR-35; BAGE; CA125.	Otte, <i>et al.</i> (2001) Cancer Res. 61:6682-6687; Lee, <i>et al.</i> (2003) Proc. Natl. Acad. Sci. USA 100:2651-2656; Sarcevic, <i>et al.</i> (2003) Oncology 64:443-449; Lin, <i>et al.</i> (2004) Clin. Cancer Res. 10:5708-5716.
GAGE-1; GAGE-2; GAGE-3; GAGE-4;	De Backer, <i>et al.</i> (1999) Cancer Res. 59:3157-3165; Scarcella, <i>et al.</i> (1999) Clin. Cancer Res. 5:335-341.

Table 1. Antigens.

Antigen	Reference
GAGE-5; GAGE-6; GAGE-7; GAGE-8; GAGE-65; GAGE-11; GAGE-13; GAGE-7B.	
HIP1R; LMNA; KIAA1416; Seb4D; KNSL6; TRIP4; MBD2; HCAC5; MAGEA3.	Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047.
DAM family of genes, e.g., DAM-1; DAM-6.	Fleishhauer, <i>et al.</i> (1998) Cancer Res. 58:2969-2972.
RCAS1.	Enjoji, <i>et al.</i> (2004) Dig. Dis. Sci. 49:1654-1656.
RU2.	Van Den Eynde, <i>et al.</i> (1999) J. Exp. Med. 190:1793-1800.
CAMEL.	Slager, <i>et al.</i> (2004) J. Immunol. 172:5095-5102; Slager, <i>et al.</i> (2004) Cancer Gene Ther. 11:227-236.
Colon cancer associated antigens, e.g., NY-CO-8; NY-CO-9; NY-CO-13; NY-CO-16; NY-CO-20; NY-CO-38; NY-CO-45; NY-CO-9/HDAC5; NY-CO-41/MBD2; NY-CO-42/TRIP4; NY-CO-95/KIAA1416; KNSL6; seb4D.	Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047.

Table 1. Antigens.

Antigen	Reference
N-Acetylglucosaminyl-transferase V (GnT-V).	Dosaka-Akita, <i>et al.</i> (2004) Clin. Cancer Res. 10:1773-1779.
Elongation factor 2 mutated (ELF2M).	Renkvist, <i>et al.</i> (2001) Cancer Immunol Immunother. 50:3-15.
HOM-MEL-40/SSX2	Neumann, <i>et al.</i> (2004) Int. J. Cancer 112:661-668; Scanlan, <i>et al.</i> (2000) Cancer Lett. 150:155-164.
BRDT.	Scanlan, <i>et al.</i> (2000) Cancer Lett. 150:155-164.
SAGE; HAGE.	Sasaki, <i>et al.</i> (2003) Eur. J. Surg. Oncol. 29:900-903.
RAGE.	See, e.g., Li, <i>et al.</i> (2004) Am. J. Pathol. 164:1389-1397; Shirasawa, <i>et al.</i> (2004) Genes to Cells 9:165-174.
MUM-1 (melanoma ubiquitous mutated); MUM-2; MUM-2 Arg-Gly mutation; MUM-3.	Gueguen, <i>et al.</i> (1998) J. Immunol. 160:6188-6194; Hirose, <i>et al.</i> (2005) Int. J. Hematol. 81:48-57; Baurain, <i>et al.</i> (2000) J. Immunol. 164:6057-6066; Chiari, <i>et al.</i> (1999) Cancer Res. 59:5785-5792.
LDLR/FUT fusion protein antigen of melanoma.	Wang, <i>et al.</i> (1999) J. Exp. Med. 189:1659-1667.
NY-REN series of renal cancer antigens.	Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047; Scanlan, <i>et al.</i> (1999) Cancer Res. 83:456-464.
NY-BR series of breast cancer antigens, e.g., NY-BR-62; NY-BR-75; NY-BR-85; NY-BR-62; NY-BR-85.	Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047; Scanlan, <i>et al.</i> (2001) Cancer Immunity 1:4.
BRCA-1; BRCA-2.	Stolier, <i>et al.</i> (2004) Breast J. 10:475-480; Nicoletto, <i>et al.</i> (2001)

Table 1. Antigens.

Antigen	Reference
	Cancer Treat Rev. 27:295-304.
DEK/CAN fusion protein.	Von Lindern, <i>et al.</i> (1992) Mol. Cell. Biol. 12:1687-1697.
Ras, e.g., wild type ras, ras with mutations at codon 12, 13, 59, or 61, e.g., mutations G12C; G12D; G12R; G12S; G12V; G13D; A59T; Q61H. K-RAS; H-RAS; N-RAS.	GenBank Acc. Nos. P01112; P01116; M54969; M54968; P01111; P01112; K00654. See also, e.g., GenBank Acc. Nos. M26261; M34904; K01519; K01520; BC006499; NM_006270; NM_002890; NM_004985; NM_033360; NM_176795; NM_005343.
BRAF (an isoform of RAF).	Tannapfel, <i>et al.</i> (2005) Am. J. Clin. Pathol. 123:256-260; Tsao and Sober (2005) Dermatol. Clin. 23:323-333.
Melanoma antigens, including HST-2 melanoma cell antigens.	GenBank Acc. No. NM_206956; NM_206955; NM_206954; NM_206953; NM_006115; NM_005367; NM_004988; AY148486; U10340; U10339; M77481. See, e.g., Suzuki, <i>et al.</i> (1999) J. Immunol. 163:2783-2791.
Survivin	GenBank Acc. No. AB028869; U75285 (see also, e.g., Tsuruma, <i>et al.</i> (2004) J. Translational Med. 2:19 (11 pages); Pisarcv, <i>et al.</i> (2003) Clin. Cancer Res. 9:6523-6533; Siegel, <i>et al.</i> (2003) Br. J. Haematol. 122:911-914; Andersen, <i>et al.</i> (2002) Histol. Histopathol. 17:669-675).
MDM-2	<u>NM_002392</u> ; <u>NM_006878</u> (see also, e.g., Mayo, <i>et al.</i> (1997) Cancer Res. 57:5013-5016; Demidenko and Blagosklonny (2004) Cancer Res. 64:3653-3660).
Methyl-CpG-binding	Muller, <i>et al.</i> (2003) Br. J. Cancer 89:1934-1939; Fang, <i>et al.</i> (2004)

Table 1. Antigens.

Antigen	Reference
proteins (MeCP2; MBD2).	World J. Gastroenterol. 10:3394-3398.
NA88-A.	Moreau-Aubry, <i>et al.</i> (2000) J. Exp. Med. 191:1617-1624.
Histone deacetylases (HDAC), e.g., HDAC5.	Waltregny, <i>et al.</i> (2004) Eur. J. Histochem. 48:273-290; Scanlan, <i>et al.</i> (2002) Cancer Res. 62:4041-4047.
Cyclophilin B (Cyp-B).	Tamura, <i>et al.</i> (2001) Jpn. J. Cancer Res. 92:762-767.
CA 15-3; CA 27.29.	Clinton, <i>et al.</i> (2003) Biomed. Sci. Instrum. 39:408-414.
Heat shock protein Hsp70.	Faure, <i>et al.</i> (2004) Int. J. Cancer 108:863-870.
GAGE/PAGE family, e.g., PAGE-1; PAGE-2; PAGE-3; PAGE-4; XAGE-1; XAGE-2; XAGE-3.	Brinkmann, <i>et al.</i> (1999) Cancer Res. 59:1445-1448.
MAGE-A, B, C, and D families. MAGE-B5; MAGE-B6; MAGE-C2; MAGE-C3; MAGE-3; MAGE-6.	Lucas, <i>et al.</i> (2000) Int. J. Cancer 87:55-60; Scanlan, <i>et al.</i> (2001) Cancer Immun. 1:4.
Kinesin 2; TATA element modulatory factor 1; tumor protein D53; NY	Scanlan, <i>et al.</i> (2001) Cancer Immun. 30:1-4.
Alpha-fetoprotein (AFP)	Grimm, <i>et al.</i> (2000) Gastroenterol. 119:1104-1112.

Table 1. Antigens.

Antigen	Reference
SART1; SART2; SART3; ART4.	Kumamuru, <i>et al.</i> (2004) <i>Int. J. Cancer</i> 108:686-695; Sasatomi, <i>et al.</i> (2002) <i>Cancer</i> 94:1636-1641; Matsumoto, <i>et al.</i> (1998) <i>Jpn. J. Cancer Res.</i> 89:1292-1295; Tanaka, <i>et al.</i> (2000) <i>Jpn. J. Cancer Res.</i> 91:1177-1184.
Preferentially expressed antigen of melanoma (PRAME).	Matsushita, <i>et al.</i> (2003) <i>Leuk. Lymphoma</i> 44:439-444; Oberthuer, <i>et al.</i> (2004) <i>Clin. Cancer Res.</i> 10:4307-4313.
Carcinoembryonic antigen (CEA), CAP1-6D enhancer agonist peptide.	GenBank Acc. No. <u>M29540</u> ; E03352; X98311; M17303 (see also, e.g., Zaremba (1997) <i>Cancer Res.</i> 57:4570-4577; Sarobe, <i>et al.</i> (2004) <i>Curr. Cancer Drug Targets</i> 4:443-454; Tsang, <i>et al.</i> (1997) <i>Clin. Cancer Res.</i> 3:2439-2449; Fong, <i>et al.</i> (2001) <i>Proc. Natl. Acad. Sci. USA</i> 98:8809-8814).
HER-2/neu.	Disis, <i>et al.</i> (2004) <i>J. Clin. Immunol.</i> 24:571-578; Disis and Cheever (1997) <i>Adv. Cancer Res.</i> 71:343-371.
Cdk4; cdk6; p16 (INK4); Rb protein.	Ghazizadeh, <i>et al.</i> (2005) <i>Respiration</i> 72:68-73; Ericson, <i>et al.</i> (2003) <i>Mol. Cancer Res.</i> 1:654-664.
TEL; AML1; TEL/AML1.	Stams, <i>et al.</i> (2005) <i>Clin. Cancer Res.</i> 11:2974-2980.
Telomerase (TERT).	Nair, <i>et al.</i> (2000) <i>Nat. Med.</i> 6:1011-1017.
707-AP.	Takahashi, <i>et al.</i> (1997) <i>Clin. Cancer Res.</i> 3:1363-1370.
Annexin, e.g., Annexin II.	Zimmerman, <i>et al.</i> (2004) <i>Virchows Arch.</i> 445:368-374.
BCR/ABL; BCR/ABL p210; BCR/ABL p190; CML-66; CML-28.	Cobaldda, <i>et al.</i> (2000) <i>Blood</i> 95:1007-1013; Hakansson, <i>et al.</i> (2004) <i>Leukemia</i> 18:538-547; Schwartz, <i>et al.</i> (2003) <i>Semin. Hematol.</i> 40:87-96; Lim, <i>et al.</i> (1999) <i>Int. J. Mol. Med.</i> 4:665-667.
BCL2; BLC6; CD10 protein.	Iqbal, <i>et al.</i> (2004) <i>Am. J. Pathol.</i> 165:159-166.
CDC27 (this is a melanoma antigen).	Wang, <i>et al.</i> (1999) <i>Science</i> 284:1351-1354.
Sperm protein 17	Arora, <i>et al.</i> (2005) <i>Mol. Carcinog.</i> 42:97-108.

Table 1. Antigens.

Antigen	Reference
(SP17); 14-3-3-zeta; MEMD; KIAA0471; TC21.	
Tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2).	GenBank Acc. No. NM_001922. (see also, e.g., Bronte, <i>et al.</i> (2000) Cancer Res. 60:253-258).
Gp100/pmel-17.	GenBank Acc. Nos. AH003567 ; U31798 ; U31799 ; U31807 ; U31799 (see also, e.g., Bronte, <i>et al.</i> (2000) Cancer Res. 60:253-258).
TARP.	See, e.g., Clifton, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:10166-10171; Virok, <i>et al.</i> (2005) Infection Immunity 73:1939-1946.
Tyrosinase-related proteins 1 and 2 (TRP-1 and TRP-2).	GenBank Acc. No. NM_001922. (see also, e.g., Bronte, <i>et al.</i> (2000) Cancer Res. 60:253-258).
Melanocortin 1 receptor (MC1R); MAGE-3; gp100; tyrosinase; dopachrome tautomerase (TRP-2); MART-1.	Salazar-Onfray, <i>et al.</i> (1997) Cancer Res. 57:4348-4355; Reynolds, <i>et al.</i> (1998) J. Immunol. 161:6970-6976; Chang, <i>et al.</i> (2002) Clin. Cancer Res. 8:1021-1032.
MUC-1; MUC-2.	See, e.g., Davies, <i>et al.</i> (1994) Cancer Lett. 82:179-184; Gambus, <i>et al.</i> (1995) Int. J. Cancer 60:146-148; McCool, <i>et al.</i> (1999) Biochem. J. 341:593-600.
Spas-1.	U.S. Published Pat. Appl. No. 20020150588 of Allison, <i>et al.</i>
CASP-8; FLICE; MACH.	Mandruzzato, <i>et al.</i> (1997) J. Exp. Med. 186:785-793.
CEACAM6; CAP-1.	Duxbury, <i>et al.</i> (2004) Biochem. Biophys. Res. Commun. 317:837-843; Morse, <i>et al.</i> (1999) Clin. Cancer Res. 5:1331-1338.
HMGB1 (a DNA binding protein and	Brezniceanu, <i>et al.</i> (2003) FASEB J. 17:1295-1297.

Table 1. Antigens.

Antigen	Reference
cytokine).	
ETV6/AML1.	Codrington, <i>et al.</i> (2000) Br. J. Haematol. 111:1071-1079.
Mutant and wild type forms of adenomatous polyposis coli (APC); beta-catenin; c-met; p53; E-cadherin; cyclooxygenase-2 (COX-2).	Clements, <i>et al.</i> (2003) Clin. Colorectal Cancer 3:113-120; Gulmann, <i>et al.</i> (2003) Appl. Immunohistochem. Mol. Morphol. 11:230-237; Jungck, <i>et al.</i> (2004) Int. J. Colorectal. Dis. 19:438-445; Wang, <i>et al.</i> (2004) J. Surg. Res. 120:242-248; Abutaily, <i>et al.</i> (2003) J. Pathol. 201:355-362; Liang, <i>et al.</i> (2004) Br. J. Surg. 91:355-361; Shirakawa, <i>et al.</i> (2004) Clin. Cancer Res. 10:4342-4348.
Renal cell carcinoma antigen bound by mAB G250.	Mulders, <i>et al.</i> (2003) Urol. Clin. North Am. 30:455-465; Steffens, <i>et al.</i> (1999) Anticancer Res. 19:1197-1200.
EphA2	See, e.g., U.S. Patent Publication No. 2005/0281783 A1; Genbank Accession No. NM_004431 (human); Genbank Accession No. NM_010139 (Mouse); Genbank Accession No. AB038986 (Chicken, partial sequence); GenBank Accession Nos. NP_004422, AAH37166, and AAA53375 (human); GenBank Accession Nos. NP_034269 (mouse), AAH06954 (mouse), XP_345597 (rat), and BAB63910 (chicken).
EGFRvIII	See, e.g., WO/2012/068360
<i>Francisella tularensis</i> antigens	
<i>Francisella tularensis</i> A and B.	Complete genome of subspecies Schu S4 (GenBank Acc. No. AJ749949); of subspecies Schu 4 (GenBank Acc. No. NC_006570). Outer membrane protein (43 kDa) Bevanger, <i>et al.</i> (1988) J. Clin. Microbiol. 27:922-926; Porsch-Ozcurumez, <i>et al.</i> (2004) Clin. Diagnostic. Lab. Immunol. 11:1008-1015). Antigenic components of <i>F. tularensis</i> include, e.g., 80 antigens, including 10 kDa and 60 kDa chaperonins (Havlasova, <i>et al.</i> (2002) Proteomics 2:857-86), nucleoside diphosphate kinase, isocitrate dehydrogenase, RNA-binding protein Hfq, the chaperone ClpB (Havlasova, <i>et al.</i>

Table 1. Antigens.

Antigen	Reference
	(2005) <i>Proteomics</i> 5:2090-2103). See also, e.g., Oyston and Quarry (2005) <i>Antonie Van Leeuwenhoek</i> 87:277-281; Isherwood, <i>et al.</i> (2005) <i>Adv. Drug Deliv. Rev.</i> 57:1403-1414; Biagini, <i>et al.</i> (2005) <i>Anal. Bioanal. Chem.</i> 382:1027-1034.
Malarial antigens	
Circumsporozoite protein (CSP); SSP2; HEP17; Exp-1 orthologs found in <i>P. falciparum</i> ; and LSA-1.	See, e.g., Haddad, <i>et al.</i> (2004) <i>Infection Immunity</i> 72:1594-1602; Hoffman, <i>et al.</i> (1997) <i>Vaccine</i> 15:842-845; Oliveira-Ferreira and Daniel-Ribeiro (2001) <i>Mem. Inst. Oswaldo Cruz, Rio de Janeiro</i> 96:221-227. CSP (see, e.g., GenBank Acc. No. AB121024). SSP2 (see, e.g., GenBank Acc. No. AF249739). LSA-1 (see, e.g., GenBank Acc. No. Z30319).
Ring-infected erythrocyte surface protein (RESA); merozoite surface protein 2 (MSP2); Spf66; merozoite surface protein 1 (MSP1); 195A; BVp42.	See, e.g., Stirnadel, <i>et al.</i> (2000) <i>Int. J. Epidemiol.</i> 29:579-586; Krzych, <i>et al.</i> (1995) <i>J. Immunol.</i> 155:4072-4077. See also, Good, <i>et al.</i> (2004) <i>Immunol. Rev.</i> 201:254-267; Good, <i>et al.</i> (2004) <i>Ann. Rev. Immunol.</i> 23:69-99. MSP2 (see, e.g., GenBank Acc. No. X96399; X96397). MSP1 (see, e.g., GenBank Acc. No. X03371). RESA (see, e.g., GenBank Acc. No. X05181; X05182).
Apical membrane antigen 1 (AMA1).	See, e.g., Gupta, <i>et al.</i> (2005) <i>Protein Expr. Purif.</i> 41:186-198. AMA1 (see, e.g., GenBank Acc. No. A`13; AJ494905; AJ490565).
Viruses and viral antigens	
Hepatitis A	GenBank Acc. Nos., e.g., NC_001489; AY644670; X83302; K02990; M14707.
Hepatitis B	Complete genome (see, e.g., GenBank Acc. Nos. AB214516; NC_003977; AB205192; AB205191; AB205190; AJ748098; AB198079; AB198078; AB198076; AB074756).
Hepatitis C	Complete genome (see, e.g., GenBank Acc. Nos. NC_004102;

Table 1. Antigens.

Antigen	Reference
	AJ238800; AJ238799; AJ132997; AJ132996; AJ000009; D84263).
Hepatitis D	GenBank Acc. Nos, e.g. NC_001653; AB118847; AY261457.
Human papillomavirus, including all 200+ subtypes (classed in 16 groups), such as the high risk subtypes 16, 18, 30, 31, 33, 45.	See, e.g., Trimble, <i>et al.</i> (2003) Vaccine 21:4036-4042; Kim, <i>et al.</i> (2004) Gene Ther. 11:1011-1018; Simon, <i>et al.</i> (2003) Eur. J. Obstet. Gynecol. Reprod. Biol. 109:219-223; Jung, <i>et al.</i> (2004) J. Microbiol. 42:255-266; Damasus-Awatai and Freeman-Wang (2003) Curr. Opin. Obstet. Gynecol. 15:473-477; Jansen and Shaw (2004) Annu. Rev. Med. 55:319-331; Roden and Wu (2003) Expert Rev. Vaccines 2:495-516; de Villiers, <i>et al.</i> (2004) Virology 324:17-24; Hussain and Paterson (2005) Cancer Immunol. Immunother. 54:577-586; Molijn, <i>et al.</i> (2005) J. Clin. Virol. 32 (Suppl. 1) S43-S51. GenBank Acc. Nos. AY686584; AY686583; AY686582; NC_006169; NC_006168; NC_006164; NC_001355; NC_001349; NC_005351; NC_001596).
Human T-cell lymphotropic virus (HTLV) types I and II, including the HTLV type I subtypes Cosmopolitan, Central African, and Austro-Melanesian, and the HTLV type II subtypes Iia, Iib, Iic, and Iid.	See, e.g., Capdepon, <i>et al.</i> (2005) AIDS Res. Hum. Retrovirus 21:28-42; Bhigjee, <i>et al.</i> (1999) AIDS Res. Hum. Restrovirus 15:1229-1233; Vandamme, <i>et al.</i> (1998) J. Virol. 72:4327-4340; Vallejo, <i>et al.</i> (1996) J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 13:384-391. HTLV type I (see, e.g., GenBank Acc. Nos. AY563954; AY563953. HTLV type II (see, e.g., GenBank Acc. Nos. L03561; Y13051; AF139382).
Coronaviridae, including Coronaviruses, such as SARS-coronavirus (SARS-CoV), and Toroviruses.	See, e.g., Brian and Baric (2005) Curr. Top. Microbiol. Immunol. 287:1-30; Gonzalez, <i>et al.</i> (2003) Arch. Virol. 148:2207-2235; Smits, <i>et al.</i> (2003) J. Virol. 77:9567-9577; Jamieson, <i>et al.</i> (1998) J. Infect. Dis. 178:1263-1269 (GenBank Acc. Nos. AY348314; NC_004718; AY394850).

Table 1. Antigens.

Antigen	Reference
Rubella virus.	GenBank Acc. Nos. NC_001545; AF435866.
Mumps virus, including the genotypes A, C, D, G, H, and I.	See, e.g., Orvell, <i>et al.</i> (2002) J. Gen. Virol. 83:2489-2496. See, e.g., GenBank Acc. Nos. AY681495; NC_002200; AY685921; AF201473.
Coxsackie virus A including the serotypes 1, 11, 13, 15, 17, 18, 19, 20, 21, 22, and 24 (also known as Human enterovirus C; HEV-C).	See, e.g., Brown, <i>et al.</i> (2003) J. Virol. 77:8973-8984. GenBank Acc. Nos. AY421768; AY790926; X67706.
Coxsackie virus B, including subtypes 1-6.	See, e.g., Ahn, <i>et al.</i> (2005) J. Med. Virol. 75:290-294; Patel, <i>et al.</i> (2004) J. Virol. Methods 120:167-172; Rezig, <i>et al.</i> (2004) J. Med. Virol. 72:268-274. GenBank Acc. No. X05690.
Human enteroviruses including, e.g., human enterovirus A (HEV-A, CAV2 to CAV8, CAV10, CAV12, CAV14, CAV16, and EV71) and also including HEV-B (CAV9, CBV1 to CBV6, E1 to E7, E9, E11 to E21, E24 to E27, E29 to E33, and EV69 and E73), as well as HEV.	See, e.g., Oberste, <i>et al.</i> (2004) J. Virol. 78:855-867. Human enterovirus A (GenBank Acc. Nos. NC_001612); human enterovirus B (NC_001472); human enterovirus C (NC_001428); human enterovirus D (NC_001430). Simian enterovirus A (GenBank Acc. No. NC_003988).
Polioviruses including PV1, PV2, and PV3.	See, e.g., He, <i>et al.</i> (2003) J. Virol. 77:4827-4835; Hahsido, <i>et al.</i> (1999) Microbiol. Immunol. 43:73-77. GenBank Acc. No. AJ132961

Table 1. Antigens.

Antigen	Reference
	(type 1); AY278550 (type 2); X04468 (type 3).
Viral encephalitides viruses, including equine encephalitis, Venezuelan equine encephalitis (VEE) (including subtypes IA, IB, IC, ID, IIC, IID), Eastern equine encephalitis (EEE), Western equine encephalitis (WEE), St. Louis encephalitis, Murray Valley (Australian) encephalitis, Japanese encephalitis, and tick-born encephalitis.	See, e.g., Hoke (2005) <i>Mil. Med.</i> 170:92-105; Estrada-Franco, <i>et al.</i> (2004) <i>Emerg. Infect. Dis.</i> 10:2113-2121; Das, <i>et al.</i> (2004) <i>Antiviral Res.</i> 64:85-92; Aguilar, <i>et al.</i> (2004) <i>Emerg. Infect. Dis.</i> 10:880-888; Weaver, <i>et al.</i> (2004) <i>Arch. Virol. Suppl.</i> 18:43-64; Weaver, <i>et al.</i> (2004) <i>Annu. Rev. Entomol.</i> 49:141-174. Eastern equine encephalitis (GenBank Acc. No. NC_003899; AY722102); Western equine encephalitis (NC_003908).
Human herpesviruses, including cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus-1 (HHV-1), HHV-2, HHV-3, HHV-4, HHV-5, HHV-6, HHV-7, HHV-8, herpes B virus, herpes simplex virus types 1 and 2 (HSV-1,	See, e.g., Studahl, <i>et al.</i> (2000) <i>Scand. J. Infect. Dis.</i> 32:237-248; Padilla, <i>et al.</i> (2003) <i>J. Med. Virol.</i> 70 (Suppl. 1) S103-S110; Jankittivong and Langlais (1998) <i>Oral Surg. Oral Med.</i> 85:399-403. GenBank Nos. NC_001806 (herpesvirus 1); NC_001798 (herpesvirus 2); X04370 and NC_001348 (herpesvirus 3); NC_001345 (herpesvirus 4); NC_001347 (herpesvirus 5); X83413 and NC_000898 (herpesvirus 6); NC_001716 (herpesvirus 7). Human herpesviruses types 6 and 7 (HHV-6; HHV-7) are disclosed by, e.g., Padilla, <i>et al.</i> (2003) <i>J. Med. Virol.</i> 70 (Suppl. 1) S103-S110. Human herpesvirus 8 (HHV-8), including subtypes A-F, are disclosed in, e.g., Treurnicht, <i>et al.</i> (2002) <i>J. Med. Virol.</i> 66:235-240.

Table 1. Antigens.

Antigen	Reference
HSV-2), and varicella zoster virus (VZV).	
HIV-1 including group M (including subtypes A to J) and group O (including any distinguishable subtypes) (HIV-2, including subtypes A-E.	See, e.g., Smith, <i>et al.</i> (1998) J. Med. Virol. 56:264-268. See also, e.g., GenBank Acc. Nos. DQ054367; NC_001802; AY968312; DQ011180; DQ011179; DQ011178; DQ011177; AY588971; AY588970; AY781127; AY781126; AY970950; AY970949; AY970948; X61240; AJ006287; AJ508597; and AJ508596.
Epstein-Barr virus (EBV), including subtypes A and B.	See, e.g., Peh, <i>et al.</i> (2002) Pathology 34:446-450. Epstein-Barr virus strain B95-8 (GenBank Acc. No. V01555).
Reovirus, including serotypes and strains 1, 2, and 3, type 1 Lang, type 2 Jones, and type 3 Dearing.	See, e.g., Barthold, <i>et al.</i> (1993) Lab. Anim. Sci. 43:425-430; Roner, <i>et al.</i> (1995) Proc. Natl. Acad. Sci. USA 92:12362-12366; Kedl, <i>et al.</i> (1995) J. Virol. 69:552-559. GenBank Acc. No. K02739 (sigma-3 gene surface protein).
Cytomegalovirus (CMV) subtypes include CMV subtypes I-VII.	See, e.g., Chern, <i>et al.</i> (1998) J. Infect. Dis. 178:1149-1153; Vilas Boas, <i>et al.</i> (2003) J. Med. Virol. 71:404-407; Trincado, <i>et al.</i> (2000) J. Med. Virol. 61:481-487. GenBank Acc. No. X17403.
Rhinovirus, including	Human rhinovirus 2 (GenBank Acc. No. X02316); Human

Table 1. Antigens.

Antigen	Reference
all serotypes.	rhinovirus B (GenBank Acc. No. NC_001490); Human rhinovirus 89 (GenBank Acc. No. NC_001617); Human rhinovirus 39 (GenBank Acc. No. AY751783).
Adenovirus, including all serotypes.	AY803294; NC_004001; AC_000019; AC_000018; AC_000017; AC_000015; AC_000008; AC_000007; AC_000006; AC_000005; AY737798; AY737797; NC_003266; NC_002067; AY594256; AY594254; AY875648; AJ854486; AY163756; AY594255; AY594253; NC_001460; NC_001405; AY598970; AY458656; AY487947; NC_001454; AF534906; AY45969; AY128640; L19443; AY339865; AF532578.
Filoviruses, including Marburg virus and Ebola virus, and strains such as Ebola-Sudan (EBO-S), Ebola-Zaire (EBO-Z), and Ebola-Reston (EBO-R).	See, e.g., Geisbert and Jahrling (1995) <i>Virus Res.</i> 39:129-150; Hutchinson, <i>et al.</i> (2001) <i>J. Med. Virol.</i> 65:561-566. Marburg virus (see, e.g., GenBank Acc. No. NC_001608). Ebola virus (see, e.g., GenBank Acc. Nos. NC_006432; AY769362; NC_002549; AF272001; AF086833).
Arenaviruses, including lymphocytic choriomeningitis (LCM) virus, Lassa virus, Junin virus, and Machupo virus.	Junin virus, segment S (GenBank Acc. No. NC_005081); Junin virus, segment L (GenBank Acc. No. NC_005080).
Rabies virus.	See, e.g., GenBank Acc. Nos. NC_001542; AY956319; AY705373; AF499686; AB128149; AB085828; AB009663.
Arboviruses, including West Nile virus, Dengue viruses 1 to 4, Colorado tick fever virus, Sindbis virus,	Dengue virus type 1 (see, e.g., GenBank Acc. Nos. AB195673; AY762084). Dengue virus type 2 (see, e.g., GenBank Acc. Nos. NC_001474; AY702040; AY702039; AY702037). Dengue virus type 3 (see, e.g., GenBank Acc. Nos. AY923865; AT858043). Dengue virus type 4 (see, e.g., GenBank Acc. Nos. AY947539; AY947539;

Table 1. Antigens.

Antigen	Reference
Togaviridae, Flaviviridae, Bunyaviridae, Reoviridae, Rhabdoviridae, Orthomyxoviridae, and the like.	AF326573). Sindbis virus (see, e.g., GenBank Acc. Nos. NC_001547; AF429428; J02363; AF103728). West Nile virus (see, e.g., GenBank Acc. Nos. NC_001563; AY603654).
Poxvirus including orthopoxvirus (variola virus, monkeypox virus, vaccinia virus, cowpox virus), yatapoxvirus (tanapox virus, Yaba monkey tumor virus), parapoxvirus, and molluscipoxvirus.	Variola virus (see, e.g., GenBank Acc. Nos. NC_001611; Y16780; X72086; X69198).
Yellow fever.	See, e.g., GenBank Acc. No. NC_002031; AY640589; X03700.
Hantaviruses, including serotypes Hantaan (IITN), Seoul (SEO), Dobrava (DOB), Sin Nombre (SN), Puumala (PUU), and Dobrava-like Saaremaa (SAAV).	See, e.g., Elgh, <i>et al.</i> (1997) J. Clin. Microbiol. 35:1122-1130; Sjolander, <i>et al.</i> (2002) Epidemiol. Infect. 128:99-103; Zeier, <i>et al.</i> (2005) Virus Genes 30:157-180. GenBank Acc. No. NC_005222 and NC_005219 (Hantavirus). See also, e.g., GenBank Acc. Nos. NC_005218; NC_005222; NC_005219.
Flaviviruses, including Dengue virus, Japanese encephalitis virus, West Nile virus, and yellow	See, e.g., Mukhopadhyay, <i>et al.</i> (2005) Nature Rev. Microbiol. 3:13-22. GenBank Acc. Nos NC_001474 and AY702040 (Dengue). GenBank Acc. Nos. NC_001563 and AY603654.

Table 1. Antigens.

Antigen	Reference
fever virus.	
Measles virus.	See, e.g., GenBank Acc. Nos. AB040874 and AY486084.
Human parainfluenzaviruses (HPV), including HPV types 1-56.	Human parainfluenza virus 2 (see, e.g., GenBank Acc. Nos. AB176531; NC003443). Human parainfluenza virus 3 (see, e.g., GenBank Acc. No. NC_001796).
Influenza virus, including influenza virus types A, B, and C.	Influenza nucleocapsid (see, e.g., GenBank Acc. No. AY626145). Influenza hemagglutinin (see, e.g., GenBank Acc. Nos. AY627885; AY555153). Influenza neuraminidase (see, e.g., GenBank Acc. Nos. AY555151; AY577316). Influenza matrix protein 2 (see, e.g., GenBank Acc. Nos. AY626144). Influenza basic protein 1 (see, e.g., GenBank Acc. No. AY627897). Influenza polymerase acid protein (see, e.g., GenBank Acc. No. AY627896). Influenza nucleoprotein (see, e.g., GenBank Acc. No. AY627895).
Influenza A virus subtypes, e.g., swine viruses (SIV): H1N1 influenzaA and swine influenza virus.	Hemagglutinin of H1N1 (GenBank Acc. No. S67220). Influenza A virus matrix protein (GenBank Acc. No. AY700216). Influenza virus A H5H1 nucleoprotein (GenBank Acc. No. AY646426). H1N1 haemagglutinin (GenBank Acc. No. D00837). See also, GenBank Acc. Nos. BD006058; BD006055; BD006052. See also, e.g., Wentworth, <i>et al.</i> (1994) J. Virol. 68:2051-2058; Wells, <i>et al.</i> (1991) J.A.M.A. 265:478-481.
Respiratory syncytial virus (RSV), including subgroup A and subgroup B.	Respiratory syncytial virus (RSV) (see, e.g., GenBank Acc. Nos. AY353550; NC_001803; NC001781).
Rotaviruses, including human rotaviruses A to E, bovine rotavirus, rhesus monkey rotavirus, and	Human rotavirus C segment 8 (GenBank Acc. No. AJ549087); Human rotavirus G9 strain outer capsid protein (see, e.g., GenBank Acc. No. DQ056300); Human rotavirus B strain non-structural protein 4 (see, e.g., GenBank Acc. No. AY548957); human rotavirus A strain major inner capsid protein (see, e.g., GenBank Acc. No. AY601554).

Table 1. Antigens.

Antigen	Reference
human-RVV reassortments.	
Polyomavirus, including simian virus 40 (SV40), JC virus (JCV) and BK virus (BKV).	See, e.g., Engels, <i>et al.</i> (2004) J. Infect. Dis. 190:2065-2069; Vilchez and Butel (2004) Clin. Microbiol. Rev. 17:495-508; Shivapurkar, <i>et al.</i> (2004) Cancer Res. 64:3757-3760; Carbone, <i>et al.</i> (2003) Oncogene 2:5173-5180; Barbanti-Brodano, <i>et al.</i> (2004) Virology 318:1-9 (SV40 complete genome in, e.g., GenBank Acc. Nos. NC_001669; AF168994; AY271817; AY271816; AY120890; AF345344; AF332562).
Coltivirus, including Colorado tick fever virus, Eyach virus.	Attoui, <i>et al.</i> (1998) J. Gen. Virol. 79:2481-2489. Segments of Eyach virus (see, e.g., GenBank Acc. Nos. AF282475 ; AF282472 ; AF282473 ; AF282478 ; AF282476 ; NC_003707 ; NC_003702 ; NC_003703 ; NC_003704 ; NC_003705 ; NC_003696 ; NC_003697 ; NC_003698 ; NC_003699 ; NC_003701 ; NC_003706 ; NC_003700 ; AF282471 ; AF282477).
Calciviruses, including the genogroups Norwalk, Snow Mountain group (SMA), and Saaporo.	Snow Mountain virus (see, e.g., GenBank Acc. No. AY134748).
Parvoviridae, including dependovirus, parvovirus (including parvovirus B19), and erythrovirus.	See, e.g., Brown (2004) Dev. Biol. (Basel) 118:71-77; Alvarez-Lafuente, <i>et al.</i> (2005) Ann. Rheum. Dis. 64:780-782; Ziyaeyan, <i>et al.</i> (2005) Jpn. J. Infect. Dis. 58:95-97; Kaufman, <i>et al.</i> (2005) Virology 332:189-198.

Other organisms for which suitable antigens are known in the art include, but are not limited to, *Chlamydia trachomatis*, *Streptococcus pyogenes* (Group A Strep), *Streptococcus agalactia* (Group B Strep), *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Vibrio cholerae*, *Salmonella* species (including *typhi*, *typhimurium*), enterica (including

Helicobacter pylori, *Shigella flexneri* and other Group D *shigella* species), *Burkholderia mallei*, *Burkholderia pseudomallei*, *Klebsiella pneumonia*, *Clostridium* species (including *C. difficile*), *Vibrio parahaemolyticus* and *V. vulnificus*. This list is not meant to be limiting.

[0092] In certain embodiments, antigen sequence(s) may be expressed as a single polypeptide fused to an amino-terminal portion of the *L. monocytogenes* ActA protein which permits expression and secretion of a fusion protein from the bacterium within the vaccinated host. In these embodiments, the antigenic construct may be a polynucleotide comprising a promoter operably linked to a nucleic acid sequence encoding a fusion protein, wherein the fusion protein comprises (a) modified ActA and (b) one or more antigenic epitopes to be expressed as a fusion protein following the modified ActA sequence.

[0093] By “modified ActA” is meant a contiguous portion of the *L. monocytogenes* ActA protein which comprises at least the ActA signal sequence, but does not comprise the entirety of the ActA sequence, or that has at least about 80 % sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 98% sequence identity to such an ActA sequence. The ActA signal sequence is MGLNRFMRAMMVVFITANCITINPDIIFA (SEQ ID NO: 2). In some embodiments, the promoter is *ActA* promoter from WO07/103225; and WO07/117371, each of which is incorporated by reference in its entirety herein.

[0094] By way of example, the modified ActA may comprise at least the first 59 amino acids of ActA, or a sequence having at least about 80 % sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 98% sequence identity to at least the first 59 amino acids of ActA. In some embodiments, the modified ActA comprises at least the first 100 amino acids of ActA, or a sequence having at least about 80 % sequence identity, at least about 85% sequence identity, at least about 90% sequence identity, at least about 95% sequence identity, or at least about 98% sequence identity to the first 100 amino acids of ActA. In other words, in some embodiments, the modified ActA sequence corresponds to an N-terminal fragment of ActA (including the ActA signal sequence) that is truncated at residue 100 or thereafter.

[0095] ActA-N100 has the following sequence (SEQ ID NO: 3):

VGLNRFMRAM MVVFITANCI TINPDIIFAA TDSEDSSLNT DEWEEEEKTEE50
 QPSEVNTGPR YETAREVSSR DIEELEKSNK VKNTNKADLI AMLKAKAEKG
 100

[0096] In this sequence, the first residue is depicted as a valine; the polypeptide is synthesized by *Listeria* with a methionine in this position. Thus, ActA-N100 may also have the following sequence (SEQ ID NO:4):

MGLNRFMRAM MVVFITANCI TINPDIIFAA TDSEDSSLNT DEWEEEEKTEE 50
 QPSEVNTGPR YETAREVSSR DIEELEKSNK VKNTNKADLI AMLKAKAEKG
 100

[0097] ActA-N100 may also comprise one or more additional residues lying between the C-terminal residue of the modified ActA and the antigen sequence. In the following sequences, ActA-N100 is extended by two residues added by inclusion of a BamHI site (SEQ ID NO: 5):

VGLNRFMRAM MVVFITANCI TINPDIIFAA TDSEDSSLNT DEWEEEEKTEE50
 QPSEVNTGPR YETAREVSSR DIEELEKSNK VKNTNKADLI AMLKAKAEKG
 100

GS

which when synthesized with a first residue methionine has the sequence (SEQ ID NO: 6):

MGLNRFMRAM MVVFITANCI TINPDIIFAA TDSEDSSLNT DEWEEEEKTEE 50
 QPSEVNTGPR YETAREVSSR DIEELEKSNK VKNTNKADLI AMLKAKAEKG
 100

GS .

[0098] As sequences encoded by one organism are not necessarily codon optimized for optimal expression in a chosen vaccine platform bacterial strain, the present invention also provides nucleic acids that are altered by codon optimized for expressing by a bacterium such as *L. monocytogenes*.

[0099] In various embodiments, at least one percent of any non-optimal codons are changed to provide optimal codons, more normally at least five percent are changed, most normally at least ten percent are changed, often at least 20% are changed, more often at least 30% are changed, most often at least 40%, usually at least 50% are changed, more usually at least 60% are changed, most usually at least 70% are changed, optimally at least 80% are changed, more optimally at least 90% are changed, most optimally at least 95% are changed, and conventionally 100% of any non-optimal codons are codon-optimized for *Listeria* expression (Table 2).

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

Amino Acid	A	R	N	D	C	Q	E	G	H	I
Optimal <i>Listeria</i> codon	GCA	CGU	AAU	GAU	UGU	CAA	GAA	GGU	CAU	AUU
Amino Acid	L	K	M	F	P	S	T	W	Y	V
Optimal <i>Listeria</i> codon	UUA	AAA	AUG	UUU	CCA	AGU	ACA	UGG	UAU	GUU

[00100] The invention supplies a number of *Listeria* species and strains for making or engineering an attenuated bacterium of the present invention. The *Listeria* of the present invention is not to be limited by the species and strains disclosed in Table 3.

Table 3. Strains of *Listeria* suitable for use in the present invention, e.g., as a vaccine or as a source of nucleic acids.

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

<i>L. monocytogenes</i> CS-L0002 (delta ActA-delta lplA).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> CS-L0003 (L461T-delta lplA).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> DP-L4038 (delta ActA-LLO L461T).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> DP-L4384 (S44A-LLO L461T).	Brockstedt, <i>et al.</i> (2004) Proc. Natl. Acad. Sci. USA 101:13832-13837; supporting information.
<i>L. monocytogenes</i> . Mutation in lipoate protein ligase (LplA1).	O’Riordan, <i>et al.</i> (2003) Science 302:462-464.
<i>L. monocytogenes</i> DP-L4017 (10403S hly (L461T) point mutation in hemolysin gene.	U.S. Provisional Pat. Appl. Ser. No. 60/490,089 filed July 24, 2003.
<i>L. monocytogenes</i> EGD.	GenBank Acc. No. AL591824.
<i>L. monocytogenes</i> EGD-e.	GenBank Acc. No. NC_003210. ATCC Acc. No. BAA-679.
<i>L. monocytogenes</i> strain EGD, complete genome, segment 3/12	GenBank Acc. No. AL591975
<i>L. monocytogenes</i> .	ATCC Nos. 13932; 15313; 19111-19120; 43248-43251; 51772-51782.
<i>L. monocytogenes</i> DP-L4029 deleted in <i>uvrAB</i> .	U.S. Provisional Pat. Appl. Ser. No. 60/541,515 filed February 2, 2004; U.S. Provisional Pat. Appl. Ser. No. 60/490,080 filed July 24, 2003.
<i>L. monocytogenes</i> DP-L4029 deleted in <i>uvrAB</i> treated with a psoralen.	U.S. Provisional Pat. Appl. Ser. No. 60/541,515 filed February 2, 2004.
<i>L. monocytogenes</i> delta <i>actA</i> delta <i>inlB</i> delta <i>uvrAB</i>	Brockstedt (2005) Nature Medicine and KBMA patent
<i>L. monocytogenes</i> delta <i>actA</i> delta <i>inlB</i> delta <i>uvrAB</i> treated with psoralen	Brockstedt (2005) Nature Medicine and KBMA patent

<i>L. monocytogenes</i> delta <i>actA</i> delta <i>inlB</i> delta <i>uvrAB prfA(G155S)</i>	Lauer et al, (2008) Infect. Immun. And WO 2009/143085
<i>L. monocytogenes</i> delta <i>actA</i> delta <i>inlB</i> delta <i>uvrAB prfA(G155S)</i> treated with psoralen	Lauer et al, (2008) Infect. Immun. And WO 2009/143085
<i>L. monocytogenes</i> ActA-/inlB- double mutant.	Deposited with ATCC on October 3, 2003, Acc. No. PTA-5562.
<i>L. monocytogenes</i> lplA mutant or hly mutant.	U.S. Pat. Applic. No. 20040013690 of Portnoy, <i>et al.</i>
<i>L. monocytogenes</i> DAL/DAT double mutant.	U.S. Pat. Applic. No. 20050048081 of Frankel and Portnoy.
<i>L. monocytogenes</i> str. 4b F2365.	GenBank Acc. No. NC_002973.
<i>Listeria ivanovii</i>	ATCC No. 49954
<i>Listeria innocua</i> Clip1 1262.	GenBank Acc. No. NC_003212; AL592022.
<i>Listeria innocua</i> , a naturally occurring hemolytic strain containing the PrfA-regulated virulence gene cluster.	Johnson, <i>et al.</i> (2004) Appl. Environ. Microbiol. 70:4256-4266.
<i>Listeria seeligeri</i> .	Howard, <i>et al.</i> (1992) Appl. Environ. Microbiol. 58:709-712.
<i>Listeria innocua</i> with <i>L. monocytogenes</i> pathogenicity island genes.	Johnson, <i>et al.</i> (2004) Appl. Environ. Microbiol. 70:4256-4266.
<i>Listeria innocua</i> with <i>L. monocytogenes</i> internalin A gene, e.g., as a plasmid or as a genomic nucleic acid.	See, e.g., Lingnau, <i>et al.</i> (1995) Infection Immunity 63:3896-3903; Gaillard, <i>et al.</i> (1991) Cell 65:1127-1141).

The present invention encompasses reagents and methods that comprise the above *Listerial* strains, as well as these strains that are modified, e.g., by a plasmid and/or by genomic integration, to contain a nucleic acid encoding one of, or any combination of, the following genes: hly (LLO; listeriolysin); iap (p60); inlA; inlB; inlC; dal (alanine racemase); daaA (dat; D-amino acid aminotransferase); plcA; plcB; ActA; or any nucleic acid that mediates growth, spread, breakdown of a single walled vesicle, breakdown of a double walled vesicle, binding to a host cell, uptake by a host cell. The present invention is not to be limited by the particular strains disclosed above.

[00101] 4. Therapeutic compositions.

[0100] The vaccine compositions described herein can be administered to a host, either alone or in combination with a pharmaceutically acceptable excipient, in an amount sufficient to induce an appropriate immune response. The immune response can comprise, without limitation, specific immune response, non-specific immune response, both specific and non-specific response, innate response, primary immune response, adaptive immunity, secondary immune response, memory immune response, immune cell activation, immune cell proliferation, immune cell differentiation, and cytokine expression. The vaccines of the present invention can be stored, e.g., frozen, lyophilized, as a suspension, as a cell paste, or complexed with a solid matrix or gel matrix.

[0101] In certain embodiments, after the subject has been administered an effective dose of a first vaccine to prime the immune response, a second vaccine is administered. This is referred to in the art as a “prime-boost” regimen. In such a regimen, the compositions and methods of the present invention may be used as the “prime” delivery, as the “boost” delivery, or as both a “prime” and a “boost.” Any number of “boost” immunizations can be delivered in order to maintain the magnitude or effectiveness of a vaccine-induced immune response.

[0102] As an example, a first vaccine comprised of killed but metabolically active *Listeria* that encodes and expresses the antigen polypeptide(s) may be delivered as the “prime,” and a second vaccine comprised of attenuated (live or killed but metabolically active) *Listeria* that encodes the antigen polypeptide(s) may be delivered as the “boost.” It should be understood, however, that each of the prime and boost need not utilize the

methods and compositions of the present invention. Rather, the present invention contemplates the use of other vaccine modalities together with the bacterial vaccine methods and compositions of the present invention. The following are examples of suitable mixed prime-boost regimens: a DNA (*e.g.*, plasmid) vaccine prime/bacterial vaccine boost; a viral vaccine prime/bacterial vaccine boost; a protein vaccine prime/bacterial vaccine boost; a DNA prime/bacterial vaccine boost plus protein vaccine boost; a bacterial vaccine prime/DNA vaccine boost; a bacterial vaccine prime/viral vaccine boost; a bacterial vaccine prime/protein vaccine boost; a bacterial vaccine prime/bacterial vaccine boost plus protein vaccine boost; etc. This list is not meant to be limiting

[0103] The prime vaccine and boost vaccine may be administered by the same route or by different routes. The term “different routes” encompasses, but is not limited to, different sites on the body, for example, a site that is oral, non-oral, enteral, parenteral, rectal, intranode (lymph node), intravenous, arterial, subcutaneous, intradermal, intramuscular, intratumor, peritumor, infusion, mucosal, nasal, in the cerebrospinal space or cerebrospinal fluid, and so on, as well as by different modes, for example, oral, intravenous, and intramuscular.

[0104] An effective amount of a prime or boost vaccine may be given in one dose, but is not restricted to one dose. Thus, the administration can be two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, twenty, or more, administrations of the vaccine. Where there is more than one administration of a vaccine or vaccines in the present methods, the administrations can be spaced by time intervals of one minute, two minutes, three, four, five, six, seven, eight, nine, ten, or more minutes, by intervals of about one hour, two hours, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 hours, and so on. In the context of hours, the term “about” means plus or minus any time interval within 30 minutes. The administrations can also be spaced by time intervals of one day, two days, three days, four days, five days, six days, seven days, eight days, nine days, ten days, 11 days, 12 days, 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, and combinations thereof. The invention is not limited to dosing intervals that are spaced equally in time, but encompass doses at non-equal intervals, such as a priming schedule consisting of administration at 1 day, 4 days, 7 days, and 25 days, just to provide a non-limiting example.

[0105] In certain embodiments, administration of the boost vaccination can be initiated at about 5 days after the prime vaccination is initiated; about 10 days after the prime vaccination is initiated; about 15 days; about 20 days; about 25 days; about 30 days; about 35 days; about 40 days; about 45 days; about 50 days; about 55 days; about 60 days; about 65 days; about 70 days; about 75 days; about 80 days, about 6 months, and about 1 year after administration of the prime vaccination is initiated. Preferably one or both of the prime and boost vaccination comprises delivery of a composition of the present invention.

[0106] A “pharmaceutically acceptable excipient” or “diagnostically acceptable excipient” includes but is not limited to, sterile distilled water, saline, phosphate buffered solutions, amino acid based buffers, or bicarbonate buffered solutions. An excipient selected and the amount of excipient used will depend upon the mode of administration. Administration may be oral, intravenous, subcutaneous, dermal, intradermal, intramuscular, mucosal, parenteral, intraorgan, intralesional, intranasal, inhalation, intraocular, intramuscular, intravascular, intranodal, by scarification, rectal, intraperitoneal, or any one or combination of a variety of well-known routes of administration. The administration can comprise an injection, infusion, or a combination thereof.

[0107] Administration of the vaccine of the present invention by a non-oral route can avoid tolerance. Methods are known in the art for administration intravenously, subcutaneously, intradermally, intramuscularly, intraperitoneally, orally, mucosally, by way of the urinary tract, by way of a genital tract, by way of the gastrointestinal tract, or by inhalation.

[0108] An effective amount for a particular patient may vary depending on factors such as the condition being treated, the overall health of the patient, the route and dose of administration and the severity of side effects. Guidance for methods of treatment and diagnosis is available (see, e.g., Maynard, et al. (1996) A Handbook of SOPs for Good Clinical Practice, Interpharm Press, Boca Raton, FL; Dent (2001) Good Laboratory and Good Clinical Practice, Urch Publ., London, UK).

[0109] The vaccines of the present invention can be administered in a dose, or dosages, where each dose comprises at least 100 bacterial cells/kg body weight or more; in certain embodiments 1000 bacterial cells/kg body weight or more; normally at least 10,000 cells; more normally at least 100,000 cells; most normally at least 1 million cells; often at least 10

million cells; more often at least 100 million cells; typically at least 1 billion cells; usually at least 10 billion cells; conventionally at least 100 billion cells; and sometimes at least 1 trillion cells/kg body weight. The present invention provides the above doses where the units of bacterial administration is colony forming units (CFU), the equivalent of CFU prior to psoralen treatment, or where the units are number of bacterial cells.

[0110] The vaccines of the present invention can be administered in a dose, or dosages, where each dose comprises between 10^7 and 10^8 bacteria per 70 kg body weight (or per 1.7 square meters surface area; or per 1.5 kg liver weight); 2×10^7 and 2×10^8 bacteria per 70 kg body weight (or per 1.7 square meters surface area; or per 1.5 kg liver weight); 5×10^7 and 5×10^8 bacteria per 70 kg body weight (or per 1.7 square meters surface area; or per 1.5 kg liver weight); 10^8 and 10^9 bacteria per 70 kg body weight (or per 1.7 square meters surface area; or per 1.5 kg liver weight); between 2.0×10^8 and 2.0×10^9 bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 5.0×10^8 to 5.0×10^9 bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^9 and 10^{10} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 2×10^9 and 2×10^{10} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 5×10^9 and 5×10^{10} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^{11} and 10^{12} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 2×10^{11} and 2×10^{12} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 5×10^{11} and 5×10^{12} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^{12} and 10^{13} bacteria per 70 kg (or per 1.7 square meters surface area); between 2×10^{12} and 2×10^{13} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 5×10^{12} and 5×10^{13} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^{13} and 10^{14} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 2×10^{13} and 2×10^{14} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); 5×10^{13} and 5×10^{14} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 10^{14} and 10^{15} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); between 2×10^{14} and 2×10^{15} bacteria per 70 kg (or per 1.7 square meters surface area, or per 1.5 kg liver weight); and so on, wet weight.

[0111] Also provided is one or more of the above doses, where the dose is administered by way of one injection every day, one injection every two days, one injection every three days, one injection every four days, one injection every five days, one injection every six days, or one injection every seven days, where the injection schedule is maintained for, e.g., one day only, two days, three days, four days, five days, six days, seven days, two weeks, three weeks, four weeks, five weeks, or longer. The invention also embraces combinations of the above doses and schedules, e.g., a relatively large initial bacterial dose, followed by relatively small subsequent doses, or a relatively small initial dose followed by a large dose.

[0112] A dosing schedule of, for example, once/week, twice/week, three times/week, four times/week, five times/week, six times/week, seven times/week, once every two weeks, once every three weeks, once every four weeks, once every five weeks, and the like, is available for the invention. The dosing schedules encompass dosing for a total period of time of, for example, one week, two weeks, three weeks, four weeks, five weeks, six weeks, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, and twelve months.

[0113] Provided are cycles of the above dosing schedules. The cycle can be repeated about, e.g., every seven days; every 14 days; every 21 days; every 28 days; every 35 days; 42 days; every 49 days; every 56 days; every 63 days; every 70 days; and the like. An interval of non dosing can occur between a cycle, where the interval can be about, e.g., seven days; 14 days; 21 days; 28 days; 35 days; 42 days; 49 days; 56 days; 63 days; 70 days; and the like. In this context, the term “about” means plus or minus one day, plus or minus two days, plus or minus three days, plus or minus four days, plus or minus five days, plus or minus six days, or plus or minus seven days.

[0114] The present invention encompasses a method of administering *Listeria* that is oral. Also provided is a method of administering *Listeria* that is intravenous. Moreover, what is provided is a method of administering *Listeria* that is oral, intramuscular, intravenous, intradermal and/or subcutaneous. The invention supplies a *Listeria* bacterium, or culture or suspension of *Listeria* bacteria, prepared by growing in a medium that is meat based, or that contains polypeptides derived from a meat or animal product. Also supplied by the present invention is a *Listeria* bacterium, or culture or suspension of *Listeria* bacteria, prepared by growing in a medium that does not contain meat or animal products, prepared by growing on a medium that contains vegetable polypeptides, prepared by growing on a

medium that is not based on yeast products, or prepared by growing on a medium that contains yeast polypeptides.

[0115] Methods for co-administration with an additional therapeutic agent are well known in the art (Hardman, et al. (eds.) (2001) Goodman and Gilman's The Pharmacological Basis of Therapeutics, 10th ed., McGraw-Hill, New York, NY; Poole and Peterson (eds.) (2001) Pharmacotherapeutics for Advanced Practice: A Practical Approach, Lippincott, Williams & Wilkins, Phila., PA; Chabner and Longo (eds.) (2001) Cancer Chemotherapy and Biotherapy, Lippincott, Williams & Wilkins, Phila., PA).

[0116] Additional agents which are beneficial to raising a cytolytic T cell response may be used as well. Such agents are termed herein carriers. These include, without limitation, B7 costimulatory molecule, interleukin-2, interferon- γ , GM-CSF, CTLA-4 antagonists, OX-40/OX-40 ligand, CD40/CD40 ligand, sargramostim, levamisole, vaccinia virus, Bacille Calmette-Guerin (BCG), liposomes, alum, Freund's complete or incomplete adjuvant, detoxified endotoxins, mineral oils, surface active substances such as lipolecithin, pluronic polyols, polyanions, peptides, and oil or hydrocarbon emulsions. Carriers for inducing a T cell immune response which preferentially stimulate a cytolytic T cell response versus an antibody response are preferred, although those that stimulate both types of response can be used as well. In cases where the agent is a polypeptide, the polypeptide itself or a polynucleotide encoding the polypeptide can be administered. The carrier can be a cell, such as an antigen presenting cell (APC) or a dendritic cell. Antigen presenting cells include such cell types as macrophages, dendritic cells and B cells. Other professional antigen-presenting cells include monocytes, marginal zone Kupffer cells, microglia, Langerhans' cells, interdigitating dendritic cells, follicular dendritic cells, and T cells. Facultative antigen-presenting cells can also be used. Examples of facultative antigen-presenting cells include astrocytes, follicular cells, endothelium and fibroblasts. The carrier can be a bacterial cell that is transformed to express the polypeptide or to deliver a polynucleotide which is subsequently expressed in cells of the vaccinated individual. Adjuvants, such as aluminum hydroxide or aluminum phosphate, can be added to increase the ability of the vaccine to trigger, enhance, or prolong an immune response. Additional materials, such as cytokines, chemokines, and bacterial nucleic acid sequences, like CpG, a toll-like receptor (TLR) 9 agonist as well as additional agonists for TLR 2, TLR 4, TLR 5, TLR 7, TLR 8, TLR9, including lipoprotein, LPS, monophosphoryl lipid A, lipoteichoic acid, imiquimod,

resiquimod, and other like immune modulators such as cyclic dinucleotide STING agonists including c-di-GMP, c-di-AMP, c-di-IMP, and c-AMP-GMP, used separately or in combination with the described compositions are also potential adjuvants. Other representative examples of adjuvants include the synthetic adjuvant QS-21 comprising a homogeneous saponin purified from the bark of *Quillaja saponaria* and *Corynebacterium parvum* (McCune et al., Cancer, 1979; 43:1619). It will be understood that the adjuvant is subject to optimization. In other words, the skilled artisan can engage in routine experimentation to determine the best adjuvant to use.

[0117] An effective amount of a therapeutic agent is one that will decrease or ameliorate the symptoms normally by at least 10%, more normally by at least 20%, most normally by at least 30%, typically by at least 40%, more typically by at least 50%, most typically by at least 60%, often by at least 70%, more often by at least 80%, and most often by at least 90%, conventionally by at least 95%, more conventionally by at least 99%, and most conventionally by at least 99.9%.

[0118] The reagents and methods of the present invention provide a vaccine comprising only one vaccination; or comprising a first vaccination; or comprising at least one booster vaccination; at least two booster vaccinations; or at least three booster vaccinations. Guidance in parameters for booster vaccinations is available. See, e.g., Marth (1997) *Biologicals* 25:199-203; Ramsay, et al. (1997) *Immunol. Cell Biol.* 75:382-388; Gherardi, et al. (2001) *Histol. Histopathol.* 16:655-667; Leroux-Roels, et al. (2001) *Acta Clin. Belg.* 56:209-219; Greiner, et al. (2002) *Cancer Res.* 62:6944-6951; Smith, et al. (2003) *J. Med. Virol.* 70:Suppl.1:S38-S41; Sepulveda-Amor, et al. (2002) *Vaccine* 20:2790-2795).

[0119] Formulations of therapeutic agents may be prepared for storage by mixing with physiologically acceptable carriers, excipients, or stabilizers in the form of, e.g., lyophilized powders, slurries, aqueous solutions or suspensions (see, e.g., Hardman, et al. (2001) Goodman and Gilman's *The Pharmacological Basis of Therapeutics*, McGraw-Hill, New York, NY; Gennaro (2000) *Remington: The Science and Practice of Pharmacy*, Lippincott, Williams, and Wilkins, New York, NY; Avis, et al. (eds.) (1993) *Pharmaceutical Dosage Forms: Parenteral Medications*, Marcel Dekker, NY; Lieberman, et al. (eds.) (1990) *Pharmaceutical Dosage Forms: Tablets*, Marcel Dekker, NY; Lieberman, et al. (eds.) (1990) *Pharmaceutical Dosage Forms: Disperse Systems*, Marcel Dekker, NY; Weiner and Kotkoskie (2000) *Excipient Toxicity and Safety*, Marcel Dekker, Inc., New York, NY).

[0120] Examples

[0121] The following examples serve to illustrate the present invention. These examples are in no way intended to limit the scope of the invention.

[0122] Example 1. Construction of Lm RIID (*Listeria monocytogenes* Recombinase Induced Intracellular Death) strains

[0123] DNA manipulations, purification and vector assembly.

[0124] Recombinase recognition sites (*loxP*, *lox66* and *lox71* variants, or *frt*) were inserted in the intergenic regions 5' and 3' of essential *Listeria monocytogenes* (Lm) gene sets using allelic exchange (Camilli, 1993). Recombinase sites were added to allelic exchange vectors by PCR using 10403S (Bishop, 1987) DNA as template. PCR was performed using the high-fidelity enzyme Phusion DNA polymerase (NEB, Ipswich, MA) in a MyCycler thermocycler (BioRad, Hercules CA). The oligonucleotides (oligos) (Integrated DNA Technologies, Coralville, IA) used for cloning are listed in the following table.

Table 4- Oligonucleotides

Oligo name	Sequence (SOE overlap underlined)	RE site
PL1536	tttCGGCCGatgagtaacctattaactgttcat (SEQ ID NO: 7)	EagI
PL1537	tttGGATCCttagctccatcttctaataat (SEQ ID NO: 8)	BamHI
PL2861	tttGGTACCggtcatgatgacattaatacaaca (SEQ ID NO: 9)	KpnI
PL2862	tttGAGCTCtatacctaaalggctttatatacagt (SEQ ID NO: 10)	SacI
PL2863	tttAAGCTTttggaaattcgattaccccact (SEQ ID NO: 11)	HindIII
PL2864	tttGGTACCtggttatttttcgtgaataactgcc (SEQ ID NO: 12)	KpnI
PL2865	tttGGTACCtttGAGCTCtttttagtaaaaaacgccagagaagc (SEQ ID NO: 13)	KpnI/SacI
PL2866	tttGAATTCtccgttggtgcaatatteget (SEQ ID NO: 14)	EcoRI
PL2876	tttAAGCTTtccctgaagaagaagtagcaatta (SEQ ID NO: 15)	HindIII
PL2868	tttGGTACCagettgattttattctctatgtcgc (SEQ ID NO: 16)	KpnI
PL2869	tttGGTACCtttGAGCTCggaaatgactctaatttgcgaat (SEQ ID NO: 17)	KpnI/SacI
PL2870	tttGAATTCccatgtataccaatcggttagga (SEQ ID NO: 18)	EcoRI
PL3139	<u>ataacttcgtatagcatatacgaacggttagg</u> aaatgactctaatttgcgaat (SEQ ID NO: 19)	
PL3140	tttGAATTCccatgtataccaatcggttagga (SEQ ID NO: 20)	EcoRI

PL3141	tttAAGCTTtcctgaagaagaagtagcaatta (SEQ ID NO: 21)	HindIII
PL3142	<u>taccgttcgtataatgtatgctatacgaagtta</u> tagcttgattttattctctatgctgc (SEQ ID NO: 22)	
PL3246	tttGAATTCacagaaggagattgtgaaatg (SEQ ID NO: 23)	EcoRI
PL3247	gggTCTAGAttGGTACCaatagaagcgtagcgact (SEQ ID NO: 24)	KpnI/XbaI
PL3248	gggTCTAGAgtttcacgtgaaacattcta (SEQ ID NO: 25)	XbaI
PL3249	tttAAGCTTcggaattgggtcaagactgg (SEQ ID NO: 26)	HindIII
PL3339	attGGTACCtcgaggagttaaactcccaa (SEQ ID NO: 27)	KpnI
PL3340	aacTCTAGAcaccgcgggtggcgccgataa (SEQ ID NO: 28)	XbaI
PL3378	<u>tatGCGCCCGC</u> gggaagcagttgggttaact (SEQ ID NO: 29)	NotI
PL3379	<u>aacTCTAGActtagtctccattcttaata</u> (SEQ ID NO: 30)	XbaI
PL3469	<u>aaaggaaagtccctattctctagaaagtata</u> ggaacttctgcggaaatgactctaattgc (SEQ ID NO: 31)	
PL3470	<u>gcagaagttccctatactttctagagaatagga</u> acttcccttagcttgattttattctct (SEQ ID NO: 32)	
PL3471	<u>aaaggaaagtccctattctctagaaagtata</u> ggaacttctgcttttaglaaaaaaacgcca (SEQ ID NO: 33)	
PL3472	<u>gcagaagttccctatactttctagagaatagga</u> acttcccttttggtattttctgctgaata (SEQ ID NO: 34)	
PL3546	tttCTGCAGgtggatagaactcataaaggac (SEQ ID NO: 35)	PstI
PL3547	tttGGTACCtcagttaaccccaactgettc (SEQ ID NO: 36)	KpnI
PL3548	tttGGTACCttAGATCTaaacacaguacgaaagaaaag (SEQ ID NO: 37)	KpnI/BglII
PL3549	tttGAATTCccagtaggtccactgtatc (SEQ ID NO: 38)	EcoRI
PL3552	tttCTGCAGtccatgtatacccaatcggttagga (SEQ ID NO: 39)	PstI
PL3553	tttAAGCTTtcctgaagaagaagtagcaatta (SEQ ID NO: 40)	HindIII
PL4017	tttAGATCTaatgggttttctctataa (SEQ ID NO: 41)	BglII
PL4018	tAGATCTataactcgtatagcatatatacgaacgggtattcgaaaattattgcgtta (SEQ ID NO: 42)	BglII
PL4075	tttCTGCAGatgattcaatccttcttgc (SEQ ID NO: 43)	PstI
PL4076	gggTCTAGAgagtatacaaacgggaata (SEQ ID NO: 44)	XbaI

[0125] The following table provides a list of sequences used in the following examples.

Table 5- Sequences

Name	Sequence
<i>lox66</i>	Ataacttcgtatagcatatcattatatacgaacggta (SEQ ID NO: 45)
<i>lox71</i>	Taccgttcgtatagcatatcattatatacgaagttat (SEQ ID NO: 46)
<i>loxP</i>	Ataacttcgtataatgtatgctatatacgaagttat (SEQ ID NO: 47)
<i>actA promoter</i>	gggaagcagttggggtaactgattaacaaatgtagagaaaaattaattctcaagtgatattcttaaa ataattcatgaatatttttcttatattagctaattaagaagataattaactgctaataccaatttttaacgg aataaattagtgaaaatgaaggccgaattttccttggtctaaaaagggtgtattagcgtatcacgaggag ggagtataa (SEQ ID NO: 48)
Cre (codon optimized)	atgagtaacctattaactgttcatcaaaattaccagcattaccagtggtgcaacatcagatgaagtaa gaaaaaatttaattggtatgtttagagaccgacaagccttttcggagcatatcggaatgttattatc tgttttagatcatgggcagcatggtgcaacttaacaatagaaaatggtttcagcagaaccagaaga tgtacgagattatttattatcctcaagcaagaggattagcagtaaaaaccattcaacaacatttagga caattaaatattgtacatagacgatcaggattaccaagacctagcgattctaacgcagtttagtttagta tgagaagaattagaaaagaaaatgtcgtatgcaggcgaacgagcaaaacaagcactagcatttgaacg tacagatttcgaccaagtaagatcattaatggaaaatagcgaccgttgtcaagacatccgaaacttagc tttttaggaatagcatacaacacattattaagaatagcagaaatagccagaattagagtaaaagacat tagtagaacagatggaggaagaatgttaattcatattggaagaacaaaaacatttagtatcaacagccg gggtagaaaaagcgttatcattaggagttacaaaattagtagaacgatggatttcagtttcaggagtg cagatgacccaaataattatttttttagagtacgaaaaacggagtagcagcaccttcagcaacaa gtcaattaagtacaagagcattagaaggaatattcgaagcaacacatcgactaatttacggagcaaaa gatgatagtggaacacgatatttagcttgagtggaacagtgccgagtaggagcagcaagagatat ggcaagagcgggagttagtataccagaaataatgcaagcaggaggatggacaaatgtaaatattgta atgaattatattagaaatttagatagtgaaaccggtgcaatggtacgattattagaagatggagactaa (SEQ ID NO: 49)
<i>frt</i>	Gaagttcctattctctagaagtagtaggaacttc (SEQ ID NO: 50)
FLP (codon optimized)	atgtcgaatttgatatactatgtaaaaactccacctaagtagtagtcgctcaatttggtaacgttttgaa cgaccaagtggcgaaaagatagcttcctgtgccggaacttacttactgtgttgatgattacacata atggcactgcaatcaaaagagcaacattcatgtcatacaacaccatcatttctaattctttatcattgat

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attgttaacaaaagttacaattcaaatacaaaactcaaaaagcgacgattcttgaagctagttgaaa
aagttaatcccagcatgggagttaccatcattccttacaatggacagaaacaccaatccgacattaca
gacattgtttctagtttacaactacaatttgaaagcagtgaagaagcggataaagggaactcacattcg
aagaaaatgttaaaggctttgtatctgaaggagaatctatctgggagattacagaaaagattctaac
tcttttgagtatacttcacgctttactaaaacaaaacggtataccagtttcttttctagctacattcatta
actgcggtcgatttagtgacattaagaatgtagatcctaatacggtcaagttagtcctaaacaagtatct
agggtgcatcattcaatgcttagttacggaaacaaaaacgagtgaagtagacatatctattctttctg
ctagaggtagaattgatccgtgtatacttagatgaatttctacgtaattcagagccggtgcttaaacgc
gttaatcgtagcaggaaatagctcaagcaataagcaagaatatcaactttgaaagacaatttggtgcgt
agctataacaaagcggttaagaagaatgcaccatatccgatttcgccataaaaacgggccaaaatc
ccacattggtcgccatctatgactagcttcttctgatgaaaggattaacggagttaacaaatgtggta
ggtaattggtccgacaaaagagcgagtgctgtagcacgaacgacatatcacatcagattacagctatt
ccagatcactactttgcattagttagtagatactatgcatatgatccaatttcaaagaaatgattgctctt
aaagatgaaacaaatccaatagaagaatggcaacatatcgacaactaaaggatcggcagaaggct
ctatacgttatcctgcatggaatgggtatcatttctcaagaagtttagactattgtcaagctatatcaatc
gtcgcatttaa (SEQ ID NO: 51)

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[0126] PCR products were purified with QIAquick purification columns (Qiagen, Valencia, CA), digested with appropriate restriction enzymes (NEB) and ligated to pKSVoriT, a derivative of the temperature sensitive allelic exchange vector pKSV7 (Smith, 1992), using T4 DNA ligase (NEB, Ipswich, MA). All vectors used for cloning were treated with calf intestinal alkaline phosphatase, (CIP, NEB, Ipswich MA)). Bacterial strains used for cloning, conjugation and the Lm parental strains are listed in Table 6 hereinafter. *Escherichia coli* SM10 was made chemically competent with the Z-Competent Kit (Zymo Research, Irvine CA). Transformations of both XL1-blue and SM10 were cultured in Luria-Bertani broth (LB) at 37°C with appropriate antibiotic selection (pKSVoriT: 75 µg/ml carbenicillin; pPI.2-based vectors: 20 µg/ml chloramphenicol. Lm strains were grown in vegetable peptone phosphate broth (VPP) (Basingstoke, UK). Lm was selected on VPP plates supplemented with 7.5 µg/ml chloramphenicol and 200 µg/ml streptomycin (Teknova, Hollister CA). Allelic exchange vectors were confirmed by diagnostic colony PCR, restriction digest, and sequencing (SeqXcel, San Diego CA). Confirmed plasmids were transformed into SM10 cells and conjugated into Lm as described. Recombinase genes were cloned downstream of the *actA* in the site-specific integration vector pPI.2 or derivatives thereof (Lauer, 2002).

[0127] Example 2. Engineering of Cre/lox-mediated Lm RIID strains

[0128] A non-limiting example that is illustrative of Lm RIID strains was based on targeting bacterial genes involved in the winding/unwinding of the bacterial genome during DNA replication, *gyrB* (*lmo0006*) and *gyrA* (*lmo0007*), encoding the two subunits of the Lm gyrase. The first step in the construction involved the placement of *lox71* (Albert et al., 1995) between coding sequences of *MATE efflux* (*lmo0003*) and *yaaA* (*lmo0004*), upstream of *recF* (*lmo0005*), and was accomplished by allelic exchange, as described previously (Camilli et al., 1993). To engineer the allelic exchange vector used to generate the Lm RIID strain, the upstream flanking region of the target genes was amplified by PCR using primers PL2863/PL2864, and the downstream flanking region with primers PL2865/PL2866. The PCR products were then cloned into a derivative of the shuttle vector plasmid pKSV7 (Smith et al., 1992) that was modified to contain an *oriT* (pKSVoriT), resulting in plasmid pBHE1937. The *lox71* sequence was inserted between the *lmo0003/lmo0004* flanking regions, by amplifying a 400 bp region containing *lox71* from an existing vector by PCR and subsequent cloning into pBHE1937, resulting in plasmid pBHE2099. The plasmid was sequence verified and transformed into *E. coli* SM10 for conjugation into Lm. The SM10 strain was mated with Lm strain BH1959, a live-attenuated derivative of DP-L4056 in which the *actA*, *inlB*, *uvrAB* coding sequences were deleted. In addition, this strain contains an antigen expression cassette at the *inlB* locus (as described in Lauer et al., 2008) for monitoring immunogenicity of the suicidal strain and its non-suicidal control. Allelic exchange was performed on transconjugants ultimately resulting in Lm strain BH3141.

[0129] The second mutant *loxP* site (*lox66*) was introduced downstream of *lox71* (Table 5) between *gyrA* (*lmo0007*) and *cardiolipin synthase* (*lmo0008*) using splicing by overlap extension (SOE) PCR as described previously (Horton, 1993). The region adjacent to *gyrA* was amplified with primers PL3141/PL3142 and the downstream region (adjacent to *lmo0008*) was amplified with PL3139/PL3140. The *lox66* sequence was included in primers PL3139 and PL3142. The primary SOE PCR products were then combined by a second PCR step, and this SOE product was then cloned into pKSV7oriT, resulting in the plasmid pBHE2193. Allelic exchange with pBHE2193 into the *lox71*-containing strain BH3141 was performed, resulting in Lm strain BH3210. This strain contained the genomic region composed of *yaaA-recF-gyrB-gyrA* flanked by *lox71* and *lox66* (Figure 2B).

[0130] The final step in generating the Lm-RIID strain involved the cloning and integration of a plasmid encoding Cre recombinase that was functionally linked to the *actA* promoter, restricting recombinase gene expression to the cytosol of infected cells in the vaccinated recipient. The nucleotide sequence of Cre was codon optimized for expression in Lm (Blue Heron, Bothell, WA). The Cre sequence was amplified with primers PL1536/PL1537 and cloned as an *EagI*/*BamHI* fragment into a pPL2-based plasmid (allowing site-specific integration at the *tRNA^{Arg}* locus) containing the *actA* promoter (221 bp, Table 5), resulting in plasmid pBHE2130. This plasmid was conjugated into both the parental strain (BH1959) and the *lox71/lox66*-containing variant (BH3210), resulting in BH3099 (non-suicidal control) and BH3226 (Lm RIID), respectively.

[0131] As a second non-limiting illustrative example of Lm-RIID vaccines, a second set of essential genes for excision was targeted by placing a *lox66* site on the other side of the bacterial origin in the strain BH3141, which contains a *lox71* between BH3141 *lmo0003* and *lmo0004*. The region deleted in this second strain includes *lmo2587*, the origin of replication (*oriC*), the essential replication initiator genes *dnaA* and *dnaN* (encoding a subunit of DNA polymerase III), and *lmo0003* (Figure 2C). The allelic exchange vector, pBHE2724, was engineered by first amplifying a region extending from the 3' end of *rnpA* through 150 bp downstream of *rpmH* by PCR, using oligo set PL4075/PL4017 (flanking region 1). The second flanking region, composed of the majority of *lmo2857*, was amplified using oligo set PL4018/PL4076 (flanking region 2). The *lox66* sequence was included in the forward primer PL4018. The two flanking regions were assembled into the vector pKSVoriT using restriction enzyme mediated three-way ligation. The same allelic exchange process was repeated to embed *lox66* in the intergenic region between *rpmH* and *lmo2857*. After confirming insertion of the *lox66*-containing sequence in strain BH3141, the new strain was designated BH3901. pBHE2130 (containing the *PactA*-Cre cassette) was introduced by conjugation resulting in the Lm RIID strain BH3908.

Using similar methods, a third non-limiting illustrative example of Lm-RIID vaccines was generated through targeting a deletion spanning the 14 kilobase region from *lmo2582* through *gyrA* (Figure 2D). The first flanking region spanning 1031 bp including *lmo2851* was amplified by PCR using the primers PL3246/PL3247. The second flanking region spanning 1029 bp including *lmo2852* and most of *lmo2853*, was amplified by PCR using oligos PL3248/PL3249. Both fragments were cloned into pKSVoriT in the vector

pBHE2292. *Lox71* from pBHE2099 (described above) was amplified by PCR using the primers PL3339/PL3340 and cloned into the KpnI/XbaI sites of pBHE2292, resulting in pBHE2358. To allow the simultaneous incorporation of both the *lox71* site and the *PactA*-Cre cassette in the same allelic exchange, the Cre cassette of pBHE2130 was amplified with PL3378/PL3379 and cloned into pBHE2358 between *lox71* and the *lmo2852* flanking region, resulting in allelic exchange vector pBHE2382. The *lox66* site was introduced into the live attenuated Lm11 strain (live-attenuated Lm $\Delta actA \Delta inlB$ vaccine strain) using pBHE2193 (described above), resulting in intermediate strain BH3291. A second allelic exchange was performed to insert *lox71* and *PactA*-Cre between *lmo2851* and *lmo2852* with that contained in pBHE2382 in BH3291 resulting in the Lm-RIID strain BH3410.

A fourth Lm-RIID strain was constructed in the Lm $\Delta actA \Delta inlB$ strain background, which allowed various vaccine antigen compositions to be subsequently introduced at the *tRNA^{Arg}* locus and tested and tested in immunogenicity studies. The same sequential allelic exchange process that gave rise to BH3210 was repeated in the Lm11 strain background. First, pBHE2193 was used to insert the *lox66* site downstream of *gyrA*, then pBHE2099 was used to insert the *lox71* site upstream of *yaaA*. The strain containing the *lox71-yaaA-recF-gyrB-gyrA-lox66* composition (Figure 2B) was designated BH3339. The *PactA*-Cre cassette was introduced by allelic exchange at the deleted *actA* locus of the Lm $\Delta actA \Delta inlB$ strain, allowing vaccine antigen expression cassettes to be subsequently introduced at the *tRNA^{Arg}* locus to generate vaccine strains for evaluation in animal studies. The *actA*-upstream flanking region including 825 bp of the *mpl* gene was amplified by PCR using primers PL3546/PL3547. The *actA*-downstream flanking region that includes 797 bp of the *plcB* gene was amplified by PCR with primers PL3548/PL3549. The PCR products were cloned into pKSVoriT by three-way ligation resulting in the vector pBHE2519. The pBHE2519 plasmid vector contained unique KpnI and BglII sites between the two flanking regions. The *PactA*-Cre cassette was subcloned from pBHE2130 into pBHE2519 resulting in pBHE2525. Allelic exchange was used to introduce the *PactA*-Cre cassette at the $\Delta actA$ locus, resulting in Lm-RIID strain BH3618.

[0132] Example 3. of FLP/*frt*-based RIID strains

[0133] It will be apparent to the skilled artisan that additional recombinase methods can be used to selectively delete Lm genes required for multiplication in the cytosol of the infected host cell. A non-limiting example is to utilize the yeast recombinase FLP with the

frt recombination sites instead of the *Cre/loxP* system to generate Lm-RIID vaccines. A strain that was analogous in composition to BH3226 (Figure 2B) was generated by replacing the *lox66* and *lox71* sites with *frt* recombination sites (Figure 2E) and utilizing FLP recombinase instead of Cre recombinase. An allelic exchange vector analogous to pBHE1937 (described in example 1) was constructed by inserting a *frt* site between *lmo0003* and *lmo0004* as follows: PCR was performed on 10403S DNA with primer pairs PL2863/PL3472 (*MATE efflux, lmo0003*) and PL3471/PL2866 (*yaaA, lmo0004*). Primers PL3471 and PL3472 added *frt* sequences to the native *Listeria* sequences. Secondary SOE PCR was used to combine PCR products into a single DNA fragment that contained the two flanking regions with an intervening *frt* site. This construct was cloned into pKSV7oriT as a PstI/EcoRI fragment, giving rise to pBHE2503. pBHE2503 was introduced into Lm11 by conjugation and allelic exchange was performed, resulting in BH3558. A second set of SOE PCR reactions were used to assemble the allelic exchange vector, pBHE2522 which introduced a *frt* recombination site between *gyrA* and *lmo0008*. Similar flanking regions found in pBHE2193 (described in example 1), were amplified with primer sets PI.3553/PI.3470 (*gyrA*) and PI.3469/PI.3552 (*lmo0008*). Primers PI.3469 and PI.3470 added a *frt* sequence to the native *Listeria* sequence. SOE PCR was used to assemble a DNA fragment with the two flanking regions with an intervening *frt* site, and the PCR product was cloned into pKVS7oriT as a HindIII/PstI fragment, resulting in pBHE2522. pBHE2522 was introduced into BH3558 by conjugation and, after allelic exchange, resulted in the strain where the gyrase region is flanked with *frt* sites, BH3578 (Figure 4E). Finally, a vector was constructed to express FLP recombinase from the *actA* promoter. The FLP recombinase coding sequence was codon optimized for expression in *Listeria* (DNA2.0, Menlo Park CA) and synthesized de novo and cloned, resulting in the vector pJ201:64349 as a ClaI/EagI fragment. The *actA* promoter was cloned into an erythromycin resistant derivative of pPL2 (Lauer, 2008), and the FLP coding sequence was subcloned from pJ201:64349 downstream of the *actA* promoter, resulting vector pBHE2516. pBHE2516 was sequence verified, introduced into strain BH3578 by conjugation, and integrated at the *tRNA^{Arg}* locus, resulting in the FLP/*frt*-based Lm RIID strain BH3602.

[0134] Example 4. Laboratory analysis methods

[0135] *In vitro* growth curves

[0136] Cultures grown overnight in BHI (BD Difco, Franklin Lakes, NJ) were diluted 1:100 into fresh BHI and grown at 37°C shaking at 250 rpm. Optical density (OD_{600 nm}) was measured in a BioRad SmartSpec 3000 (BioRad, Hercules, CA). Alternatively, overnight BHI cultures were diluted 1:100 and 150 µL/well was aliquoted into a 96-well, flat bottomed plate and monitored for growth using a Versa max microplate reader (Molecular Devices, Sunnyvale CA).

[0137] Intracellular growth curves

[0138] Growth curves in the dendritic cell line DC2.4 (Shen, 1997) were performed in 24-well tissue-culture plates (Costar 3524, Corning, NY). 2×10^5 DC2.4 cells were seeded per well in RPMI 1640 (Thermo Scientific, Waltham, MA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 23.8 mM sodium bicarbonate (Sigma), 1x non-essential amino acids (Cellgro), 2mM L-glutamine (Cellgro), 1×10^{-2} M HEPES buffer (Gibco), 1 mM sodium pyruvate (Sigma), and 50 µM β mercaptoethanol (Sigma, St. Louis, MO). For infection, Lm strains were grown overnight in BHI at 30°C without agitation, then diluted into RPMI to a final concentration 4×10^6 cfu/mL. 0.5 mL was used to infect each well for a final multiplicity of infection (moi) of 20. Cells were infected for 45 min, washed with 1 ml DPBS (Hyclone, South Logan UT), and RPMI complete medium containing 50 µg/mL gentamycin was added to prevent growth of extracellular bacteria. Intracellular bacterial growth was monitored at several time-points by aspiration of the media, washing cell monolayers with DPBS and lysing the cell monolayer hypotonically with 1 mL of sterile water. Serial 10 fold dilutions were plated on BHI agar plates supplemented with 200 µg/ml streptomycin (Teknova, Hollister CA). Plates were incubated overnight at 37°C and enumerated for calculation of cfu/well.

[0139] Western blots

[0140] Semi-quantitative intracellular Western blots were performed in DC2.4 cells. 3×10^5 DC2.4 cells were seeded in each well of a 12-well tissue culture plate and incubated overnight. Cells were infected with 6×10^6 of test Lm strains per well (MOI 20). Cells were incubated for 1 hour, rinsed with DPBS, and RPMI complete medium containing 50 µg/mL gentamycin was added. Cells were cultured seven additional hours at 37°C/5% CO₂ before washing each well with 1 mL DPBS and lysing cells in 150 µL 1x LDS (250 µL 4X LDS buffer (Life Technologies, Grand Island NY), 650 µL TE buffer (Fisher Scientific, Waltham

MA) 100 μ L sample reducing agent (Life Technologies, Grand Island NY)). Cell lysates were heated in a block at 95°C for 10 min and 20 μ L aliquots were run on 4-12% Bis-Tris PAGE gels in 1x MES buffer (Invitrogen) and transferred to nitrocellulose membranes for detection. Membranes were incubated in Odyssey blocking buffer (Li-Cor, Lincoln NE). Heterologous antigens were detected using the A18K polyclonal rabbit antibody which recognizes the mature 18 amino acid amino terminus of the ActA protein, used as a fusion for antigen expression at 1:4000 dilution. Expression levels were normalized to the *Listeria* P60 protein using a monoclonal antibody MAB8001 at 1:4000 dilution (Fisher Scientific). P60 expression level correlates with cfu. Secondary antibodies, α -MS 800 Cw and α -Rb 680 (Li-Cor) were used at 1:1000 dilutions. Westerns were scanned and quantitated on a Li-Cor Odyssey system.

[0141] Animals

[0142] C57BL/6, BALB/c, CD1, CD1^{nu/nu} and SCID beige mice were obtained from Charles River Laboratories (Wilmington, MA). Mice were treated according to National Institutes of Health guidelines. All animal protocols were approved by the Aduro BioTech IACUC. All vaccinations of *Listeria* were given intravenously at 5×10^6 cfu unless otherwise stated.

[0143] ELISpot assay

[0144] Immunogenicity was monitored during the experiments by ELISpot assays performed with lymphocytes isolated from whole mouse blood using Lympholyte-Mammal (Cedarlane Labs, Burlington, NC) and a murine IFN- γ ELISpot pair (BD Biosciences, San Jose, CA). At the termination of the experiments, ELISpot assays were performed on splenocytes. 2×10^3 cells/well were incubated with the appropriate peptide overnight at 37°C in anti-murine IFN- γ coated ELISpot plate (Millipore, Billerica, MA). Cells were incubated with no peptide as a negative control. Murine ELISpots were developed using alkaline phosphatase detection reagents (Invitrogen, Carlsbad, CA) and scanned and quantified using Immunospot plate reader and software (CTL Ltd, Cleveland, OH).

[0145] WT *Listeria* challenge

[0146] C57BL/6 mice were vaccinated once with 5×10^6 cfu of each Lm strain. 34 days later, mice were challenged with $2 \times \text{LD}_{50}$ of the WT Lm strain DP-L4056 (Lauer, 2002).

Three days later, spleens and livers were harvested and homogenized. Dilutions were plated on BHI plates containing 200 µg/mL streptomycin to determine cfu/organ.

[0147] Vaccinia challenge

[0148] C57BL/6 mice were vaccinated on day 0 and day 29 with 5×10^6 cfu of various Lm strains. Mice were challenged 49 days post second vaccination with WT vaccinia intraperitoneally with 1×10^6 pfu. Ovaries were harvested 5 days later and plaque assays were performed to determine the level of protection (Brockstedt, 2005).

[0149] Tumor studies

[0150] BALB/c mice were challenged intravenously on day 0 with 2×10^5 the CT26 tumor cell line engineered to express human mesothelin. Mice were vaccinated 4 days later with 5×10^6 cfu Lm RIID, along with appropriate controls, and boosted with the same vaccination dose 14 days later. Mice were weighed and monitored daily. At the time of death, lungs were harvested and metastases enumerated.

[0151] Example. 5. Results

[0152] To be effective, vaccines require a balance between immunogenicity, potency and safety. For practical reasons, it is further desirable that vaccines can be easily manufactured at large scale using established fermentation methods. Lm constitutes an immunologically potent vaccine platform due to its properties of cytosolic access, where it multiplies, and can be engineered further to express and secrete encoded antigens which are subsequently processed and presented on MHC class I molecules, and priming of antigen-specific CD8+ T cell responses (Brockstedt and Dubensky, 2008). Wild-type or live-attenuated Lm strains such as Lm $\Delta actA/\Delta inlB$ grow and multiply in broth culture and also grow and multiply in cells where they can also deliver antigenic target proteins to the host cytosol inducing an immune response. Lm-RIID vaccines have been developed to readily grow in broth culture and to deliver vaccine antigens to the host cell, but have been further engineered to initiate a program within the cytosol of the infected cell to “commit suicide,” preventing bacterial multiplication and increasing the safety of the vaccine platform (Fig 1).

[0153] To evaluate the growth characteristics of the Lm-RIID vaccine platform, the Lm-RIID strains BH3226 and BH3618 that delete the gyrase region, termed Deletion 1 (Figure

3A) to the parent strain BH3210 (which contains both *lox66* and *lox71* sites but lacks the Cre recombinase expression cassette; genotypes and strain characteristics for all strains are listed in Table 6), were compared for growth characteristics in vitro (fermentation in broth culture) and in host cells. Growth in vitro was measured following optical density over time in BHI broth culture over the course of 8 hours (as described in Example 1). In vitro growth curves were very similar for all strains (Figure 3A), demonstrating that both the parent strain and Lm-RIID strain can be propagated by conventional fermentation methods. Next, the intracellular growth kinetics for the BH3210 parental Lm strain and the Lm-RIID strains BH3226 and BH3618 were in the DC2.4 mouse dendritic cell line were compared. BH3210 grew like a typical wild-type Lm strain, multiplying approximately 2 logs over the course of 7 hours (Portnoy et al., 1988). In contrast, both BH3226 and BH3618 Lm-RIID strains lost the ability to generate colony forming units over the same time frame by 2 to 4 logs (Figure 3C). These results demonstrate that while Lm-RIID strains can be propagated by conventional methods in fermentation culture, following infection of host mammalian cells, the expression of Cre recombinase is induced, resulting in the subsequent excision of the region containing the essential gyrase genes *gyrB* and *gyrA*, and loss of viability.

[0154] As an additional non-limiting illustration of the approach, the kinetics of growth in broth culture and intracellular growth of Lm-RIID strains that targeted a second set of essential genes were determined. The Lm-RIID Deletion 2 strain that was engineered to delete the chromosomal origin of replication (*oriC*), as well as the essential replication initiation genes *dnaA* and *dnaN* (Figure 4A). Lm-RIID BH3908 strain contains *lox66* and *lox71* sites flanking *oriC*, *dnaA*, and *dnaN*, and grew in broth culture with the same kinetics as did the parental BH1959 strain (Figure 4B). In contrast, following infection of DC2.4 mouse dendritic cells, the Lm-RIID BH3908 strain rapidly lost viability (>2 logs in cfu) over the 7 hour time course, while the parental Lm BH1959 strain multiplied by >2 logs in cfu, a 4 to 5-log difference compared to the Lm-RIID strain. These results provide a second non-limiting example that while Lm-RIID strains can be propagated by conventional methods in fermentation culture, following infection of host mammalian cells, the expression of Cre recombinase is induced, resulting in the subsequent excision of the region containing the essential *oriC*, *dnaA*, and *dnaN* genes and loss of viability.

[0155] As yet a further non-limiting illustration of the approach, the kinetics of growth in broth culture and intracellular growth of Lm-RIID strains that targeted a third set of

essential genes were determined. Lm-RIID strains were constructed that contained a Deletion 3 composition, which spanned both Deletion 1 and Deletion 2 described herein, and also included 5 additional identified open reading frames in the *Listeria* genome (*lmo2852* through *rpmH*) (Fig 5A). The growth characteristics of Lm-RIID strain BH3410 in broth culture was compared to the parental strain BH3408, which contained a single *lox71* site between *lmo2851* and *lmo2852* and the *PactA*-Cre cassette inserted in the same intergenic region. The growth characteristics between the Lm-RIID strain and the parental strain were indistinguishable, demonstrating once again that Lm-RIID strains can be propagated by conventional broth culture fermentation (Fig 5B). Additionally, intracellular growth curves closely approximated the data from strains Deletion 1 and Deletion 2. In contrast, following infection of DC2.4 mouse dendritic cells, the Lm-RIID BH3410 strain rapidly lost viability (approximately 3 logs in cfu) over the 7 hour time course, while the Lm BH3408 parental strain multiplied by >2 logs in cfu, a 5-log difference compared to the Lm-RIID strain (Figure 5C).

[0156] The skilled artisan will recognize that alternative recombinase systems can be used to selectively delete Lm genes required for multiplication in the cytosol of the infected host cell. The growth characteristics of Lm-RIID strain BH3602 in broth culture and after infection of DC2.4 cells was compared to the live-attenuated parental strain Lm11 (Lm Δ actA/ Δ inlB). BH3602 targeted essential genes *yaaA*, *recF*, *gyrB*, *gyrA* for deletion, which was similar to the genes targeted in Deletion 1 of Lm-RIID strains BH3226 and BH3618, except Lm-RIID strain BH3602 utilizes the alternate recombinase system FLP/*frt* (Figure 6A). The in vitro growth kinetics of the Lm-RIID strain BH3602 and the Lm11 parental strain were indistinguishable. In contrast, following infection of DC2.4 mouse dendritic cells, the Lm-RIID strain BH3602 rapidly lost viability (approximately 2 logs in cfu) over the 7 hour time course, while the Lm 11 parental strain multiplied by >2 logs in cfu, a 4-log difference compared to the Lm-RIID strain (Figure 5C).

[0157] The following table provides a list of bacterial strains prepared as described herein:

Table 6. Bacterial strains.

Strain	Genotype	Parent Strain	Recombinase expr. cassette & locus	Antigen expr. cassette & locus
Lm11	$\Delta actA$, $\Delta inlB$		none	none
BH1959	$\Delta actA$, $\Delta inlB$, $\Delta uvrAB$		none	<i>inlB</i> ::ActAN100-QuadVac
BH1960	$\Delta actA$, $\Delta inlB$, $\Delta uvrAB$, <i>prfAG155S</i>		none	<i>inlB</i> ::ActAN100-QuadVac
BH3141	$\Delta actA$, $\Delta inlB$, $\Delta uvrAB$, <i>lmo0003-lox71-yaaA</i>	BH1959	none	<i>inlB</i> ::ActAN100-QuadVac
BH3210	$\Delta actA$, $\Delta inlB$, $\Delta uvrAB$, <i>lmo0003-lox71-yaaA</i> , <i>gyrA-lox66-lmo0008</i>	BH3141	none	<i>inlB</i> ::ActAN100-QuadVac
BH3226	$\Delta actA$, $\Delta inlB$, $\Delta uvrAB$, <i>lmo0003-lox71-yaaA</i> , <i>gyrA-lox66-lmo0008</i>	BH3210	<i>tRNA^{Arg}::PactA-Cre</i>	<i>inlB</i> ::ActAN100-QuadVac
BH3099	$\Delta actA$, $\Delta inlB$, $\Delta uvrAB$	BH1959	<i>tRNA^{Arg}::PactA-Cre</i>	<i>inlB</i> ::ActAN100-QuadVac
BH3291	$\Delta actA$, $\Delta inlB$, <i>gyrA-lox66-lmo0008</i>	Lm11	none	none
BH3339	$\Delta actA$, $\Delta inlB$, <i>lmo0003-lox71-yaaA</i> , <i>gyrA-lox66-lmo0008</i>	BH3291	none	none
BH3618	$\Delta actA$, $\Delta inlB$, <i>lmo0003-lox71-yaaA</i> , <i>gyrA-lox66-lmo0008</i>	BH3339	$\Delta actA$::PactA-Cre	none
BH3408	$\Delta actA$, $\Delta inlB$, <i>lmo2851-lox71-lmo2852</i>	Lm11	<i>lmo2851-PactA-Cre-lmo2852</i>	none
BH3410	$\Delta actA$, $\Delta inlB$, <i>lmo2851-lox71-lmo2852</i> , <i>gyrA-lox66-lmo0008</i>	BH3291	<i>lmo2851-PactA-Cre-lmo2852</i>	none
BH3558	$\Delta actA$, $\Delta inlB$, <i>lmo0003-frt-yaaA</i>	Lm11	none	none
BH3578	$\Delta actA$, $\Delta inlB$, <i>lmo0003-frt-yaaA</i> , <i>gyrA-frt-lmo0008</i>	BH3558	none	none
BH3602	$\Delta actA$, $\Delta inlB$, <i>lmo0003-frt-yaaA</i> , <i>gyrA-frt-lmo0008</i>	BH3578	<i>tRNA^{Arg}::PactA-ITLP</i>	none
BH3943	$\Delta actA$, $\Delta inlB$	Lm11	none	<i>tRNA^{Arg}::ActAN100-NY-ESO-1-MAGE A3</i>
BH3953	$\Delta actA$, $\Delta inlB$, <i>lmo0003-lox71-yaaA</i> , <i>gyrA-lox66-lmo0008</i>	BH3816	$\Delta actA$::PactA-Cre	<i>tRNA^{Arg}::ActAN100-NY-ESO-1-MAGE A3</i>

Strain	Genotype	Parent Strain	Recombinase expr. cassette & locus	Antigen expr. cassette & locus
BH3947	$\Delta actA$, $\Delta inlB$	Lm11	none	<i>iRNA^{Arg}::ActAN100-HBV Polymerase-HBxAg</i>
BH3957	$\Delta actA$, $\Delta inlB$, <i>lmo0003-lox71-yaaA</i> , <i>gyrA-lox66-lmo0008</i>	BH3816	$\Delta actA::PactA$ -Cre	<i>iRNA^{Arg}::ActAN100-HBV Polymerase-HBxAg</i>
BH3951	$\Delta actA$, $\Delta inlB$	Lm11	none	<i>iRNA^{Arg}::ActAN100-PfCSP</i>
BH3961	$\Delta actA$, $\Delta inlB$, <i>lmo0003-lox71-yaaA</i> , <i>gyrA-lox66-lmo0008</i>	BH3816	$\Delta actA::PactA$ -Cre	<i>iRNA^{Arg}::ActAN100-PfCSP</i>
BH892	$\Delta actA$, $\Delta inlB$	Lm11	none	<i>iRNA^{Arg}::ActAN100-OVA</i>
BH3709	$\Delta actA$, $\Delta inlB$, <i>lmo0003-lox71-yaaA</i> , <i>gyrA-lox66-lmo0008</i>	BH3816	$\Delta actA::PactA$ -Cre	<i>iRNA^{Arg}::ActAN100-AH1-OVA</i>

[0158] Example 6. Use as an antigen expression platform

[0159] A prerequisite for vaccine potency is delivery of the target antigen in an immunologically relevant context. For Lm-based vaccines, antigens must be expressed and delivered to the host antigen presenting cell (e.g., dendritic cells) cytosol, where encoded antigens are expressed, secreted from the bacterium into the cytosol, where subsequent antigen processing and presentation on MHC class I molecules results in CD8+ T cell priming. It will be appreciated by the skilled artisan that the magnitude of antigen expression can impact the magnitude of the vaccine-induced immune response. The intracellular level of encoded heterologous antigen expression by Lm-RIID vaccines following infection of mouse DC2.4 dendritic cells was measured. As a non-limiting example, the antigen expression level of four independent Lm-RIID strains encoding distinct heterologous was measured. All antigen expression cassette constructs encoded a fusion protein consisting of the amino terminal 100 amino acids of the *Listeria* ActA protein (ActAN100) cloned in-frame with the heterologous antigen, as described previously (Lauer et al., 2008). Detection of intracellular antigen expression was by Western blot analysis of infected cell lysates, using a polyclonal antibody raised against the mature amino terminus of ActA (described in Example 1). Expression of all antigens was functionally linked the *actA* promoter, which is minimally expressed in broth culture, and induced approximately 200-fold in the host cell cytosol (Shetron-Rama et al., 2002). The *actA* promoter was also used for Cre or FLP recombinase expression, to link the temporal

expression of the vaccine antigen with the intracellular death of the bacterium in the cytosol of the infected host cell of the vaccinated recipient. All antigen expression cassettes were cloned into a derivative of the site-specific integration vector pPL2 and integrated at the *tRNA^{Arg}* locus of both Lm11 (live-attenuated) and BH3618 (Lm-RIID) strains, as described previously (Lauer et. al., 2008).

[0160] Genes for all antigens were codon-optimized for expression in *Listeria* and synthesized de novo (DNA2.0, Menlo Park, CA). Fusions were then assembled by PCR and cloning for a fusion of the cancer antigens NY-ESO-1 and MAGE A3 (Figure 7B), and the infectious disease antigens HBV polymerase and HBxAg (Figure 7C) or the *Plasmodium falciparum* circumsporozoite protein (PfCSP) (Figure 7D). Expression was normalized to the *Listeria* P60 protein as described in Example 1. Relative expression from Lm-RIID was calculated as the ratio of expression measured from the Lm-RIID strain divided by the expression from the paired live attenuated control strain. The measured ratio of expression were 1x for NY-ESO-1-MAGE A3, 1.1x for the HBV polymerase-HBxAg fusion, and 1.8x for the Pf-CSP antigen construct. These results demonstrate that concurrently with intracellular bacterial death, Lm-RIID strains expressed robust levels of encoded vaccine antigens and delivered them to the host-cell for processing and the induction of an immune response, significantly at levels that were comparable with that observed with parental recombinant live-attenuated Lm vaccine strains.

[0161] Example 7. Clearance of Lm-RIID vaccine strains in vivo

[0162] The principal organs that are infected in the mouse following intravenous (IV) administration of *L. monocytogenes* (Lm) are the liver and spleen (Portnoy et al., 2002). The acute toxicity determined by median lethality of the live-attenuated Lm Δ actA/ Δ inlB vaccine platform is attenuated by more than 3 logs in the mouse listeriosis model as compared to wild-type Lm (WT Lm), which is reflected by rapid clearance in the liver and spleen in infected mice, measured by quantification of colony forming units (cfu) in cell lysates prepared from harvested organs (Brockstedt et al., 2004).

[0163] As shown in the examples contained herein, Lm-RIID can be derived from the live-attenuated Lm Δ actA/ Δ inlB (i.e., deletion or mutation of actA and inlB virulence genes) vaccine strain. However, in contrast to Lm Δ actA/ Δ inlB, Lm-RIID vaccines are pre-programmed (i.e., engineered) to spontaneously initiate its own suicide within the context of

infected cells of the vaccinated mammal. To illustrate the increased safety profile of vaccine strains based on Lm-RIID vaccines compared to Lm Δ actA/ Δ inlB vaccines, the kinetics of clearance in both immune competent C57BL/6 mice and in immune deficient SCID-Beige mice lacking functional innate and adaptive immunity were compared. Groups of 5 female C57BL/6 mice were given 5×10^6 cfu of Lm-RIID (BH3226) or Lm Δ actA/ Δ inlB (BH3099) vaccine strains by IV injection, and the bacterial burden in the livers and spleens were determined by plating on nutrient agar serial dilutions of clarified lysates processed from harvested organs at 0.2, 4 and 24 hours post injection. The amount of Lm Δ actA/ Δ inlB (BH3099) vaccine strain measured in the liver or spleen was at similar levels at the 4 and 24 hour time points; i.e., the Lm Δ actA/ Δ inlB (BH3099) vaccine strain bacterial burden did not diminish during this period (Fig 8A). In striking contrast, the quantity of Lm-RIID (BH3226) rapidly decreased at 4 hours post injection as compared to the 0.2 hours post injection time point, and no Lm-RIID (BH3226) colonies could be detected by culture at the final time point evaluated at 24 hours post infection (Fig 8A).

[0164] These results provide unequivocal evidence that Lm-RIID (BH3226) is cleared significantly more rapidly from the livers and spleens of mice following IV administration as compared to live-attenuated Lm Δ actA/ Δ inlB (BH3099). To assess the dependence of the host immune response on the clearance of live-attenuated Lm Δ actA/ Δ inlB and Lm-RIID vaccine strains, in a separate experiment, groups of 4 female SCID/Beige immune deficient mice were given 5×10^6 cfu of Lm-RIID (BH3226) or Lm Δ actA/ Δ inlB (BH3099) vaccine strains by IV injection, and the bacterial burden in the spleens were determined by plating on nutrient agar serial dilutions of clarified lysates processed from organs harvested at 20 minutes, 1, 2 and 4 days post injection. The bacterial burden measured in the spleens of SCID/Beige immune mice given live-attenuated Lm Δ actA/ Δ inlB (BH3099) increased at 1 day compared to the level measured at 20 minutes after injection, indicating multiplication of the input bacteria. The peak level of bacteria observed at 1 day post injection with Lm Δ actA/ Δ inlB (BH3099) was at least 5×10^6 cfu per spleen, and then decreased over the next 2 time points evaluated to a level of about 5×10^4 cfu per spleen measured at the last 4 day post injection time point evaluated (Fig 8B). In striking contrast, no multiplication of bacteria, as determined by an increase in cfu between time points, could be measured in the spleens of SCID/Beige immune mice given Lm-RIID (BH3226) vaccine strain; the bacterial burden rapidly declined following the first time point evaluated at 20 minutes post injection, and by the 4 day post injection time point, no Lm-RIID (BH3226) colonies could be detected

in the spleens (Fig 8B). Taken together, the results shown in Figs 8A and 8B demonstrate that Lm-RIID vaccine strains spontaneously initiate its own clearance from the vaccinated host, and, essentially, a functioning immune response is not required for clearance. The skilled artisan will recognize that this characteristic of spontaneous clearance is a desirable feature for broad application of Lm-RIID based strains for both prophylactic and therapeutic vaccines.

[0165] Example 8. Immunogenicity of Lm-RIID strains

[0166] Recombinant *L. monocytogenes* has been shown to induce potent CD4+ and CD8+ T cell immunity to encoded heterologous antigens in mice and in humans (Brockstedt et al., 2004; Le et al., 2012). The immunogenicity of Lm-RIID in mice was compared to live-attenuated *Lm* $\Delta actA/\Delta inlB$ and Killed But Metabolically Active (KBMA; *Lm* $\Delta actA/\Delta inlB/\Delta uvrAB$) was compared. To enable the immunogenicity of the Lm-RIID, *Lm* $\Delta actA/\Delta inlB$, and KBMA vaccines to be compared, each vaccine strain contained the same Ag expression cassette encoding five defined H-2^b-restricted major histocompatibility complex (MHC) class I epitopes that have previously been shown to elicit a range of CD8+ T-cell responses in mice, when encoded by live-attenuated *Lm* $\Delta actA/\Delta inlB$, and KBMA vaccines (Lauer et al., 2008). The use of an array of precise class I restricted epitopes provides an optimized method for assessing immunogenicity *in vivo*, simplifying comparisons of the Lm-RIID, *Lm* $\Delta actA/\Delta inlB$, and KBMA vaccine platforms.

[0167] The Ag expression cassette construct encodes four tandemly spaced vaccinia virus (A42R, C4L, K3L, and B8R) class I epitopes and the chicken OVA (SL8) epitope, and was synthesized and then cloned under the control of the PrfA-regulated *actA* promoter as a fusion protein with the 100 N-terminal amino acids of ActA (Lauer et al., 2008). The construct is known as Quadvac and was cloned into a derivative of the pPL2 integration vector and then integrated into the tRNA^{Arg} site of Lm-RIID, *Lm* $\Delta actA/\Delta inlB$, and KBMA vaccine strains as described previously (Lauer et al., 2002; Lauer et al., 2008).

[0168] To evaluate relative immunogenicity, groups of 5 female C57BL/6 (H-2^b) mice were immunized twice at an interval of 36 days with the Lm-RIID, *Lm* $\Delta actA/\Delta inlB$, and KBMA vaccine strains each encoding Quadvac, at a dose level of 5×10^6 colony forming units (CFU). The KBMA vaccine dose level was measured prior to photochemical inactivation, as described previously (Brockstedt et al., 2005). Five days following the

second immunization, spleens were harvested from vaccinated mice, and the magnitude of the CD8+ T cell responses specific for the 5 encoded epitopes was measured by ELISpot analysis of splenocytes following overnight stimulation with 1 μ M of peptides corresponding to each of the MHC class I epitopes having the amino acid sequence as follows, as described previously: A42R₈₈₋₉₆, YAPVSPIVI; C4L₁₂₅₋₁₃₂, LNFRFENV; K3L₆₋₁₅, YSLPNAGDVI; B8R₂₀₋₂₇, TSYKFESV; and, SL8₂₅₇₋₂₆₄, SIINFEKL (Lauer et al., 2008; Moutaftsi et al., 2006). Lm-RIID vaccines induced CD8+ T cell responses specific for the 5 MHC class I epitopes that were comparable in magnitude to mice immunized with live-attenuated *Lm* Δ actA/ Δ inlB vaccine strains, and that were significantly higher than the magnitude of the CD8+ T cell responses measured in mice immunized with KBMA vaccines (Fig 9).

[0169] These results demonstrate that although Lm-RIID vaccines are engineered initiate a program to commit suicide within infected cells of the vaccinated recipient and do not require a functional immune response for clearance, surprisingly, Lm-RIID vaccines retain the capacity to induce a robust specific CD8 T cell response that is comparable to live-attenuated *Lm* vaccines. The skilled artisan will understand that these results demonstrate that Lm-RIID vaccines have general utility for preventative or therapeutic vaccination in humans to induce a T cell response specific for desired Ags, due to the characteristics of having similar immunologic potency to live-attenuated *Lm* vaccines, but having a significantly improved safety profile due to its feature of immune-response independent spontaneous clearance.

[0170] Example 9. Protective immunity afforded by vaccination with Lm-RIID based vaccine strains

[0171] The Examples provided herein demonstrate that Lm-RIID vaccines self-initiate a pre-programmed suicide within the host cell, do not require a functioning immune response for clearance from the vaccinated host, yet retain the capacity to induce cellular immunity specific for an encoded antigen that is comparable to vaccination with live-attenuated *Lm* Δ actA/ Δ inlB vaccine strains. However, the skilled artisan will recognize that one important measure of effective immunization is whether a vaccine candidate can confer protection against subsequent challenge with a virulent pathogen.

[0172] It is well-recognized in the field that a single immunization of mice with sub-lethal doses of wild-type *Lm* (WT *Lm*) or appropriate live-attenuated strains affords life-

long protection against lethal challenge with WT Lm, measured by bacterial burden in the liver or spleen at three days post challenge (Bahjat et al., 2006). The correlates of protection are CD4+ and CD8+ T cell immunity, and humoral immunity plays no role in protection (Berche et al., 1987). To evaluate the relative capacity of Lm-RIID vaccination to provide protective immunity, groups of 5 female C57BL/6 mice were vaccinated with Hanks-balanced salt solution (HBSS) as a negative control, or with 5×10^6 cfu of Lm-RIID or live-attenuated Lm Δ actA/ Δ inlB vaccines, or with 1×10^8 cfu (determined prior to photochemical inactivation) of KBMA vaccines. Vaccinated and control mice were challenged at 41 days post vaccination in order to assess the capacity of the vaccine-induced memory T cell response to provide protection against lethal bacterial challenge with 20 LD₅₀ doses (2×10^5 cfu) of WT Lm.

[0173] Vaccination with Lm-RIID provided 4 logs of protection against WT Lm challenge as compared to the HBSS negative control group (Fig 10A). While the level of protection afforded by Lm-RIID was approximately 20-fold less than vaccination with Lm Δ actA/ Δ inlB, it was more than 3 logs better compared to vaccination with KBMA vaccines (Fig 10A). As a second measure to assess the functional capacity of Lm-RIID vaccine-induced T cell responses to afford protection against pathogen challenge, we utilized the vaccinia virus challenge model. With this model, protection is measured by quantitating challenge virus in the ovaries of mice by plaque assay 5 days following challenge with vaccinia virus encoding an antigen that is cognate to the test vaccine used for immunization (Belyakov et al., 1998; Brockstedt et al., 2005). To assess protective immunity, mice were vaccinated twice with an interval of 29 days with 5×10^6 cfu of Lm-RIID or live-attenuated Lm Δ actA/ Δ inlB vaccines, or with 1×10^8 cfu (determined prior to photochemical inactivation) of KBMA vaccines. All test vaccines encoded the Quadvac vaccinia virus CD8+ T cell epitopes and SL8 CD8+ T cell epitope described in Example 9. 49 days following the boost vaccination, mice were challenged by intraperitoneal challenge with 1×10^6 plaque forming units (pfu) with vaccinia virus encoding chicken Ovalbumin, and pfu in the ovaries was measured 4 days later by plaque assay on BSC cells of serial dilutions of clarified processed organ homogenates. S

[0174] Strikingly, the level of protection afforded by immunization with Lm-RIID or live-attenuated Lm Δ actA/ Δ inlB vaccines was indistinguishable, and was greater than 1000-fold compared to the HBSS negative control group, and was more than 100-fold better than the

level of protection afforded by vaccination with KBMA vaccines (Fig 10B). The skilled artisan will readily recognize that the Lm-RIID vaccines have a desirable safety profile compared to live-attenuated Lm Δ actA/ Δ inlB vaccines, yet provide comparable immunologic potency, as measured by the extent of protection against pathogen challenge.

[0175] Example 10. Lm-RIID vaccine induced therapeutic anti-tumor efficacy

[0176] Having demonstrated that Lm-RIID vaccines stimulate CD4+ and CD8+ T cell immunity specific for vector encoded antigens which function to provide protection against challenge with a pathogen encoding the cognate antigen, that is comparable to live-attenuated Lm Δ actA/ Δ inlB vaccine strains, the skilled artisan will recognize that Lm-RIID vaccines will also have application for cancer immunotherapy. It is well-appreciated that barriers to effective immune therapies include tolerance to the targeted tumor-associated antigen(s) that can limit induction of cytotoxic CD8+ T cells of appropriate magnitude and function, poor trafficking of the generated T cells to sites of malignant cells, and poor persistence of the induced T cell response (Blankenstein et al., 2012; Topalian et al., 2011). One measure of potency is to evaluate the capacity of a selected platform to reverse tumor progression and increase survival time compared to controls, when administered to tumor-bearing animals.

[0177] The CT-26 murine colon tumor cell line was used to test the benefit of therapeutic vaccination with Lm-RIID vaccines (Slansky et al., 2000). The CT-26 tumor cells were given by intravenous (IV) inoculation, which results in the establishment of lung tumor nodules that can be quantitated in the lungs harvested from treated mice at desired time points post implantation, or, alternatively, mice can be monitored for survival, and sacrificed when moribund, due to excessive tumor growth preventing adequate respiration. Therapeutic efficacy in the CT26 tumor model requires breaking of self-tolerance to the H-2L^d-restricted immunodominant epitope AH1 from the gp70 endogenous tumor antigen (Slansky et al., 2000). Lm-RIID vaccines were constructed that expressed the altered peptide ligand AH1-A5 epitope (SPSYAYHQF), containing a heteroclitic valine to alanine change in the fifth position of the MHC class I epitope, embedded in-frame within a unique *Ava II* restriction endonuclease site in chicken ovalbumin (OVA), as described previously (Brockstedt et al., 2004). To evaluate the therapeutic efficacy of Lm-RIID, 30 Female BALB/c mice were each given 2×10^5 CT26 cells suspended in 100 μ l of HBSS by tail vein injection, and then randomized to 3 groups of 10 mice. 4 days later, the 3 groups of mice were given 100 μ l of HBSS, or 5×10^6 cfu of live-attenuated Lm Δ actA/ Δ inlB expressing OVA (BJI892), or 5×10^6 cfu of Lm RIID expressing OVA-

AH1-A5 (BH3709). Fourteen days later, the same injections were given to all 3 groups of mice, and all mice were then monitored daily for survival.

[0178] Tumor-bearing animals in negative controls groups treated with HBSS or live-attenuated *Lm* Δ actA/ Δ inlB expressing OVA had a median survival time of about 24 days post CT-26 tumor cell implantation (Fig. 11). In striking contrast, the group of mice given *Lm* RIID expressing OVA-AH1-A5 had a median survival time of 55 days, that was significantly longer than either control group ($p < 0.001$, log-rank Mantel-Cox test) (Fig 11). Furthermore, 30% of the animals treated with *Lm*-RIID vaccines expressing OVA-AH1-A5 were long-term survivors, when the experiment was terminated 160 days following tumor cell implantation (Fig 11). These results provide unequivocal evidence that *Lm*-RIID vaccines although pre-programmed to commit suicide within host cells of the vaccinated recipient, retain the capacity to initiate a functional tumor-specific CD8+ T cell response in tumor-bearing animals, and provide significant benefit and overall survival, as compared to negative controls. The skilled artisan will recognize that *Lm*-RIID vaccines are a platform with direct application to clinical cancer immunotherapy, due to the combined properties of safety and therapeutic efficacy.

[0179] Example 11. References

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[0202] One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The examples provided herein are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention.

[0203] It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[0204] All patents and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[0205] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms “comprising”, “consisting essentially of” and “consisting of” may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

[0206] Other embodiments are set forth within the following claims.

We claim:

1. A method for configuring a bacterium to delete one or more genes in the bacterial genome which are essential for multiplication of the bacterium, the deletion occurring preferentially when the bacterium is introduced into a mammalian host, comprising:
 - (i) introducing into the bacterium a nucleic acid encoding a recombinase heterologous to the bacterium operably connected to regulatory sequences which preferentially induce expression of the recombinase in the mammalian host, and
 - (ii) introducing in the bacterial genome a first attachment site for the recombinase upstream from the gene(s) essential for multiplication of the bacterium, and a second attachment site for the recombinase downstream from the gene(s) essential for multiplication of the bacterium, such that the recombinase, when expressed in the mammalian host, catalyzes a site specific recombination event which deletes the gene(s) essential for multiplication of the bacterium flanked by the first and second attachment sites, wherein the bacterium is deficient for multiplication following the site specific recombination event.
2. A method according to claim 1, wherein the bacterium is an intracellular pathogen, and the regulatory sequences preferentially induce expression of the recombinase when the bacterium is in a mammalian host cell.
3. A method according to claim 1, wherein the bacterium is of a genus selected from the group consisting of *Listeria*, *Neisseria*, *Mycobacterium*, *Francisella*, *Bacillus*, *Salmonella*, *Shigella*, *Yersinia*, *Brucella*, *Legionella*, *Rickettsia*, and *Chlamydia*.
4. A method according to claim 1, wherein the bacterium is *Listeria monocytogenes*.
5. A method according to claim 4, wherein the bacterium is *Listeria monocytogenes*., wherein the *Listeria monocytogenes* is $\Delta ActA/\Delta InlB$.
6. A method according to claim 4, wherein the regulatory sequences comprise a *Listeria monocytogenes* promoter which is PrfA-dependent.

7. A method according to claim 6, wherein PrfA-dependent promoter is selected from the group consisting of the *inlA* promoter, the *inlB* promoter, the *inlC* promoter, the *hpt* promoter, the *hly* promoter, the *plcA* promoter, the *mpl* promoter, and the *actA* promoter.
8. A method according to claim 6, wherein the PrfA-dependent promoter is an *actA* promoter.
9. A method according to claim 1, wherein the recombinase is selected from the group consisting of ϕ C31 integrase, R4 integrase, TP901 integrase, ϕ BT1 integrase, BxB1 integrase, PSA integrase, Cre recombinase, Flp recombinase, XerC recombinase, λ integrase, HK022 integrase, P22 integrase, HP1 integrase, L5 integrase, $\gamma\delta$ recombinase, Tn3 recombinase, gin recombinase, RV integrase, SPBc integrase, TG1 integrase, ϕ C1 integrase, MR11 integrase, ϕ 370 integrase, ϕ K38 integrase, W β integrase, and BL3 integrase.
10. A method according to claim 1, wherein the first attachment site is an *attB* site and the second attachment site is an *attP* site.
11. A method according to claim 1, wherein the one or more genes essential for multiplication of the bacterium comprise at least one gene involved in DNA replication.
12. A method according to claim 11, wherein the at least one gene involved in DNA replication is selected from the group consisting of *ori*, *dnaA*, *dnaN*, *gyrA*, *gyrB*, *polC*, *dnaE*, *ftsK*, *ftsZ*, *ligA*, *dnaG*, *parC*, *parE*, *holB*, *dnaX*, *SMC*, and *ftsY*.
13. A method according to claim 1, wherein the one or more genes essential for multiplication of the bacterium are part of an operon, and the first attachment site is upstream of all or a portion of the operon, and the second attachment site is downstream of all or a portion of the operon.
14. A method according to claim 13, wherein the first attachment site is upstream of the operon, and the second attachment site is downstream of the operon.
15. A method according to claim 1, wherein the recombinase is a Cre recombinase, the regulatory sequences comprise an *actA* promoter, the first and second attachment sites comprise loxP sites, and the gene(s) essential for multiplication of the bacterium flanked by the first and second attachment sites are involved in DNA replication.

16. A method according to claim 1, wherein the first and second attachment sites flank a nucleic acid sequence about 20kb in length or less.
17. A method according to claim 16, wherein the nucleic acid sequence is about 10kb in length or less.
18. A method according to claim 16, wherein the nucleic acid sequence is about 6kb in length.
19. A method according to claim 1, wherein the bacterium comprises within the bacterial genome an exogenous nucleic acid sequence encoding a heterologous polypeptide, wherein the exogenous nucleic acid sequence is operably connected to regulatory sequences which preferentially induce expression of the heterologous polypeptide when the bacterium is in a mammalian host.
20. A method of deleting one or more genes in a bacterial genome which are essential for multiplication of a bacterium, comprising:
- introducing the bacterium according to one of claims 1-19 to a mammalian host under conditions wherein the recombinase is expressed by the bacterium, and wherein the expressed recombinase deletes the one or more genes essential for multiplication of the bacterium by the site specific recombination event.
21. A method according to claim 20, wherein the bacterium comprises within the bacterial genome an exogenous nucleic acid sequence encoding a heterologous polypeptide, wherein the exogenous nucleic acid sequence is operably connected to regulatory sequences which preferentially induce expression of the heterologous polypeptide when the bacterium is in the mammalian host, and wherein the bacterium expresses the heterologous polypeptide within the mammalian host.

22. A bacterium comprising:
- (i) a nucleic acid encoding a recombinase heterologous to the bacterium operably connected to regulatory sequences which preferentially induce expression of the recombinase when the bacterium is in a mammalian host, and
 - (ii) a first attachment site for the recombinase introduced upstream from one or more genes in the bacterial genome which are essential for multiplication of the bacterium, and a second attachment site for the recombinase introduced downstream from the gene(s) genes in the bacterial genome which are essential for multiplication of the bacterium, wherein the first and second attachment sites are configured to provide recombinase-catalyzed removal of the one or more genes in the bacterial genome which are essential for multiplication of the bacterium upon expression of the recombinase.
23. A bacterium according to claim 22, wherein the bacterium is an intracellular pathogen, and the regulatory sequences preferentially induce expression of the recombinase when the bacterium is in a mammalian host cell.
24. A bacterium according to claim 22, wherein the bacterium is of a genus selected from the group consisting of *Listeria*, *Neisseria*, *Mycobacterium*, *Francisella*, *Bacillus*, *Salmonella*, *Shigella*, *Yersinia*, *Brucella*, *Legionella*, *Rickettsia*, and *Chlamydia*.
25. A bacterium according to claim 22, wherein the bacterium is *Listeria monocytogenes*.
26. A bacterium according to claim 25, wherein the bacterium is *Listeria monocytogenes*., wherein the *Listeria monocytogenes* is $\Delta ActA/\Delta InlB$.
27. A bacterium according to claim 22, wherein the regulatory sequences comprise a *Listeria monocytogenes* promoter which is PrfA-dependent.
28. A bacterium according to claim 27, wherein PrfA-dependent promoter is selected from the group consisting of the *inlA* promoter, the *inlB* promoter, the *inlC* promoter, the *hpt* promoter, the *hly* promoter, the *plcA* promoter, the *mpl* promoter, and the *actA* promoter.
29. A bacterium according to claim 27, wherein the PrfA-dependent promoter is an *actA* promoter.

30. A bacterium according to claim 22, wherein the recombinase is selected from the group consisting of ϕ C31 integrase, R4 integrase, TP901 integrase, ϕ BT1 integrase, BxB1 integrase, PSA integrase, Cre recombinase, Flp recombinase, XerC recombinase, λ integrase, HK022 integrase, P22 integrase, HP1 integrase, L5 integrase, $\gamma\delta$ recombinase, Tn3 recombinase, gin recombinase, RV integrase, SPBc integrase, TG1 integrase, ϕ C1 integrase, MR11 integrase, ϕ 370 integrase, ϕ K38 integrase, W β integrase, and BL3 integrase.
31. A bacterium according to claim 22, wherein the first attachment site is an *attB* site and the second attachment site is an *attP* site.
32. A bacterium according to claim 22, wherein the one or more genes essential for multiplication of the bacterium comprise at least one gene involved in DNA replication.
33. A bacterium according to claim 30, wherein the at least one gene involved in DNA replication is selected from the group consisting of *ori*, *dnaA*, *dnaN*, *gyrA*, *gyrB*, *polC*, *dnaE*, *ftsK*, *ftsZ*, *ligA*, *dnaG*, *parC*, *parE*, *holB*, *dnaX*, *SMC*, and *ftsY*.
34. A bacterium according to claim 22, wherein the one or more genes essential for multiplication of the bacterium are part of an operon, and the first attachment site is upstream of all or a portion of the operon, and the second attachment site is downstream of all or a portion of the operon.
35. A bacterium according to claim 34, wherein the first attachment site is upstream of the operon, and the second attachment site is downstream of the operon.
36. A bacterium according to claim 22, wherein the recombinase is a Cre recombinase, the regulatory sequences comprise an *actA* promoter, the first and second attachment sites comprise loxP sites, and the gene(s) essential for multiplication of the bacterium flanked by the first and second attachment sites are involved in DNA replication.
37. A bacterium according to claim 22, wherein the first and second attachment sites flank a nucleic acid sequence about 20kb in length or less.
38. A bacterium according to claim 37, wherein the nucleic acid sequence is 10kb in length or less.
39. A bacterium according to claim 38, wherein the nucleic acid sequence is about 6kb in length.

40. A bacterium according to claim 22, wherein the bacterium comprises within the bacterial genome an exogenous nucleic acid sequence encoding a heterologous polypeptide, wherein the exogenous nucleic acid sequence is operably connected to regulatory sequences which preferentially induce expression of the heterologous polypeptide when the bacterium is in a mammalian host.
41. A method according to claim 1, wherein the bacterium is a facultative intracellular bacterium.
42. A bacterium according to claim 22, wherein the bacterium is a facultative intracellular bacterium.