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(54) **Title:** ATTENUATED FRANCISELLA MUTANTS AND METHODS OF USE

(57) **Abstract:** Francisella tularensis is the bacterial pathogen that causes tularemia in humans and a number of animals. To date, no approved vaccine exists for this widespread and life-threatening disease. The present disclosure provides attenuated Francisella mutants that include genetic inactivations in two genes. The Francisella mutants contain a genetic inactivation in at least one gene selected from dsbB, FTT0742, pdpB, fumA, and carB, and at least one gene selected from tolB, htrB, ipxH, ostA1, fimT, ipcC, manB, manC, nusA, wzx, kdtA, FTN0408, FTN0757 and FTN1254. Also provided are immunogenic compositions that include the attenuated bacteria. Methods are provided for treatment (e.g., treatment of tularemia) using the attenuated Francisella mutants.



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ATTENUATED FRANCISELLA MUTANTS AND METHODS OF USE

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of priority of U.S. Provisional
5 Application No. 61/292,550, filed January 6, 2010, which is herein incorporated by
reference in its entirety.

FIELD

This disclosure concerns attenuated *Francisella* bacteria and methods of
10 their use, for example to stimulate an immune response in a mammal.

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This invention was made with government support under R41 AI072906-02
and U54 AI057141, awarded by the National Institute of Allergy and Infectious and
15 Diseases, National Institutes of Health. The government has certain rights in the
invention.

BACKGROUND

Francisella tularensis is a gram-negative facultative intracellular pathogen
20 and the causative agent of tularemia. Four subspecies of *F. tularensis* are commonly
recognized (Pechous *et al.*, *PLoS ONE* 3:e2487, 2008): *F. tularensis* subsp.
tularensis (type A), *F. tularensis* subsp. *holarctica* (type B), *F. tularensis* subsp.
mediasiatica, and *F. tularensis* subsp. *novicida* (denoted as FTN). All of these
subspecies exhibit more than 95% DNA sequence identity (Forsman *et al.*, *Int J Syst*
25 *Bacteriol* 44:38-46, 1994). The ability of *Francisella* to grow within cells,
particularly macrophages, is an essential virulence factor (Oyston *et al.*, *Nat Rev*
Microbiol 2:967-78, 2004). *F. tularensis* strains can replicate intracellularly and
eventually kill both primary and cultured macrophage cell lines (Anthony *et al.*,
Infect Immun 59:3291-3296, 1991; Brotcke *et al.*, *Infect Immun* 74:6642-6655,
30 2006; Lai *et al.*, *Infect Immun* 69:4691-4694, 2001; Maier *et al.*, *Infect Immun*
75:5376-5389, 2007; Tempel *et al.*, *Infect Immun* 74:5095-5105, 2006; Weiss *et al.*,
Proc Natl Acad Sci USA 104:6037-6042, 2007).

Francisella research has historically been hampered by the paucity of available genetic tools to make mutants. Since the introduction of transposons in *Francisella*, ten *Francisella* transposon (Tn) libraries (banks) have been described and partially characterized (Brotcke *et al.*, *Infect Immun* 74:6642-6655, 2006; 5 Buchan *et al.*, *Appl Environ Microbiol* 74:2637-2645, 2008; Gallagher *et al.*, *Proc Natl Acad Sci USA* 104:1009-1014, 2007; Gray *et al.*, *FEMS Microbiol Lett* 215:53-56, 2002; Kawula *et al.*, *Appl Environ Microbiol* 70:6901-6904, 2004; Maier *et al.*, *Infect Immun* 75:5376-5389, 2007; Meibom *et al.*, *Mol Microbiol* 67:1384-13401, 2008; Qin and Mann, *BMC Microbiol* 6:69, 2006; Su *et al.*, *Infect Immun* 75:3089-10 3101, 2007; Tempel *et al.*, *Infect Immun* 74:5095-5105, 2006; Weiss *et al.*, *Proc Natl Acad Sci USA* 104:6037-6042, 2007). Gallagher *et al.* (*Proc Natl Acad Sci USA* 104:1009-14, 2007) were the first to have created a comprehensive library by selecting and directly sequencing approximately 17,000 independent insertions using a high-throughput approach. This library is provided to the community as an 15 ordered array containing two-allelic insertion mutations in each non-essential gene in *F. novicida*.

While *F. novicida* is not generally considered a human pathogen, it displays a similar, if not greater, degree of virulence in mice as other *F. tularensis* subspecies. Moreover, *F. novicida* is much easier to manipulate genetically than *F.* 20 *tularensis*. In addition to their considerable genomic similarity, the close relationship between *F. novicida* and *F. tularensis* is further highlighted by their nearly identical 16S rDNA sequences. This degree of genetic identity indicates that the two organisms utilize similar virulence genes, and that *F. novicida* is an apt platform for the development of attenuated *Francisella* bacteria that can be used in 25 immunogenic compositions, such as a tularemia vaccine.

Francisella tularensis has been designated as a Class A bioterrorism agent by the Centers for Disease Control and Prevention and is acknowledged as a potential threat to national security. Thus, a need exists for the development of effective immunogenic compositions that can be used to treat (such as prevent) 30 tularemia. Described herein is a comprehensive analysis of a *F. novicida* transposon library to identify essential virulence factors on a genome-wide scale by screening for morphological changes in infected macrophages. Thirteen mutants were

identified that exhibit a hypercytopathogenic phenotype in macrophages, but are attenuated *in vivo*.

SUMMARY

5 Using an *F. novicida* transposon library, a comprehensive screen was performed to identify essential *Francisella* virulence factors by evaluating morphological changes in infected macrophages. The screen identified 13 genes in which genetic inactivation conferred hypercytotoxicity in macrophages and attenuation in mice. Six of these genes appear to be directly or indirectly related to lipopolysaccharide (LPS) modification or biosynthesis. Genetic inactivation of a
10 seventh gene involved in LPS biosynthesis is also shown herein to enhance killing of macrophages by *F. novicida*. These results indicate that functionally deleting one or more of these genes in other *F. tularensis* subspecies can be used to generate immunogenic compositions for use against pathogenic subspecies. A previous study
15 found that genetic inactivation of one or more of the *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB* genes led to a decrease in replication *in vitro* and an attenuated phenotype (see PCT Publication No. WO 2007/097789).

 Provided herein are isolated *Francisella* bacteria, which are attenuated by functionally deleting or inactivating at least two genes. The provided bacteria
20 contain a genetic inactivation in at least one gene selected from *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB* and at least one gene selected from *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254. One skilled in the art will appreciate that any species or variety of *Francisella* can be used, such as *Francisella tularensis*, for example *Francisella tularensis* subspecies
25 *tularensis* or *Francisella tularensis* subspecies *novicida*. Methods of generating attenuated *Francisella* bacterium with the desired genes functionally deleted (or otherwise inactivated) are known in the art, and can include complete or partial deletion mutation or insertional mutation.

 These genetic inactivations attenuate the bacterium, and reduce the risk of
30 the bacterium reverting to a virulent form. Ideally, such genetic inactivations retain the ability of the isolated *Francisella* bacterium to stimulate a sufficient immune response in a mammal (such as a rodent or human) to provide the desired protection

or treatment. For example, an effective amount of the disclosed attenuated *Francisella* bacteria can produce an immune response in a subject, and in some examples can treat a subject (such as a subject exposed to *Francisella* or who may become exposed to *Francisella* in the future).

5 In particular examples, the isolated *Francisella* bacteria disclosed herein include genetic inactivations in at least two of *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB*. In some examples, the isolated *Francisella* bacteria disclosed herein include genetic inactivations in at least two of *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254. In some examples, the
10 isolated *Francisella* bacteria disclosed herein include genetic inactivations in at least two of *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB* and at least two of *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254.

Also provided by the present disclosure are immunogenic compositions that
15 include the disclosed isolated *Francisella* bacteria. In particular examples, such compositions can further include other biologically active or inactive agents, for example an adjuvant, a pharmaceutically acceptable carrier, or combinations thereof.

Methods are disclosed for eliciting an immune response against *Francisella* in a subject. In particular examples, the methods include administering a
20 therapeutically effective amount of the disclosed attenuated *Francisella* bacteria (for example in an immunogenic composition), thereby eliciting an immune response against *Francisella* in the subject. Methods of administration are routine and known to those skilled in the art. In some examples, the subject is a mammal, such as a human or veterinary subject (such as a laboratory animal, dog, cat, sheep, or cow).
25 In particular examples, the resulting immune response provides a prophylactic effect, for example in a subject who may be exposed to *Francisella* at a later date. In some examples, the resulting immune response treats tularemia in a subject, for example in a subject who was previously infected with or exposed to *Francisella*.

30 The foregoing and other objects and features of the disclosure will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A-1F are a series of images showing morphological changes of J774 macrophage-like cells under phase-contrast microscope upon infection of *F.*

5 *novicida* strains. As compared to the normal morphology of uninfected cells (A and D), cells infected with the wild-type strain U112 at 10 hours post-infection appear normal (B), whereas a representative mutant (*fopA*) kills J774 cells much earlier (C). A ten-fold reduced input of *fopA* mutant (F) causes similar intensity of cell death at 24 hours post-infection as compared with parental strain U112 (E).

10 **FIG. 2** is a table showing exemplary combinations of genetic inactivations in *Francisella* that can be used for vaccination.

FIG. 3 is a schematic drawing of a portion of the *Francisella* LPS including lipid A, the core and one O-antigen repeat (modified from Raetz *et al.*, *J Lipid Res* 50 Suppl:S103-8, 2009). Indicated in the drawing are the regions of the LPS that are
15 affected by the mutant alleles described in the studies disclosed herein. Both *wzx* and *htrB* contain transposon insertions while the remaining mutations are gene deletions.

FIGS. 4A and 4B demonstrate that the presence of cytochalasin D (2 μ M) during infection decreased LDH release in all but three of the J774 macrophage-like
20 cells infected with *F. novicida* transposon mutant strains. (A) J774 macrophages were infected with one of 12 hypercytotoxic transposon mutants or wild-type U112 either in the presence or absence of cytochalasin D (cytD). The levels of LDH in the extracellular medium were determined 12 hours post-infection (p.i.). The levels of LDH release from the mutant- or U112-infected J774 cells were normalized to the
25 level of LDH release from uninfected macrophages lysed with detergent. Parent strain U112 does not promote cell death at 12 hours p.i. (B) The level of LDH release from J774 macrophages infected with U112 at a MOI of 100 was determined 24 hours p.i. LDH release was determined from macrophages infected with U112 both in the presence and absence of cytD. In (A) and (B), each column is an average
30 of three individual infections ($\pm$ s.d.).

FIG. 5 is a graph showing that strains containing deletion mutations in *lpcC*, *manB*, and *manC* induce early cytotoxicity in primary macrophages. Bone marrow-

derived macrophages (BMDM) derived from BALB/c mice were infected with the deletion mutants or parental strain MFN245 at a MOI of 100. The level of LDH release from infected macrophages was determined 10 hours p.i. as described in FIG. 4. Each column is an average of three individual infections ($\pm$ s.d.).

5 **FIGS. 6A and 6B** are a series of representative images showing that high numbers of mutant bacteria are observed intracellularly in infected J774 macrophages even in the absence of actin polymerization. J774 macrophages were infected with the three deletion mutants or parental strain MFN245 in four-well microscope chambers for two hours at an MOI of 100 either in the absence (A) or
10 presence (B) of cytD. The cells were fixed in 4% paraformaldehyde, permeabilized, and probed with a rabbit polyclonal antibody against *Francisella* followed by a secondary goat anti-rabbit antibody conjugated with Alexa 488. J774 nuclei were identified by staining DNA with DAPI. Cells were imaged with an Applied Precision DeltaVision deconvolution microscope system. Eukaryotic cell boundary
15 can be observed in the phase-contrast images of the same fields. Scale bar 10 μ m (lower left corner). X-Z stack images show that bacteria were within cells.

FIG. 7 is a graph showing inhibiting actin polymerization did not reduce the number of intracellular mutant bacteria. J774 macrophages were infected with *Δ lpcC*, *Δ manB* or *Δ manC* deletion mutants or parental strain MFN245 at a MOI of
20 100. Cells were infected either in the presence or absence of cytD. At two hours p.i., the macrophages were washed and treated with gentamicin to kill extracellular bacteria. Cells were lysed and the lysates plated on CHA plates. Colonies were counted two days after incubation and the numbers of CFU/well were calculated and converted to a log scale. Each column is an average of three individual infections ($\pm$
25 s.d.).

FIGS. 8A and 8B demonstrate that increasing the number of internalized wild-type bacteria did not increase the cytotoxicity of the strain. (A) J774 macrophages were infected with *Δ lpcC*, *Δ manB*, *Δ manC*, or parental strain MFN245, as well as with complemented mutant strains expressing a wild-type copy
30 of the gene *in trans*. The cells were infected for 10 hours at three different MOI. The level of LDH release from infected macrophages was determined as described in FIG. 4. (B) J774 macrophages were infected with wild-type U112 at a MOI of

10,000 for two hours either in the presence or absence of cytD. *Francisella* and macrophage nuclei were visualized as described in FIG. 6.

FIGS. 9A and 9B demonstrate that dead bacteria do not promote cell death but are internalized similarly to live strains. (A) J774 macrophages were infected with formaldehyde-fixed $\Delta lpcC$ or MFN245 at various MOI. *Francisella* and macrophage nuclei were visualized as described in FIG. 6. (B) J774 macrophages were infected with live mutant or parental bacteria, and with strains that were fixed with 4% formaldehyde, at a MOI of 100. LDH release was determined 12 hours p.i. for the mutant strains and 24 hours p.i. for wild-type strain as described in FIG. 4.

FIG. 10 is a graph showing viable bacteria are required for the cell toxicity observed in the mutant strains. J774 macrophages were infected with $\Delta lpcC$, $\Delta manB$, and $\Delta manC$ mutant strains at a MOI of 100. Ciprofloxacin, a bactericidal and host cell membrane permeable antibiotic, was added concurrent with infection (0hr) or at one of six time points following initial infection (1hr-6hr). LDH release levels were determined 12 hours p.i. as described in FIG. 4 and compared to LDH release from infected macrophages not treated with ciprofloxacin (None).

FIG. 11 is an image of a gel showing lipopolysaccharides (LPS) prepared from $\Delta lpcC$, $\Delta manB$, $\Delta manC$, and $\Delta kdtA$ lack the O-antigen and contain a defect in the core. Lipopolysaccharides were purified from U112; strains containing deletions in *lpcC* (FTN1253), *manB* (FTN1417), *manC* (FTN1418), *kdtA* (FTN1469), *wbtA* (FTN1431); and strains containing transposon mutations in *wzx* (FTN1420) and *htrB* (FTN0071), and analyzed on a gradient SDS-PAGE gel. The inverted image is shown in the figure.

FIGS. 12A and 12B demonstrate that deleting LPS biosynthesis gene *kdtA* results in a cytotoxicity and localization phenotype similar to the $\Delta lpcC$, $\Delta manB$, and $\Delta manC$ mutants. (A) J774 macrophages were infected with $\Delta wbtA$ (FTN1431), $\Delta kdtA$ (FTN1469), transposon mutated FTN1420 (*wzx*), parental strain MFN245, or $\Delta kdtA$ complemented *in trans* with wild-type *kdtA* at a MOI of 100 for 10 hours either in the presence or absence of cytD. LDH release levels were determined as described in FIG. 4. (B) *Francisella* and macrophage nuclei were visualized in macrophages two hours after infection with the mutant strains as described in FIG. 7.

FIG. 13 is a graph showing the amount (Log_{10} CFU/ml) of viable bacteria following infection of J774 macrophages with either parental strain MFN245 or the $\Delta lpcC$ mutant strain at 2, 4 or 8 hours post-infection (p.i.).

FIGS. 14A and 14B are graphs showing LDH release from J774 macrophages infected with wild-type *Francisella tularensis* strain Schu S4 or Schu S4 $\Delta lpcC$ mutant at 8 hours (A) and 23 hours (B) post-infection. J774 cells were either untreated or pre-treated with 5 $\mu\text{g/ml}$ cytochalasin D (cytD) for 30 minutes prior to infection to inhibit actin polymerization. Wild-type Schu S4 or $\Delta lpcC$ (in-frame deletion in *lpcC* in Schu S4) were used to infect the macrophages at an input MOI of 10 or 100. The Schu S4 $\Delta lpcC$ mutant killed the J774 macrophages more quickly than wild-type Schu S4 (as indicated by the greater LDH release). In the presence of cytD, the cytotoxicity was reduced for wild-type Schu S4 but not for the $\Delta lpcC$ mutant.

SEQUENCE LISTING

The nucleic acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on December 30, 2010, 6.85 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NOS: 1 and 2 are the nucleotide sequences of PCR primers for amplification of the *F. novicida groE* promoter.

SEQ ID NOS: 3 and 4 are the nucleotide sequences of complementary pairs of oligonucleotides containing *Apal* and *SbfI* restriction sites.

SEQ ID NOS: 5 and 6 are the nucleotide sequences of complementary pairs of oligonucleotides containing *AscI* and *SmaI* restriction sites.

SEQ ID NOS: 7-34 are the nucleotides sequences of primer pairs specific for genes to be deleted in *F. novicida*.

DETAILED DESCRIPTION

I. Introduction

Francisella tularensis is an intracellular pathogen, the causative agent of tularemia, and infects a wide array of animals. A close relative, *F. novicida* U112, is not pathogenic for humans but retains mouse virulence allowing manipulation under BSL-2 conditions. Described herein is the screening of a comprehensive *F. novicida* transposon library to identify essential virulence factors on a genome wide scale by screening for morphological changes in infected J774 macrophages. Mutants in genes that showed differences in cell damage were tested for virulence in a mouse model. The screen identified 29 genes in which a mutation confers an increase in cytotoxicity and these mutants were heterogeneous in their mouse virulence. Thirteen of the identified genes exhibited a hypercytotoxic phenotype in macrophages, but were attenuated in mice. Six of these genes appear to be directly or indirectly related to LPS modification, suggesting that the normal function of these genes may be to block recognition of bacteria by toll like receptor 4 (TLR4). In addition, it is disclosed herein that genetic inactivation of a seventh gene involved in LPS biosynthesis enhances killing of macrophages by *F. novicida*.

II. Abbreviations

20	BMDM	Bone marrow-derived macrophages
	BSL	Biosafety level
	CFU	Colony forming units
	CHA	Cysteine heart agar
	CytD	Cytochalasin D
25	FBS	Fetal bovine serum
	FPI	<i>F. tularensis</i> pathogenicity island
	FTN	<i>Francisella tularensis</i> subspecies <i>novicida</i>
	FTT	<i>Francisella tularensis</i> subspecies <i>tularensis</i>
	IFA	Freund's incomplete adjuvant
30	i.p.	Intraperitoneal
	IVGI	<i>In vitro</i> growth index
	LD₅₀	50% lethal dose

	LDH	Lactate dehydrogenase
	LPS	Lipopolysaccharide
	MOI	Multiplicity of infection
	OD	Optical density
5	PBS	Phosphate-buffered saline
	PI	Post infection
	ssp.	Subspecies
	TCID	Tissue culture infectious dose
	TLR	Toll-like receptor
10	Tn	Transposon
	TSBC	Tryptic soy broth with 0.1% cysteine

III. Terms

Unless otherwise noted, technical terms are used according to conventional
 15 usage. Definitions of common terms in molecular biology may be found in Benjamin Lewin, *Genes V*, published by Oxford University Press, 1994 (ISBN 0-19-854287-9); Kendrew *et al.* (eds.), *The Encyclopedia of Molecular Biology*, published by Blackwell Science Ltd., 1994 (ISBN 0-632-02182-9); and Robert A. Meyers (ed.), *Molecular Biology and Biotechnology: a Comprehensive Desk Reference*,
 20 published by VCH Publishers, Inc., 1995 (ISBN 1-56081-569-8).

In order to facilitate review of the various embodiments of the disclosure, the following explanations of specific terms are provided:

Adjuvant: A compound, composition, or substance that when used in combination with an immunogenic agent (such as the attenuated *Francisella* bacteria
 25 disclosed herein) augments or otherwise alters or modifies a resultant immune response. In some examples, an adjuvant increases the titer of antibodies induced in a subject by the immunogenic agent. In another example, if the antigenic agent is a multivalent antigenic agent, an adjuvant alters the particular epitopic sequences that are specifically bound by antibodies induced in a subject.

30 Exemplary adjuvants include, but are not limited to, Freund's Incomplete Adjuvant (IFA), Freund's complete adjuvant, B30-MDP, LA-15-PH, montanide, saponin, aluminum salts such as aluminum hydroxide (Amphogel, Wyeth

Laboratories, Madison, NJ), alum, lipids, keyhole limpet protein, hemocyanin, the MF59 microemulsion, a mycobacterial antigen, vitamin E, non-ionic block polymers, muramyl dipeptides, polyanions, amphipathic substances, ISCOMs (immune stimulating complexes, such as those disclosed in European Patent EP 109942), vegetable oil, Carbopol, aluminium oxide, oil-emulsions (such as Bayol F or Marcol 52), *E. coli* heat-labile toxin (LT), Cholera toxin (CT), and combinations thereof.

In one example, an adjuvant includes a DNA motif that stimulates immune activation, for example the innate immune response or the adaptive immune response by T-cells, B-cells, monocytes, dendritic cells, and natural killer cells. Specific, non-limiting examples of a DNA motif that stimulates immune activation include CG oligodeoxynucleotides, as described in U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199.

Administration: To provide or give a subject an agent, such as an immunogenic composition disclosed herein, by any effective route. Exemplary routes of administration include, but are not limited to, oral, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), sublingual, rectal, transdermal, intranasal, intraocular, and inhalation routes.

Antibody: A molecule including an antigen binding site which specifically binds (immunoreacts with) an antigen. Examples include polyclonal antibodies, monoclonal antibodies, humanized monoclonal antibodies, or immunologically effective portions thereof. In a particular example, a subject produces antibodies when exposed to attenuated *Francisella* bacteria of the present application.

Immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

Antigen: A compound, composition, or substance that can stimulate the production of antibodies or a T-cell response in an animal, including immunogenic compositions that are administered to an animal. An antigen reacts with the products of specific humoral or cellular immunity, including those induced by

heterologous immunogens. The term “antigen” includes all related antigenic epitopes. In one example, an antigen is an attenuated *Francisella* bacterium that includes two or more functionally deleted genes selected from *dsbB*, FTT0742, *pdpB*, *fumA*, *carB*, *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*,
 5 *kdtA*, FTN0408, FTN0757 and FTN1254.

Attenuated bacterium: A bacterium having a decreased or weakened ability to produce disease (for example having reduced virulence) while retaining the ability to stimulate an immune response like that of the natural (or wild-type) bacterium. In one example, a live bacterium is attenuated by functionally deleting
 10 one or more genes of the bacterium, such as functionally deleting at least two genes. In a particular example, live *Francisella* is attenuated by functionally deleting at one or more of (such as two, three, four or five of) *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB*, and functionally deleting at one or more of (such as two, three, four, five or more of) *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408,
 15 FTN0757 and FTN1254.

Attenuated vaccine: An immunogenic composition that includes live pathogens (such as live *F. tularensis* subsp. *tularensis* having a functionally deleted *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB* gene, and a functionally deleted *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or
 20 FTN1254 gene), that have decreased virulence but are still capable of inducing a protective immune response to the virulent forms of the pathogen.

Carbamoyl phosphate synthase (*carB*): The large subunit of heterodimeric enzyme carbamoyl phosphate synthase, which is involved in pyrimidine biosynthesis (Koonin and Galperin. 2003. Sequence - evolution - function: computational approaches in comparative genomics. Kluwer Academic, Boston).
 25 The term *carB* includes any *Francisella carB* gene, cDNA, mRNA, or protein, that is a *carB* involved in pyrimidine biosynthesis, and when functionally deleted in *Francisella tularensis* subsp. *novicida*, results in a bacterium that is able to infect macrophages and protect mammals (such as mice) against challenges with the wild-type bacterium (PCT Publication No. WO 2007/097789).
 30

Francisella carB sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_170571 disclose *Francisella tularensis* subsp.

tularensis SCHU S4 *carB* nucleic acid and protein sequences, respectively.

However, one skilled in the art will appreciate that in some examples, a *carB* sequence can include variant sequences (such as allelic variants and homologs) that retain carbamoyl phosphate synthase activity and when functionally deleted in

- 5 *Francisella* results in a bacterium that is able to infect macrophages and protect mammals against challenge with wild-type *Francisella*.

Cellular immunity: An immune response mediated by cells or the products they produce, such as cytokines, rather than by an antibody. Includes, but is not limited to, delayed type hypersensitivity and cytotoxic T cells.

- 10 **DNA (deoxyribonucleic acid):** A long chain polymer which includes the genetic material of most living organisms (many viruses have genomes containing only ribonucleic acid, RNA). The repeating units in DNA polymers are four different nucleotides, each of which includes one of the four bases, adenine, guanine, cytosine and thymine bound to a deoxyribose sugar to which a phosphate
- 15 group is attached. Triplets of nucleotides, referred to as codons, in DNA molecules code for amino acid in a polypeptide. The term codon is also used for the corresponding (and complementary) sequences of three nucleotides in the mRNA into which the DNA sequence is transcribed.

- Disulfide bond formation protein B (*dsbB*):** An integral membrane protein
- 20 that is part of a pathway that leads to disulfide bond formation between cysteines in periplasmic proteins in *E. coli* and other bacteria (Kadokura *et al.*, *Annu. Rev. Biochem.* 72:111-135, 2003). The term *dsbB* includes any *Francisella dsbB* gene, cDNA, mRNA, or protein that is a *dsbB* involved in disulfide bond formation between cysteines, and when functionally deleted in *Francisella tularensis* subsp.
- 25 *novicida*, results in a bacterium that is able to infect macrophages and protect mammals (such as mice) against challenges with the wild-type bacterium (PCT Publication No. WO 2007/097789).

- Francisella dsbB* sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_169177 disclose *Francisella tularensis* subsp.
- 30 *tularensis* SCHU S4 *dsbB* nucleic acid and protein sequences, respectively.

However, one skilled in the art will appreciate that in some examples, a *dsbB* sequence can include variant sequences (such as allelic variants and homologs) that

retain the ability to promote disulfide bond formation between cysteines, and when functionally deleted in *Francisella* results in a bacterium that is able to infect macrophages and protect mammals against challenge with wild-type *Francisella*.

Epitope: Chemical groups or peptide sequences that are antigenic, that is, that elicit a specific immune response. An antibody binds a particular antigenic epitope, or a T-cell reacts with a particular antigenic epitope bound to a specific MHC molecule. In some examples, an epitope has a minimum sequence of 6-8 amino acids, and a maximum sequence of about 100 amino acids, for example, about 50, 25, or 18 amino acids in length.

***Francisella tularensis*:** A Gram-negative bacterium that is the causative agent of tularemia. Subspecies of *F. tularensis* include *tularensis* (type A), *holarctica* (type B), *novicida*, and *mediasiatica*.

***fimT*:** A type IV pilus assembly protein. The term *fimT* includes any *Francisella fimT* gene, cDNA, mRNA, or protein that is a *fimT* involved in type IV pilus assembly, and when functionally deleted in *Francisella tularensis* subsp. *novicida*, results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella fimT sequences are publicly available. For example, GenBank Accession Nos: CP000439 and YP_898310 disclose *Francisella tularensis* subsp. *novicida* U112 *fimT* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a *fimT* sequence can include variant sequences (such as allelic variants and homologs) that function as a type IV pilus assembly protein, and when functionally deleted in *Francisella*, results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice. For example, *fimT* is homologous to FTT1314c (type IV pili fiber building block protein) in *Francisella tularensis* subsp. *tularensis*.

FTN0408: Encodes a mannose-6-phosphate isomerase. The term FTN0408 includes any *Francisella* FTN0408 gene, cDNA, mRNA, or protein that is a FTN0408 protein, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella FTN0408 sequences are publicly available. For example, GenBank Accession Nos: CP000439 and ABK89311 disclose *Francisella tularensis* subsp. *novicida* U112 FTN0408 nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a FTN0408
5 sequence can include variant sequences (such as allelic variants and homologs) that retain mannose-6-phosphate isomerase activity, and when functionally deleted in *Francisella* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

FTN0757: A membrane protein of unknown function. The term FTN0757
10 includes any *Francisella* FTN0757 gene, cDNA, mRNA, or protein that is a FTN0757 membrane protein, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella FTN0757 sequences are publicly available. For example,
15 GenBank Accession Nos: CP000439 and YP_898402 disclose *Francisella tularensis* subsp. *novicida* U112 FTN0757 nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a FTN0757 sequence can include variant sequences (such as allelic variants and homologs) that function as membrane proteins, and when functionally deleted in *Francisella* results
20 in a bacterium that is hypercytotoxic in macrophages and attenuated in mice. For example, FTN0757 is homologous to FTT0584 in *Francisella tularensis* subsp. *tularensis*.

FTN1254: Encodes a hypothetical protein of 362 amino acids. The term FTN1254 includes any *Francisella* FTN1254 gene, cDNA, mRNA, or protein that
25 shares sequence similarity with FTN1254, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella FTN1254 sequences are publicly available. For example, GenBank Accession Nos: CP000439 and YP_898889 disclose *Francisella tularensis*
30 subsp. *novicida* U112 FTN1254 nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that a FTN1254 sequence can include variant sequences (such as allelic variants and homologs) that retain at least about

75% sequence identity to FTN1254, and when functionally deleted in *Francisella*, results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice. For example, FTN1254 is homologous to FTT1236 in *Francisella tularensis* subsp. *tularensis*.

5 **FTT0742:** A hypothetical lipoprotein that is predicted to have transmembrane regions, and thus may be a component of the *F. novicida* cell wall or involved in molecule transport. The term FTT0742 includes any *Francisella* FTT0742 gene, cDNA, mRNA, or protein that is a FTT0742 lipoprotein, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium
10 that has lower levels of *in vitro* replication and can protect mammals (such as mice) against challenges with the wild-type bacterium (PCT Publication No. WO 2007/097789).

Francisella FTT0742 sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_169753 disclose *Francisella*
15 *tularensis* subsp. *tularensis* SCHU S4 FTT0742 nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a FTT0742 sequence can include variant sequences (such as allelic variants and homologs) that retain the ability to function as lipoproteins, and when functionally deleted in *Francisella*, results in a bacterium that has lower levels of *in vitro*
20 replication and can protect mammals against challenges with wild-type *Francisella*.

Fumarate hydratase A (*fumA*): The enzyme of the Krebs's cycle (citric acid cycle/CAC) that converts fumarate to malate (Tseng *et al.*, *J. Bacteriol.* 183:461-7, 2001). The term *fumA* includes any *Francisella fumA* gene, cDNA, mRNA, or protein that is a *fumA* that can convert fumarate to malate, and when
25 functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that has lower levels of *in vitro* replication and can protect mammals (such as mice) against challenges with the wild-type bacterium (PCT Publication No. WO 2007/097789).

Francisella fumA sequences are publicly available. For example, GenBank
30 Accession Nos: NC_006570 and YP_170516 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *fumA* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a *fumA*

sequence can include variant sequences (such as allelic variants and homologs) that retain the ability to convert fumarate to malate, and when functionally deleted in *Francisella* results in a bacterium that has lower levels of *in vitro* replication and can protect mammals against challenges with wild-type *Francisella*.

5 **htrB:** The *htrB* gene encodes a LPS fatty acid acyltransferase. The term *htrB* includes any *Francisella htrB* gene, cDNA, mRNA, or protein that is an *htrB* having acyltransferase activity, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

10 *Francisella htrB* sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_169284 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *htrB* nucleic acid and protein sequences, respectively. In addition, GenBank Accession Nos: CP000439 and YP_897736 disclose *Francisella tularensis* subsp. *novicida* U112 *htrB* (FTN0071) nucleic acid and protein
15 sequences, respectively. However, one skilled in the art will appreciate that in some examples, an *htrB* sequence can include variant sequences (such as allelic variants and homologs) that retain acyltransferase activity, and when functionally deleted in *Francisella*, results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

20 **Genetic inactivation:** A mutation, such as a substitution, partial or complete deletion, insertion, or other variation, made to a gene sequence that significantly reduces (and in some cases eliminates) production of the gene product or renders the gene product substantially or completely non-functional. For example, a genetic inactivation of a *dsbB*, FTT0742, *pdpB*, *fumA*, *carB*, *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*,
25 *lpcC*, *manB*, *manC*, *nusA*, *wzx*, FTN0408, FTN0757 or FTN1254 gene (or combinations thereof) in *F. tularensis* results in *F. tularensis* having substantially non-functional or non-existent *dsbB*, FTT0742, *pdpB*, *fumA*, *carB*, *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, FTN0408, FTN0757 or FTN1254 protein, respectively, which results in attenuation of the *F. tularensis* pathogen.
30 Genetic activation is also referred to herein as “functional deletion.”

Humoral immunity: Immunity that can be transferred with immune serum from one subject to another. Typically, humoral immunity refers to immunity

resulting from the introduction of specific antibodies or stimulation of the production of specific antibodies, for example by administration of an attenuated *F. tularensis* disclosed herein.

Immune response: A response of a cell of the immune system, such as a B-cell, T-cell, macrophage, monocyte, or polymorphonucleocyte, to an immunogenic agent (such as the disclosed attenuated *F. tularensis*) in a subject. An immune response can include any cell of the body involved in a host defense response, such as an epithelial cell that secretes interferon or a cytokine. An immune response includes, but is not limited to, an innate immune response or inflammation.

10 The response can be specific for a particular antigen (an “antigen-specific response”). In a particular example, an immune response is a T cell response, such as a CD4+ response or a CD8+ response. In another example, the response is a B cell response, and results in the production of specific antibodies to the immunogenic agent.

15 In some examples, such an immune response provides protection for the subject from the immunogenic agent or the source of the immunogenic agent. For example, the response can protect a subject, such as a human or veterinary subject, from infection by a pathogen (such as *F. tularensis*), or interfere with the progression of an infection by a pathogen. An immune response can be active and
20 involve stimulation of the subject’s immune system, or be a response that results from passively acquired immunity.

Immunity: The state of being able to mount a protective response upon exposure to an immunogenic agent (such as the disclosed attenuated *F. tularensis*). Protective responses can be antibody-mediated or immune cell-mediated, and can be
25 directed toward a particular pathogen (such as *F. tularensis*). Immunity can be acquired actively (such as by exposure to an immunogenic agent, either naturally or in a pharmaceutical composition) or passively (such as by administration of antibodies).

Immunogen: An agent (such as a compound, composition, or substance)
30 that can stimulate or elicit an immune response by a subject’s immune system, such as stimulating the production of antibodies or a T-cell response in a subject. Immunogenic agents include, but are not limited to, pathogens (such as the disclosed

attenuated *F. tularensis*) and their corresponding proteins. One specific example of an immunogenic composition is a vaccine.

Immunogenicity: The ability of an agent to induce a humoral or cellular immune response. Immunogenicity can be measured, for example, by the ability to
5 bind to an appropriate MHC molecule (such as an MHC Class I or II molecule) and to induce a T-cell response or to induce a B-cell or antibody response, for example, a measurable cytotoxic T-cell response or a serum antibody response to a given epitope. Immunogenicity assays are well-known in the art and are described, for example, in Paul, Fundamental Immunology, 3rd ed., 243-247 (Raven Press, 1993)
10 and references cited therein.

Immunologically effective dose: A therapeutically effective amount of an immunogen (such as the disclosed attenuated *F. tularensis*) that will treat (such as prevent), lessen, or attenuate the severity, extent or duration of a disease or condition, for example, infection by a pathogen or development of a disease
15 resulting from infection (such as tularemia).

Isolated: To be significantly separated from other agents. An “isolated” biological component (such as a nucleic acid molecule or protein) has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component occurs, for example,
20 other chromosomal and extra-chromosomal DNA and RNA, and proteins. Nucleic acid molecules and proteins which have been “isolated” include nucleic acid molecules and proteins purified by standard purification methods. The term also embraces nucleic acid molecules and proteins prepared by recombinant expression in a host cell as well as chemically synthesized proteins and nucleic acids. Samples
25 of isolated biological components include samples of the biological component wherein the biological component represents greater than 90% (for example, greater than 95%, such as greater than 98%) of the sample.

An “isolated” microorganism (such as an attenuated *Francisella* bacterium) has been substantially separated or purified away from microorganisms of different
30 types, strains, or species. Microorganisms can be isolated by a variety of techniques, including serial dilution and culturing.

kdtA: The *kdtA* gene encodes a 3-deoxy-D-manno-octulosonic-acid transferase. The term *kdtA* includes any *Francisella lpcC* gene, cDNA, mRNA, or protein that is a *kdtA* gene with 3-deoxy-D-manno-octulosonic-acid transferase activity. *Francisella* strains lacking the *kdtA* gene ($\Delta kdtA$) synthesize a LPS without the core and O-antigen.

Francisella kdtA sequences are publicly available. For example, GenBank Accession No. YP_170484 discloses *Francisella tularensis* subsp. *tularensis* SCHU S4 *kdtA* nucleic acid and protein sequences. However, one skilled in the art will appreciate that in some examples, a *kdtA* sequence can include variant sequences (such as allelic variants and homologs) that retain 3-deoxy-D-manno-octulosonic-acid transferase activity and when genetically inactivated in *Francisella* results in a bacterium.....

lpcC: The *lpcC* gene encodes a glycosyltransferase group 1 family protein. Glycosyltransferases catalyze the transfer of sugar moieties from a glycosyl donor to an acceptor molecule. The term *lpcC* includes any *Francisella lpcC* gene, cDNA, mRNA, or protein that is an *lpcC* having glycosyltransferase activity, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella lpcC sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_170193 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *lpcC* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, an *lpcC* sequence can include variant sequences (such as allelic variants and homologs) that retain glycosyltransferase activity, and when functionally deleted in *Francisella* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

lpxH: The *lpxH* gene encodes a UDP-2,3-diacylglucosamine hydrolase, which plays a role in lipid A biosynthesis. The term *lpxH* includes any *Francisella lpxH* gene, cDNA, mRNA, or protein that is an *lpxH* involved in lipid A biosynthesis, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella lpxH sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_169476 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *lpxH* nucleic acid and protein sequences, respectively.

However, one skilled in the art will appreciate that in some examples, a *lpxH* sequence can include variant sequences (such as allelic variants and homologs) that retain hydrolase activity, and when functionally deleted in *Francisella* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

manB: The *manB* gene encodes a phosphomannomutase that catalyzes the conversion of mannose 6-phosphate to mannose-1-phosphate in the second of three steps in the GDP-mannose pathway. *manB* is involved in LPS synthesis. The term *manB* includes any *Francisella manB* gene, cDNA, mRNA, or protein that is a *manB* possesses phosphomannomutase activity, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella manB sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_170385 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *manB* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a *manB* sequence can include allelic variant sequences (such as allelic variants and homologs) that retain phosphomannomutase activity, and when functionally deleted in *Francisella* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

manC: The *manC* gene encodes a mannose-1-phosphate guanyltransferase. The term *manC* includes any *Francisella manC* gene, cDNA, mRNA, or protein that is a *manC* having mannose-1-phosphate guanyltransferase activity, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella manC sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_170386 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *manC* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a *manC* sequence can include variant sequences (such as allelic variants and homologs) that

retain guanyltransferase activity, and when functionally deleted in *Francisella* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Mutation: A change in a nucleic acid sequence (such as a gene sequence) or amino acid sequence, for example as compared to a nucleic acid or amino acid sequence present in a wild-type or native organism. In particular examples, a mutation in one or more genes can attenuate a pathogen, such as a *F. tularensis*. Mutations can occur spontaneously, or can be introduced, for example using molecular biology methods. In particular examples, a mutation includes one or more nucleotide substitutions, deletions, insertions, or combinations thereof. In particular examples, the presence of one or more mutations in a gene can significantly inactivate that gene.

Nucleic acid molecule: A deoxyribonucleotide or ribonucleotide polymer including, without limitation, cDNA, mRNA, genomic DNA, genomic RNA, and synthetic (such as chemically synthesized) DNA. Includes nucleic acid sequences that have naturally-occurring, modified, or non-naturally-occurring nucleotides linked together by naturally-occurring or non-naturally-occurring nucleotide linkages. Nucleic acid molecules can be modified chemically or biochemically and can contain non-natural or derivatized nucleotide bases. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with analogs, and internucleotide linkage modifications.

Nucleic acid molecules can be in any topological conformation, including single-stranded, double-stranded, partially duplexed, triplexed, hairpinned, circular, linear, and padlocked conformations. Where single-stranded, a nucleic acid molecule can be the sense strand or the antisense strand. Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known and include, for example, molecules in which peptide linkages are substituted for phosphate linkages in the backbone.

nusA: The *nusA* gene encodes a transcription elongation factor. The term *nusA* includes any *Francisella nusA* gene, cDNA, mRNA, or protein that is a *nusA* involved in transcription elongation, and when functionally deleted in *Francisella*

tularensis subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella nusA sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_169124 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *nusA* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a *nusA* sequence can include variant sequences (such as allelic variants and homologs) that retain transcriptional elongation activity, and when functionally deleted in *Francisella* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Organic solvent tolerance protein (*ostA1*): A protein involved in organic solvent tolerance in bacteria. The term *ostA1* includes any *Francisella ostA1* gene, cDNA, mRNA, or protein that is an *ostA1* involved in organic solvent tolerance, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella ostA1 sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_169505 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *ostA1* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, an *ostA1* sequence can include variant sequences (such as allelic variants and homologs) that retain the ability to confer organic solvent tolerance, and when functionally deleted in *Francisella* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Passive immunity: Immunity acquired by the introduction by immune system components into a subject rather than by stimulation.

***pdpB*:** The *pdpB* gene encodes an uncharacterized protein encoded on the *F. tularensis* pathogenicity island (FPI) that exhibits some similarity to the conserved bacterial protein IcmF. It has been shown that *icmF* is required for *Legionella pneumophila* intracellular growth, so *pdpB* may play a similar role in *F. novicida* intracellular growth. The *pdpB* sequence also has some homology to *Plasmodium rhoptry* proteins, which are involved in host cell binding and invasion. This, coupled with the reduced ability of *pdpB* mutants to enter host cells, suggests that

the gene product of *pdpB* may also play a role in host cell invasion. The term *pdpB* includes any *Francisella pdpB* gene, cDNA, mRNA, or protein that functions as *pdpB*, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that has lower levels of *in vitro* replication and can protect mammals (such as mice) against challenges with the wild-type bacterium (PCT Publication No. WO 2007/097789).

Francisella pdpB sequences are publicly available. For example, GenBank Accession No: NC_006570 discloses *Francisella tularensis* subsp. *tularensis* SCHU S4 *pdpB* nucleic acid and protein sequences (regions 1382427...1385708 and 1775771...1779052) and GenBank Accession Nos: AY293579 and AAP58967 disclose *Francisella tularensis* subsp. *novicida pdpB* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that in some examples, a *pdpB* sequence can include variant sequences (such as allelic variants and homologs) and when functionally deleted in *Francisella* results in a bacterium that has lower levels of *in vitro* replication and can protect mammals against challenges with wild-type *Francisella*.

Pharmaceutically acceptable carrier: Compositions or formulations suitable for pharmaceutical delivery of one or more therapeutic molecules, such as one or more immunogenic compositions that includes attenuated *Francisella* bacteria of the present disclosure. The pharmaceutically acceptable carriers (vehicles) useful in this disclosure are conventional (for example see Remington's Pharmaceutical Sciences, by E. W. Martin, Mack Publishing Co., Easton, PA, 15th Edition (1975)).

In general, the nature of the carrier will depend on the particular mode of administration being employed. For instance, parenteral formulations can include injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. In addition to biologically-neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan

monolaurate, sodium lactate, potassium chloride, calcium chloride, and triethanolamine oleate.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified attenuated *Francisella* bacterial preparation is one in which the bacteria are more enriched than the bacteria is in its natural environment (for example within a cell or culture medium). In one example, a preparation is purified such that the purified bacteria represent at least 50% of the total content of the preparation. In other examples, bacteria is purified to represent at least 90%, such as at least 95%, or even at least 98%, of all macromolecular species present in a purified preparation prior to admixture with other formulation ingredients, such as a pharmaceutical carrier, adjuvant or other co-ingredient. In some examples, the purified preparation is essentially homogeneous, wherein other macromolecular species are not detectable by conventional techniques.

Such purified preparations can include materials in covalent association with the active agent, such as glycoside residues or materials admixed or conjugated with the active agent, which may be desired to yield a modified derivative or analog of the active agent or produce a combinatorial therapeutic formulation, conjugate, fusion protein or the like.

Quantitating: Determining a relative or absolute quantity of a particular component in a sample. For example, in the context of quantitating antibodies in a sample of a subject's blood to detect an immune response to a pathogen (such as the attenuated *Francisella* disclosed herein), quantitating refers to determining the quantity of antibodies using an antibody assay, for example, an ELISA-assay or a T-cell proliferation assay.

Recombinant: A recombinant nucleic acid molecule or protein is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. In particular examples, this artificial combination is accomplished by chemical synthesis or by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques such as those described in Sambrook et al. (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, Cold Spring

Harbor Laboratory Press, Cold Spring Harbor, NY, 1989. The term recombinant includes nucleic acid molecules that have been altered solely by addition, substitution, or deletion of a portion of the nucleic acid molecule. Similarly, a recombinant protein can be encoded for by a recombinant nucleic acid molecule, or
 5 generated using chemical synthesis.

Replicative fitness: The ability of a pathogen to produce mature infectious progeny. In some examples, functionally deleting one or more genes of a pathogen reduces the replicative fitness of the pathogen, as compared to a pathogen containing a native gene sequence. In particular examples, functionally deleting one or more
 10 genes (such as two or more, for example two, three, four or five genes) in *F. tularensis*, such as one or more of *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB*, and one or more of *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254, reduces the replicative fitness of *F. tularensis*, as compared to *F. tularensis* containing native gene sequences. In some examples,
 15 such replicative fitness is reduced by at least 10%, such as at least 20%, at least 50%, or even at least 90% as compared to a *F. tularensis* containing native gene sequences.

Methods that can be used to determine replicative fitness and are known in the art (see, for example, PCT Publication No. WO 2007/097789, herein
 20 incorporated by reference). For example, to determine the replicative fitness of a bacterium, exemplary replicative fitness assays include assays for colony-forming activity, assays that measure survival of a mammal into which the bacterium was introduced, reduced ability of the bacteria to survive various stress conditions (such as nutrient deprivation), altered host range, enzymatic assays indicating reduced
 25 activity of a key enzyme, or assays for reduced pathogenicity due to decreased expression of an important protein (such as LPS).

Specifically bind: Refers to the ability of a particular agent (a “specific binding agent”) to specifically react with a particular analyte, for example to specifically immunoreact with an antibody, or to specifically bind to a particular
 30 peptide sequence. The binding is a non-random binding reaction, for example between an antibody molecule and an antigenic determinant. Binding specificity of an antibody is typically determined from the reference point of the ability of the

antibody to differentially bind the specific antigen and an unrelated antigen, and therefore distinguish between two different antigens, particularly where the two antigens have unique epitopes. An antibody that specifically binds to a particular epitope is referred to as a "specific antibody".

5 In particular examples, two compounds are said to specifically bind when the binding constant for complex formation between the components exceeds about 10^4 L/mol, for example, exceeds about 10^6 L/mol, exceeds about 10^8 L/mol, or exceeds about 10^{10} L/mol. The binding constant for two components can be determined using methods that are well known in the art.

10 **Subject:** Living multi-cellular organisms, a category that includes human and non-human mammals, as well as other veterinary subjects susceptible to infection by *Francisella*.

Therapeutically effective amount: An amount of a therapeutic agent (such as an immunogenic composition) that alone, or together with an additional
15 therapeutic agent(s), induces the desired response, such as a protective immune response or therapeutic response to a pathogen (such as *F. tularensis*). In one example, it is an amount of immunogen (such as attenuated *F. tularensis* having a genetic inactivation in one or more of *dsbB*, FTT0742, *pdpB*, *fumA* and *carB*, in combination with a genetic inactivation in one or more of *tolB*, *htrB*, *lpxH*, *ostA1*,
20 *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254) needed to increase resistance to, prevent, ameliorate, or treat infection and disease caused by a pathogenic infection in a subject. Ideally, a therapeutically effective amount of an immunogen provides a therapeutic effect without causing a substantial cytotoxic effect in the subject. The preparations disclosed herein are administered in
25 therapeutically effective amounts.

 In general, an effective amount of a composition administered to a human or veterinary subject will vary depending upon a number of factors associated with that subject, for example whether the subject previously has been exposed to the pathogen. An effective amount of a composition can be determined by varying the
30 dosage of the product and measuring the resulting immune or therapeutic responses, such as the production of antibodies. Effective amounts also can be determined through various *in vitro*, *in vivo* or *in situ* immunoassays. The disclosed therapeutic

agents can be administered in a single dose, or in several doses, as needed to obtain the desired response. However, the effective amount can be dependent on the source applied, the subject being treated, the severity and type of the condition being treated, and the manner of administration.

5 The disclosed therapeutic agents can be administered alone, or in the presence of a pharmaceutically acceptable carrier, or in the presence of other agents, for example an adjuvant.

 In one example, a desired response is to increase an immune response in response to infection with a pathogen (such as *F. tularensis*). For example, the
10 therapeutic agent can increase the immune response by a desired amount, for example by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 50%, at least 75%, or even at least 90%, for example as compared to an immune response in the absence of the therapeutic agent. This increase can result in decreasing or slowing the progression of a disease or condition associated
15 with a pathogenic infection (such as tularemia).

tolB: The *tolB* gene encodes a group A colicin translocase. The term *tolB* includes any *Francisella tolB* gene, cDNA, mRNA, or protein that is a *tolB* involved in colicin translocase, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated
20 in mice.

Francisella tolB sequences are publicly available. For example, GenBank Accession Nos: NC_006570 and YP_169845 disclose *Francisella tularensis* subsp. *tularensis* SCHU S4 *tolB* nucleic acid and protein sequences, respectively. However, one skilled in the art will appreciate that a *tolB* sequence can include
25 variant sequences (such as allelic variants and homologs) that retain colicin transferase activity, and when functionally deleted in *Francisella* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Treating a disease: Treatment refers to a therapeutic intervention that ameliorates a sign or symptom of a disease or pathological condition related to a
30 disease (such as tularemia), even if the underlying pathophysiology is not affected. Reducing a sign or symptom associated with a pathogenic infection can be evidenced, for example, by a delayed onset of clinical symptoms of the disease in a

susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, a reduction in the number of relapses of the disease, an improvement in the overall health or well-being of the subject, or by other parameters well known in the art that are specific to the particular disease. For
5 example, treatment of tularemia may be evidenced by a reduction or delayed onset of one or more of the following symptoms: fever, headache, chills and rigors, generalized body aches, coryza, sore throat, coughing, diarrhea, nausea, vomiting, malaise, anorexia, or weight loss.

Treatment can also induce remission or cure of a condition, such as a
10 pathogenic infection or a pathological condition associated with such an infection (such as tularemia). In particular examples, treatment includes preventing a disease, for example by reducing or even avoiding altogether the full development of a disease or condition, such as a disease associated with a pathogen, such as tularemia. Thus, prevention of pathogenic disease can include reducing the number of subjects
15 who acquire a disease associated with a pathogenic infection (such as the development of tularemia by *Francisella*) in a population of subjects receiving a preventative treatment (such as vaccination) relative to an untreated control population, or delaying the appearance of such disease in a treated population versus an untreated control population. Prevention of a disease does not require a total
20 absence of disease. For example, a decrease of at least 50% can be sufficient.

Tularemia: The disease caused by infection with *Francisella* species, such as *F. tularensis*. The primary clinical forms of tularemia can vary in severity and presentation according to virulence of the infecting organism and the site of inoculum. Primary disease presentations include ulceroglandular, glandular,
25 oculoglandular, oropharyngeal, pneumonic, typhoidal, and septic forms. The onset of tularemia is usually abrupt, with symptoms that can include fever (38-40°C), headache, chills and rigors, generalized body aches, coryza, sore throat, and coughing. Some subjects also experience diarrhea, nausea, or vomiting. As the disease progresses, subjects can experience sweats, fever, chills, progressive
30 weakness, malaise, anorexia, and weight loss. If left untreated, symptoms often persist for several weeks. In ulceroglandular tularemia, a local cutaneous papule appears that the inoculation site at about the same time as the general symptoms.

The papule ulcerates in a few days, and regional lymph nodes may become enlarged. Tularemia pneumonia, usually the result of inhaling *F. tularensis*, can be associated with pharyngitis, bronchiolitis, pleuropneumonitis, and hilar lymphadenitis.

Unit dose: A physically discrete unit containing a predetermined quantity of an active material calculated to individually or collectively produce a desired effect such as an immunogenic effect. A single unit dose or a plurality of unit doses can be used to provide the desired effect, such as an immunogenic effect. In one example, a unit dose includes a desired amount of one or more of the disclosed attenuated *F. tularensis* bacteria.

Vaccine: An immunogenic composition that can be administered to a veterinary subject or a human to confer immunity, such as active immunity, to a disease or other pathological condition (such as tularemia). Vaccines can be used therapeutically, for example prophylactically. Thus, vaccines can be used to reduce the likelihood of infection or to reduce the severity of symptoms of a disease or condition or limit the progression of the disease or condition. In one example, a vaccine includes one or more of the disclosed attenuated *F. tularensis* bacteria.

Vector: A nucleic acid molecule as introduced into a host cell (such as a *F. tularensis* bacterial cell), thereby producing a transformed host cell. In particular examples, a vector includes nucleic acid sequences that permit allelic replacement of *dsbB*, FTT0742, *pdpB*, *fumA*, *carB*, *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254 in a *Francisella* cell. A vector can transduce, transform or infect a cell, thereby causing the cell to express nucleic acid molecules or proteins other than those native to the cell. A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like.

wzx: The *wzx* gene encodes an O-antigen flippase. The term *wzx* includes any *Francisella* *wzx* gene, cDNA, mRNA, or protein that is a *wzx* protein having O-antigen flippase activity, and when functionally deleted in *Francisella tularensis* subsp. *novicida* results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Francisella *wzx* sequences are publicly available. For example, GenBank Accession Nos: AJ749949 and YP_170390 disclose *Francisella tularensis* subsp.

tularensis SCHU S4 *wzx* nucleic acid and protein sequences, respectively.

However, one skilled in the art will appreciate that in some examples, a *wzx* sequence can include variant sequences (such as allelic variants and homologs) that retain O-antigen flippase activity, and when functionally deleted in *Francisella*,
5 results in a bacterium that is hypercytotoxic in macrophages and attenuated in mice.

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. The singular terms “a,” “an,” and “the” include
10 plural referents unless context clearly indicates otherwise. Similarly, the word “or” is intended to include “and” unless the context clearly indicates otherwise. Hence “comprising A or B” means including A, or B, or A and B. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or
molecular mass values, given for nucleic acids or polypeptides are approximate, and
15 are provided for description. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. All Genbank Accession numbers mentioned herein are incorporated by reference. In case of conflict, the present specification, including explanations of terms, will control. In
20 addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

IV. *Attenuated Francisella Mutants*

The present disclosure provides isolated *Francisella* bacteria having least
25 two genes inactivated, wherein such inactivation results in attenuation of *Francisella* virulence. In some embodiments, at least one of the inactivated genes is selected from *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB*; and at least one of the inactivated genes is selected from *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254. *Francisella* bacteria having inactivated
30 *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB* have been shown to exhibit reduced replication *in vitro* and to confer protection to mammals (such as mice) against challenge with wild-type bacteria (see PCT Publication No. WO 2007/097789,

herein incorporated by reference). It is disclosed herein that genetic inactivation of either *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, FTN0408, FTN0757 or FTN1254 results in *Francisella* bacteria with a hypercytotoxic phenotype in macrophages and attenuated pathogenicity in mice. It is further
 5 disclosed herein that genetic inactivation of the *kdtA* gene ($\Delta kdtA$), kills infected macrophages as quickly as three other LPS biosynthesis mutants, $\Delta lpcC$, $\Delta manB$, and $\Delta manC$.

Contemplated herein are *Francisella* bacteria containing at least one gene that is inactivated, wherein the inactivation results in reduced replication of the
 10 bacteria *in vivo* (*dsbB*, FTT0742, *pdpB*, *fumA*, and *carB*), in combination with genetic inactivation of at least one gene which deletion results in hyperpathogenicity in macrophages (*tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254).

The inactivation of the genes described herein attenuate the *Francisella*
 15 bacterium in an amount sufficient to significantly reduce or prevent the attenuated *Francisella* bacterium from evoking severe clinical symptoms in the subject, while allowing limited replication and growth of the bacteria in the recipient to produce an immune response in a subject.

In some examples, the *Francisella* bacterium is live. One skilled in the art
 20 will appreciate that the disclosed functional mutations can be made to any genus or variety of *Francisella*. In particular examples, the disclosed attenuated *Francisella* bacterium is *Francisella tularensis*, such as *Francisella tularensis* subspecies *tularensis* or *Francisella tularensis* subspecies *novicida*. In a specific example, the attenuated bacterium is *Francisella tularensis* subspecies *tularensis* strain SCHU S4.

25 In particular examples, at least 2, at least 3, at least 4, or all 5 of the *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB* genes are genetically inactivated in *Francisella*. In some examples, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13 or all 14 of the *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and
 30 FTN1254 genes are genetically inactivated in *Francisella*. All combinations of gene inactivations are contemplated, wherein at least one gene from the first group (*dsbB*, FTT0742, *pdpB*, *fumA*, and *carB*) and at least one gene from the second group (*tolB*,

htrB, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254) is genetically inactivated.

One skilled in the art will appreciate that additional genes can also be inactivated, wherein the additional genes may or may not provide additional
5 attenuation to the bacterium. Particular examples of combinations of genes that can be inactivated are provided in table shown in FIG. 2. However, based on the teachings herein, those skilled in the art can determine other appropriate combinations.

In some embodiments, the *Francisella* bacterium comprises genetic
10 inactivation of one or more genes involved in LPS biosynthesis. In some embodiments, the one or more genes involved in LPS biosynthesis include *lpcC*, *manB*, *manC*, *lpxH*, *wzx*, *htrB* and *kdtA*. In some examples, the *Francisella* bacterium comprises genetic inactivation of one or more genes selected from *lpcC*, *manB*, *manC* and *kdtA*. The *Francisella* bacterium optionally further comprises
15 inactivation of the *pdpB* gene. In one non-limiting example, the *Francisella* bacterium comprises genetically inactivated *lpcC* and *pdpB* genes ($\Delta lpcC \Delta pdpB$).

In the context of the present disclosure, “genetic inactivation” need not be 100% genetic inactivation. In some embodiments, genetic inactivation refers to at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, or at least 95%
20 gene inactivation.

A. *Methods of functionally deleting genes*

As used herein, an “inactivated” or “functionally deleted” gene means that the gene has been mutated by insertion, deletion, or substitution (or combinations thereof) of one or more nucleotides such that the mutation substantially reduces (and
25 in some cases abolishes) expression or biological activity of the encoded gene product. The mutation can act through affecting transcription or translation of the gene or its mRNA, or the mutation can affect the peptide gene product itself in such a way as to render it substantially inactive.

Genetic inactivation of one or more genes (which in some examples is also
30 referred to as gene inactivation) can be performed using any conventional method known in the art. In one example, a strain of *Francisella* bacteria is transformed with a vector which has the effect of downregulating or otherwise inactivating the

gene. This can be done by mutating control elements such as promoters and the like which control gene expression, by mutating the coding region of the gene so that any protein expressed is substantially inactive, or by deleting the gene entirely. For example, a gene can be functionally deleted by complete or partial deletion mutation
5 (for example by deleting a portion of the coding region of the gene) or by insertional mutation (for example by inserting a sequence of nucleotides into the coding region of the gene, such as a sequence of about 1-5000 nucleotides). In particular examples, an insertional mutation includes introduction of a sequence that is in multiples of three bases (*e.g.*, a sequence of 3, 9, 12, or 15 nucleotides) to reduce the
10 possibility that the insertion will be polar on downstream genes. For example, insertion or deletion of even a single nucleotide that causes a frame shift in the open reading frame, which in turn can cause premature termination of the encoded peptide or expression of a substantially inactive peptide. Mutations can also be generated through insertion of foreign gene sequences, for example the insertion of a
15 gene encoding antibiotic resistance.

In one example, genetic inactivation is achieved by deletion of a portion of the coding region of the *dsbB*, FTT0742, *pdpB*, *fumA*, *carB*, *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254 gene. Deletion mutations reduce the risk that the mutant will revert to a virulent state. For
20 example, some, most (such as at least 50%) or virtually the entire coding region can be deleted. In particular examples, about 5% to about 100% of the gene is deleted, such as at least 20% of the gene, at least 40% of the gene, at least 75% of the gene, or at least 90% of the gene is deleted.

Deletion mutants can be constructed using any of a number of techniques
25 known in the art. In one example, allelic exchange is employed to genetically inactivate one or more genes in *Francisella* (for example, using the methods of Golovliov *et al.*, *FEMS Microbiol. Lett.* 222:273-280, 2003). A specific example of such a method is described in PCT Publication No. WO 2007/097789 (see FIG. 5). A construct that includes the flanking region of the gene to be deleted with an in-
30 frame deletion of a significant part of the gene is introduced into a pDM4 vector. This is a suicide vector in *F. tularensis*. In particular examples, pDM4 includes an antibiotic resistance marker, such as Kan^r. In particular examples, the resulting

vector is transformed into *E. coli* strain S17. The resulting transformed *E. coli* is mated with a native *Francisella* bacteria (such as a wild-type virulent strain), thereby allowing the vector to be introduced into the *Francisella* bacteria via conjugation. The pDM4 vector DNA is incorporated into the *F. tularensis* genome by recombination between the homologous gene sequences. Conjugants can be selected based on the antibiotic resistance marker, such as selection with kanamycin (and for *F. tularensis* only with polymyxin that kills *E. coli*). pDM4 also contains *sacB*, which does not permit growth in/on sucrose. By growing the conjugants with sucrose, the incorporated plasmid DNA will loop out of the *F. tularensis* genome and leave behind one copy of the gene. PCR can be used to confirm if it is the deletion or the full-length wild-type copy. This results in an avirulent strain of *F. tularensis* that carries a deletion in *dsbB*, FTT0742, *pdpB*, *fumA*, *carB*, *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254 (or combinations thereof) and is antibiotic sensitive.

In one example, a strategy using counterselectable markers can be employed which has been utilized to delete genes in many bacteria. For a review, see Reyrat *et al.* (*Infect. Immun.* 66:4011-4017, 1998). In this technique, a double selection strategy is employed wherein a plasmid is constructed encoding both a selectable and counterselectable marker, with flanking DNA sequences derived from both sides of the desired deletion. The selectable marker is used to select for bacteria in which the plasmid has integrated into the genome in the appropriate location and manner. The counterselectable marker is used to select for the very small percentage of bacteria that have spontaneously eliminated the integrated plasmid. A fraction of these bacteria will then contain only the desired deletion with no other foreign DNA present.

In another technique, the *cre-lox* system is used for site specific recombination of DNA. The system includes 34 base pair lox sequences that are recognized by the bacterial *cre* recombinase gene. If the lox sites are present in the DNA in an appropriate orientation, DNA flanked by the lox sites will be excised by the *cre* recombinase, resulting in the deletion of all sequences except for one remaining copy of the lox sequence. Using standard recombination techniques, the targeted gene of interest (*dsbB*, FTT0742, *pdpB*, *fumA*, *carB*, *tolB*, *htrB*, *lpxH*,

ostA1, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254, or combinations thereof) can be deleted in the *Francisella* genome and to replace it with a selectable marker (for example a gene coding for kanamycin resistance) that is flanked by the lox sites. Transient expression (by electroporation of a suicide
 5 plasmid containing the *cre* gene under control of a promoter that functions in *Francisella*) of the *cre* recombinase should result in efficient elimination of the lox flanked marker. This process will produce a mutant containing the desired deletion mutation and one copy of the lox sequence.

In another method, a gene sequence in the *Francisella* genome is replaced
 10 with a marker gene, such as green fluorescent protein, β -galactosidase, or luciferase. In this technique, DNA segments flanking a desired deletion are prepared by PCR and cloned into a suicide (non-replicating) vector for *Francisella*. An expression cassette, containing a promoter active in *Francisella* and the appropriate marker gene, is cloned between the flanking sequences. The plasmid is introduced into
 15 wild-type *Francisella*. Bacteria that incorporate and express the marker gene are isolated and examined for the appropriate recombination event (replacement of the wild type gene with the marker gene).

B. Measuring attenuation

Methods of determining whether a genetic inactivation in one or more of
 20 *dsbB*, FTT0742, *pdpB*, *fumA* and *carB*, in combination with a genetic inactivation in one or more of *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254, in *Francisella* attenuates the bacteria, for example in a mammal, are known in the art. Although particular examples are disclosed herein, the methods are not limiting. For example, attenuation of bacteria
 25 can be measured *in vitro* by infecting macrophages (such as a primary macrophage culture or a tissue culture cell line, for example those available from American Type Culture Collection, Manassas, VA) with the mutated *Francisella* bacteria (for example containing genetic inactivations as shown in Table 2). In particular examples, cells are infected with a multiplicity of infection (MOI) of about 1-5000,
 30 such as an MOI of at least 1, at least 10, at least 100, at least 500, at least 1000, or at least 2000, for example an MOI of about 10-100, 1000-2000, or 500-1500. The MOI is the ratio of bacteria to the number of cells being infected, and thus is

dependent on the number of macrophages present, but not necessarily the number that get infected. After the desired incubation, such as 12-48 hours (for example 24 hours), the macrophages are lysed and the resulting lysate cultured. The resulting growth of *Francisella* is monitored, for example by visual inspection of bacterial colonies. In particular examples, parallel reactions are performed for native *Francisella* bacteria of the same species and strain as the mutated bacteria. Mutated *Francisella* bacteria that exhibit smaller colonies or fewer colonies (such as an absence of colonies), for example as compared to a reference value representing native *Francisella* bacteria growth of the same species and strain, indicates that the mutated *Francisella* bacteria are attenuated. Such attenuated *Francisella* bacteria can be selected for further analysis, for example by determining attenuation *in vivo*.

Attenuation *in vivo* can be determined in a laboratory animal, such as a rodent (for example a mouse, rat, or rabbit) or non-human primate. Mutated *Francisella* bacteria are administered to the laboratory animal. A parallel set of animals can be administered native *Francisella* bacteria of the same species and strain as the mutated bacteria as a control. In particular examples, the animals are administered a dose of bacteria that is at least 50 times, such as at least 100 times, the LD₅₀ of the native bacteria in that animal. For example, for a mutated *Francisella tularensis* subsp. *tularensis*, mice can be administered 10³ to 10¹¹ cfu bacteria, and rhesus monkeys can be administered 10³ to 10¹¹ cfu bacteria. Any method of administration can be used, such as injection (for example intraperitoneal or intradermal) or inhalation. The animals are subsequently observed for survival. Animals receiving *Francisella* bacteria containing one or more genetic inactivations in *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB*, in combination with one or more genetic inactivations in *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254, that exhibit 100% survival one month following infection, is an indication that the animal received an attenuated form of the *Francisella* bacteria. Such attenuated *Francisella* bacteria can be selected for further analysis. In contrast, animals administered the same dose of the native *Francisella* bacteria should demonstrate substantially 0% survival.

C. *Measuring immune response*

Francisella bacteria having one or more genetic inactivations in *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB*, in combination with one or more genetic inactivations in *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*,
5 FTN0408, FTN0757 or FTN1254 that have been shown to be attenuated *in vitro*, *in vivo*, or both, can be examined for their ability to stimulate an immune response, for example to protect a subject from challenge with the native bacteria. Such methods are known in the art. For example, an immunogenic response of an animal to a composition that includes the attenuated *Francisella* bacteria disclosed herein can be
10 evaluated indirectly through measurement of antibody titers or lymphocyte proliferation assays, or directly through monitoring signs and symptoms after challenge with wild type strain.

For example, the ability of *Francisella* bacteria having one or more genetic inactivations in *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB*, in combination with one or
15 more genetic inactivations in *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254 to stimulate an immune response can be determined following administration of the mutated bacteria to a subject (such as a human or laboratory animal) (for example using the methods described above). Subsequently, stimulation of the immune response can be measured. In one
20 example, 7-60 days following administration of the *Francisella* bacteria having the desired genetic inactivations, a biological sample (such as blood or a fraction thereof, for example serum) can be obtained from the subject, and analyzed by an immunoassay (such as an ELISA or western blot) to determine the presence of antibodies against *Francisella* bacteria. For example, commercially available
25 antibodies that specifically recognize one or more *Francisella* antigens (such as mouse anti-*Francisella tularensis* LPS monoclonal antibody from Abcam, Cambridge, MA and GeneTex, San Antonio, TX) can be contacted with a biological sample. In one example, microagglutination using formalin-inactivated bacteria as an antigen is used to detect the presence of *Francisella* antibodies in the biological
30 sample. In a particular example, subjects having an antibody titer of $\geq 1:80$ are considered responders, while subjects having an antibody titer of $\leq 1:20$ are considered non-responders. In another example, stimulation of the immune

response can be measured by detecting levels of cytokines in a biological sample obtained from the subject following administration of the bacteria. For example, levels of IL-6 and TNF- α can be measured using commercially available kits. In one example, an at least 5-fold increase (such as at least a 6-, 7-, 8-, 9- or 10-fold increase) in the level of IL-6 or TNF- α relative to background (or relative to an amount present before administration of the *Francisella* bacteria), indicates that the subject has had an immune response.

The immunogenic response of an animal to a composition that includes the attenuated *Francisella* bacteria disclosed herein can be evaluated directly through monitoring signs and symptoms after challenge with a native *Francisella* strain. For example, the ability of *Francisella* bacteria having one or more genetic inactivations in *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB*, in combination with one or more genetic inactivations in *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254 to protect a subject from challenge with the native bacteria can be determined following administration of the mutated bacteria to a laboratory animal (for example using the methods described above). Any method of administration can be used, such as the methods described herein. Subsequently, for example 2-6 weeks (such as 4-6 weeks), the animal is administered native *Francisella* bacteria of the same subspecies and strain as the attenuated bacteria previously administered. The amount of native *Francisella* bacteria administered can be at least 1000 times the LD₅₀ observed for native infection, such as at least 5000 times, or at least 10,000 times the LD₅₀. The animals are subsequently observed for survival. Animals receiving *Francisella* bacteria containing one or more genetic inactivations in *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB*, in combination with one or more genetic inactivations in *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 or FTN1254, that exhibit 100% survival 7-28 days following challenge, indicates that the attenuated bacteria provides a protective immune response to the subject. Such attenuated *Francisella* bacteria can be selected for further analysis, for example human clinical trials. In contrast, animals not administered the attenuated *Francisella* bacteria should demonstrate substantially 0% survival.

V. *Immunogenic Compositions*

Immunogenic compositions are provided that include the disclosed attenuated *Francisella* bacteria. In particular examples, an immunogenic composition includes more than one type of attenuated *Francisella* bacteria. For
5 example, the composition can include two or more populations of attenuated *Francisella* bacteria, such as the *Francisella* bacteria of two or more groups shown in Table 2. One skilled in the art will recognize that other combinations can be selected. In particular examples, the attenuated *Francisella* bacteria are present in a therapeutically effective amount. In some embodiments, the therapeutically
10 effective amount is a dose that is at least 20-fold, at least 15-fold, at least 10-fold or at least 5-fold lower than the LD₅₀ of the attenuated *Francisella* bacteria. In particular examples, the therapeutically effective amount is a dose that is at least 10-fold lower than the LD₅₀ of the attenuated *Francisella* bacteria.

The disclosed immunogenic compositions can include other biologically
15 inactive or active agents (or both). For example, the disclosed immunogenic compositions can include adjuvants, carriers, excipients, anti-microbial agents (such as antibiotics), as well as pharmaceutically acceptable carriers (such as sterile water, saline, and preservatives).

For example, an immunogenic composition that includes the disclosed
20 attenuated *Francisella* bacteria can also include one or more adjuvants. Adjuvants are agents that can augment the resultant immune response. Particular examples of adjuvants include, but are not limited to: IFA, Freund's complete adjuvant, and oil-emulsions.

In another example, an immunogenic composition that includes the disclosed
25 attenuated *Francisella* bacteria can also include a pharmaceutically acceptable carrier. For example, a pharmaceutically acceptable carrier can be used to provide a medium in which to administer the composition into a subject. Exemplary pharmaceutical carriers include physiological saline, glycerol, and preservatives.

In some examples, an immunogenic composition that includes the disclosed
30 attenuated *Francisella* bacteria can include both a pharmaceutically acceptable carrier and an adjuvant.

The immunogenic compositions can be packaged in forms convenient for delivery. The compositions can be enclosed within a capsule, caplet, sachet, cachet, gelatin, paper, or other container. In particular examples, dosage units are packaged, in tablets, capsules, suppositories or cachets. In particular examples, the disclosed
5 immunogenic compositions are in a lyophilized form.

VI. *Methods of Stimulating an Immune Response*

Methods are provided for eliciting an immune response against *Francisella* in a subject. In particular examples, the method includes administering to the
10 subject a therapeutically effective amount of the attenuated *Francisella* bacteria disclosed herein (for example in the form of an immunogenic composition), thereby eliciting an immune response against *Francisella* in the subject. In particular examples, stimulating an immune response is used to treat tularemia in a subject previously infected with *Francisella tularensis* subsp. *tularensis* Type A or Type B.
15 In other particular examples, stimulating an immune response is used to prevent development of tularemia in a subject who may become infected or has been infected with *Francisella tularensis* subsp. *tularensis* Type A or Type B.

In particular examples, the subject is a mammal, such as a laboratory animal (for example a mouse, rat, non-human primate, or rabbit), or human subject.

20 Methods of administration are known in the art. Particular examples of administration that can be used to practice the disclosed methods include, but are not limited to: injection (such as intradermal or subcutaneous), intranasal, transdermal, or oral administration. If desired, multiple administrations can be performed over time (for example by the administration of booster doses). In one example, one,
25 two, or three additional administrations are performed, for example 1-6 months apart.

A "therapeutically effective amount" of the attenuated mutant *Francisella* bacteria is an amount effective to induce an immunogenic response in the recipient. In some examples, the immunogenic response is adequate to inhibit (including
30 prevent) or ameliorate signs or symptoms of disease, including adverse health effects or complications thereof, caused by infection with wild type *Francisella* bacteria. Either humoral immunity or cell-mediated immunity or both can be

induced by the attenuated mutant *Francisella* bacteria (for example in an immunogenic composition) disclosed herein.

The therapeutically effective amount can vary depending on the particular attenuated *Francisella* bacterium administered, the age, weight, or health of the subject, and other factors known to those skilled in the art. Ideally, the therapeutically effective amount produces a therapeutic immune response in the subject (for example by treating an existing *Francisella* infection or reducing the pathological consequences of a future *Francisella* infection), without significantly affecting the overall health of the subject.

In some examples, a therapeutically effective dose can be determined by also making reference to the LD₅₀ and ED₅₀ values for the attenuated bacterium. In one example, a therapeutically effective dose is 100-1000 fold less than the LD₅₀, and/or is at least the ED₅₀ dose.

In a specific example, the therapeutically effective amount includes at least 50 colony forming units (cfu) of the attenuated *Francisella* bacterium, such as at least 100 cfu, at least 200 cfu, at least 300 cfu, at least 500 cfu, at least 800 cfu, at least 1000 cfu, for example 100 cfu to 500 cfu, or 100 cfu to 1000 cfu, of the attenuated *Francisella* bacteria. In other particular examples, depending on the route of administration, suitable amounts of the mutant bacteria to be administered include about 10³ to 10¹¹ bacteria, such as 10⁶ to 10¹⁰, 10⁸ to 10¹⁰, or 10⁹ to 10¹⁰ attenuated *Francisella* bacteria.

Methods of determining whether an immune response has been generated can be determined using routine methods, such as indirect immunoassays or by direct clinical evaluation of the subject (for example by monitoring one or more signs of tularemia), for example as described above.

The following examples are provided to illustrate certain particular features and/or embodiments. These examples should not be construed to limit the disclosure to the particular features or embodiments described.

30

EXAMPLES

Example 1: Materials and Methods

This example describes the experimental procedures used for the studies described in Example 2.

5 *General screening procedures*

A comprehensive library was screened in several steps (FIG. 1). The library (32, 96-well plates) was used to inoculate tryptic soy broth supplemented with 0.1% cysteine (TSBC), grown overnight at 37°C with gentle shaking (200 rpm). J774 macrophage-like cells in 24-well plates (ca. 2.5×10^5 cells) were infected with 10 μ l of overnight culture and monitored for changes in cell morphology at 12 hours and at later timepoints essentially as described by Tempel *et al.* (*Infect Immun* 74:5095-105, 2006). Based on this preliminary screen, the mutants were categorized and those that either appeared to cause more or less cytopathology were examined further. In the second screen, individual mutant cultures were grown overnight and adjusted to give an approximate input multiplicity of infection (MOI) of either 50 or 500 bacteria per cell and examined at multiple time points. Those mutants that again showed phenotypic variation were tested for virulence in mice as described (Tempel *et al.*, *Infect Immun* 74:5095-105, 2006). To describe the phenotypes, mutants that showed either hyper- or hypocytopathogenicity, indicating they either killed cells more or less quickly (or in many cases not at all), respectively, than the parent strain following macrophage infection, were selected for further experiments. For mouse infection, the term hypervirulence (or attenuation) is used to mean that a mutant kills mice in a shorter (or longer) time, or at a lower (or higher) dose.

25 *Bacterial strains and growth conditions*

Francisella tularensis ssp. *novicida* type strain U112, the *F. novicida* transposon two-allele mutant library (Gallagher *et al.*, *Proc Natl Acad Sci U S A* 104:1009-1014, 2007), the restriction-deficient strain MFN245 (Gallagher *et al.*, *J Bacteriol* 190:7830-7837, 2008), and the deletion mutant strains were stored at -80°C in tryptic soy broth (Becton, Dickinson and Company, Sparks, MD) plus 0.1% cysteine (TSBC) plus 10% DMSO. *Francisella* strains were cultured at 37°C in TSBC or on cysteine heart agar (CHA, Difco/Becton, Dickinson and Company)

plates unless indicated below. Antibiotics used to select for *Francisella* transformants were kanamycin (20 µg/ml) and tetracycline (8 µg/ml). *E. coli* strains used to generate the allelic replacement and complementation plasmids were One Shot TOP10 Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA) and

5 TransforMax EC100D pir-116 Electrocompetent *E. coli* (Epicentre Biotechnologies, Madison, WI). *E. coli* transformants were grown in Luria-Bertani (LB) broth or agar containing kanamycin (60 µg/ml) or tetracycline (30 µg/ml).

Culture and infection of J774 murine macrophage-like cells and murine bone marrow-derived macrophages (BMDM)

10

The J774 murine macrophage-like cells (American Type Culture Collection, Manassas, VA) were cultured in Ham's F10 medium (Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), 1 mM nonessential amino acids (Gibco-BRL), and 0.2 mM sodium pyruvate (Gibco-BRL) at 37°C in

15 the presence of 5% CO₂. For infection, bacteria were added to 70% confluent cells in 6-, 24- or 96-well culture dishes (Corning, Corning, NY) at the MOI indicated below, and incubated at 37°C in the presence of 5% CO₂. Two hours after infection, the cells were washed twice with phosphate-buffered saline (PBS), and Ham's F10 medium containing 10 µg/ml of gentamicin was added to prevent the growth of

20 extracellular bacteria. For some experiments, J774 cells were treated with 1 µg/ml cytochalasin D (Sigma) to inhibit actin filament polymerization from 30 minutes prior to infection, and 2-hour invasion assays were performed.

BMDM were collected by flushing the femurs of BALB/c (TLR4+) mice with Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and cultured in

25 DMEM with 10% heat-inactivated FBS, 30% sterile filtered L-cell conditioned media, and penicillin/streptomycin (10,000 U/ml each) for six to seven days. The cells were split and infected as above for J774 macrophages.

Lactate dehydrogenase (LDH) release assay for cytopathogenicity

30

The LDH release assay was conducted as described (Tempel *et al.*, *Infect Immun* 74:5095-105, 2006). Briefly, J774 cells (about 4-5×10⁴/well) seeded in 96-well culture plates were infected in triplicate with either the transposon mutants or

wild-type *F. novicida* U112 at an input MOI of 50 or 500 and washed as described at 2 hours post infection (PI). At specified time points post infection, the supernatants were removed and assayed for release of LDH using the CytoTox 96™ nonradioactive cytotoxicity assay (Promega, Madison, WI). Cytotoxicity was
5 determined by calculating the amount of LDH released as a percentage of the maximal amount released from macrophages lysed with detergent.

Phase-contrast and fluorescent microscopy

J774 cells were infected as previously described (Tempel *et al.*, *Infect Immun*
10 74:5095-5105, 2006) at the indicated input MOI, in four-well chamber plates (Nalgene Nunc/Thermo Scientific, Rochester, NY). After two hours, the cells were washed twice with PBS, fixed for one hour with 4% paraformaldehyde at 4°C. After three washes for 10 minutes in phosphate-buffered saline (PBS), the cells were permeabilized with 0.5% Triton X-100 (Sigma) in PBS for 20 minutes at room
15 temperature, blocked with 5% FBS in PBS for 30 minutes, and incubated for one hour at 4°C with a polyclonal antibody against *F. tularensis* at a 1:2,000 dilution (Becton, Dickinson and Company). After three washes for 10 minutes in PBS, the cells were again blocked with 5% FBS. A goat anti-rabbit antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR) was applied to the cells at a 1:500
20 dilution for one hour at 4°C. The cells were again washed three times for 10 minutes in PBS and incubated with a 1:1,000 dilution of FM 4-64 membrane stain (Molecular Probes) and 1:1,000 dilution of DAPI DNA stain in PBS (Alexis Biochemicals, San Diego, CA) for 10 minutes at room temperature. The cells were washed twice with PBS and mounted in Fluormount-G antifade solution (Southern
25 Biotechnology, Birmingham, AL), and images were obtained with an Applied Precision DeltaVision deconvolution microscope system (Advanced Precision Instruments, Issaquah, WA). All images were taken with a 60× objective. Stacks of 10 z-plane images that were 1 µm apart were captured at 1024 × 1024 pixels and deconvolved for seven iterations. Selected images were saved in TIFF format and
30 imported into Adobe Photoshop to be formatted.

In vitro growth index assay

After OD₆₀₀ normalization of the overnight cultures, each culture was diluted in new culture medium to a starting OD₆₀₀ of 0.05. At 18 hours post inoculation, OD₆₀₀ was taken and an *in vitro* growth index was calculated as (mutant OD_{18h} – mutant OD_{0h})/(wt OD_{18h} – wt OD_{0h}) to determine if the mutant shows an obvious growth difference than the wild-type during *in vitro* growth (Su *et al.*, *Infect Immun* 75:3089-101, 2007). A value of 30% (*i.e.*, 0.3) more or less than one (arbitrary value for wild-type) is considered significantly different than the wild-type (Su *et al.*, *Infect Immun* 75:3089-101, 2007).

Mouse studies

Six- to 8-week old female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and acclimatized for one week. The animals were fed autoclaved food and water *ad libitum*. All experiments were performed in accordance with Animal Care and Use Committee guidelines. Mutants were cultured, OD normalized, and diluted with PBS. Mice were inoculated intraperitoneally (*i.p.*) with bacteria in 150 µl (total volume) of PBS and checked for signs of illness or death twice each day following infection for a total of 28 days. The 50% lethal doses (LD₅₀) were calculated by the method of Reed and Muench (Reed and Muench, *Am J Hyg* 27:493-497, 1935).

Construction and complementation of deletions in F. novicida

All deletions were generated in *F. novicida* mutant strain MFN245, a quadruple mutant that substantially reduces the restriction barrier and thereby increases the efficiency of transformation (Gallagher *et al.*, *J Bacteriol* 190:7830-7837, 2008). The cytotoxicity and virulence of this modified host strain in mice is comparable to U112. Plasmid pKD13 (Datsenko and Wanner, *Proc Natl Acad Sci USA* 97:6640-6645, 2000) was modified such that expression of kanamycin was more efficient in *Francisella* and segments of *Francisella* DNA would be easier to clone into the vector. First, the *groE* promoter was amplified from *F. novicida* genomic DNA by PCR using oligonucleotides CGCGGATCCGTATGGATTAGTCGAGC (SEQ ID NO: 1) and

CGCGGATCCTGCACGACGAACATACTC (SEQ ID NO: 2). The oligonucleotides also contained a recognition site for *Bam*HI at the 5' end. The DNA fragment containing the *groE* promoter was digested with *Bam*HI and ligated into the *Bgl*III site directly upstream of the *kan* gene in pKD13. A pKD13 plasmid with the *groE* promoter in the correct orientation and free of PCR errors was identified by sequence analysis.

Second, complementary pairs of oligonucleotides were annealed to generate DNA fragments containing *Apa*I and *Sbf*I restriction sites (TCGAGGGCCCCGCACCTGCAGGGC (SEQ ID NO: 3) and TCGACCTGCAGGTGCGGGCCC (SEQ ID NO: 4)) and *Asc*I and *Sma*I restriction sites (CTAGAGGCGCGCCGCCCGGG (SEQ ID NO: 5) and CTAGCCCGGGGCGGCGCGCCT (SEQ ID NO: 6)). After annealing, the complementary oligonucleotides had single-strand 5' overhangs that allowed the fragments to be cloned into pKD13 digested with *Sal*I and *Avr*II, respectively. In the final modified pKD13 plasmid, both the *Apa*I and *Sma*I restriction sites were most distal to the FRT sites.

For each gene to be deleted, two sets of oligonucleotide pairs were designed (Table 1). The first pair (labeled Up F and Up R) amplified the first 50-70 bp of the open reading frame (ORF) plus about 500bp upstream and includes recognition sites for *Apa*I and *Sbf*I. The second pair (labeled Down F and Down R) amplified the last 50-70 bp of the ORF plus about 500bp downstream and includes recognition sites for *Asc*I and *Sma*I.

Table 1: Oligonucleotides used in this study

Primer	Sequence	SEQ ID NO:
FTN1253 up F	GATCGGGCCCCCATCGTATAGCTTG CCAAT	7
FTN1253 up R	GATCCCTGCAGGTACCAGAAAATC TACGTCCTAGTGAT	8
FTN1253 down F	GATCGGCGCGCCTGAAGCTGAAGG GATTCAACAA	9
FTN1253 down R	GATCCCCGGGTGCTCCTACTTATGA TTGGCATC	10
FTN1417 up F	GATCGGGCCCTATTTACGCTCGCAT GATCG	11
FTN1417 up R	GATCCCTGCAGGTACCAAACCTTAC GCCGCTAGA	12

Primer	Sequence	SEQ ID NO:
FTN1417 down F	GATCGGCGCGCCGGCTAGTGATGA GCAGGCAAA	13
FTN1417 down R	GATCCCCGGGCTTTGGGTGCTGCGT AAGAT	14
FTN1418 up F	GATCGGGCCCAACAGAAAATGC TCCACA	15
FTN1418 up R	GACTCCTGCAGGATAGTGGCCATA GCCTTGAGC	16
FTN1418 down F	GATCGGGCGCGCCGCAAGTGGGAG AATATATAAGTGA	17
FTN1418 down R	GATCCCCGGGTGCTTGCTTACTAGG CTCTGG	18
<i>kdtA</i> up F	GATCGGGCCCCCTCAAACCTGATTTT AATGTTCTTGACGC	19
<i>kdtA</i> up R	GATCCCTGCAGGGCGAATCTCTCA GCCCATCTTTTCTG	20
<i>kdtA</i> down F	GATCGGCGCGCCTTAAAAAGCCAT AGTGATGTACTCGAAAAACAG	21
<i>kdtA</i> down R	GATCCCCGGGCCTCAATATCTAGTT GTTGACCACCAACC	22
<i>wbtA</i> up F	GATCGGGCCCAACACCTTAGCACT GGTGATGAAGAAGTAAC	23
<i>wbtA</i> up R	GATCCCTGCAGGACTATTATTACCA CGAAATTAAGCGTTCTATTATCG	24
<i>wbtA</i> down F	GATCGGCGCGCCCAGCTTGTGATAT TAAAGAAAATTGTTCCG	25
<i>wbtA</i> down R	GATCCCCGGGCTTGTAGAACTAC CTAAACTTTCAGCAGCATC	26
FTN1253 complete F	GATCGCGGCCGCTTTACCATCGTAT AGCTTGCCAATAGTCG	27
FTN1253 complete R	GATCGGGCCCTGATAATAATGAAA ATCTTGTCACTAAAGTCACCC	28
FTN1417/18 complete F	GATCGCGGCCGCATGAATATAAAC CAGAAAATGCTCCACATTC	29
FTN1417/18 complete R	GATCGGGCCCCGAAAATGAAAGGC TCACTAACTAATGAAGAGTTC	30
FTN1418 complete F	GATCGCGGCCGCTGTAACTAATG GATGAATATAAACCAGAAAATGC	31
FTN1418 complete R	GATCGGGCCCCGCCGAAACAAGAC CTCTAACTCCACTG	32
<i>kdtA</i> complete F	GATCGCGGCCGCTCCTGACGCTGAT GAAATTG	33
<i>kdtA</i> complete R	GATCGGGCCCCGCCGCTAAGATTG CAGTAG	34

For one-step allelic replacement, pKD13 containing the *Francisella* fragments was digested with *Apa*I and *Sma*I and transformed into MFN245 as

described previously (Ludu *et al.*, *FEMS Microbiol Lett* 278:86-93, 2008).

Kanamycin resistant transformants were streaked for single colonies and correct integration of only the linear fragment was verified by PCR. Although the gene deletions marked with kanamycin were in-frame, the kanamycin resistance gene was

5 removed by transforming the temperature-sensitive plasmid pFFLP (Gallagher *et al.*, *J Bacteriol* 190:7830-7837, 2008), which expresses the flippase recombination enzyme, into the kanamycin resistant colonies by electroporation as described in Maier *et al.* (*Appl Environ Microbiol* 70:7511-7519, 2004). Briefly, a 10 ml culture in Mueller-Hinton (MH) broth containing 0.1% glucose, 0.025% ferric
10 pyrophosphate, and 2% IsoVitaleXTM (Becton Dickinson) was inoculated to an OD of approximately 0.15 using overnight cultures of each of the deletion mutants. After approximately four hours of growth the cultures (OD between 0.3 to 0.5) were washed twice with 10-15 ml of 0.5 M sucrose (Fisher Scientific) and suspended in 200 μ l of 0.5 M sucrose. One microliter pFFLP (500 ng/ μ l) was mixed with 200 μ l
15 of cells, incubated at room temperature for 10 minutes, transferred to a 0.2-mm cuvette and electroporated using a GenePulser (BioRad) at 2.5 kV, 25 μ F, and 600 Ω . Following electroporation, cells were suspended in 1 ml of MH broth and incubated at 30°C for two hours before plating on CHA plates containing 8 μ g/ml tetracycline. As detailed in Gallagher *et al.* (*J Bacteriol* 190:7830-7837, 2008), tetracycline
20 resistant transformants were streaked for single colonies on tetracycline and grown at 30°C. Single colonies were transferred to CHA, grown at 37°C and tested for kanamycin sensitivity. Kanamycin sensitive colonies were streaked again for single colonies on CHA, grown at 37°C and tested for tetracycline sensitivity. The tetracycline sensitive colonies, which indicate loss of the pFFLP plasmid, were used
25 in the experimental analyses. The resolved deletion was confirmed by PCR and sequencing.

The gene deletions in $\Delta lpcC$, $\Delta manB$, $\Delta manC$ and $\Delta kdtA$ strains were complemented *in trans* by transforming into the mutant strains plasmids that express the wild-type gene. The promoter and ORF of *lpcC* (FTN1253), *manC* (FTN1418)
30 and *kdtA* (FTN1469) were amplified by PCR from *F. novicida* genomic DNA using oligonucleotides complementary to about 500 bp upstream of the start codon and 100-300 bp downstream of the stop codon. *manB* (FTN1417) lies downstream of

manC in an operon; in order to express *manB* from its endogenous promoter, oligonucleotides were designed that amplified 503 bp upstream of *manC* and 403 bp downstream of *manB*. The resulting FTN1417/18 PCR fragment contains the entire operon. The oligonucleotides used to amplify the wild-type copies of *lpcC*, *manB*,
 5 *manC* and *kdtA* are shown in Table 1 and contain recognition sites for *NotI* and *ApaI*. DNA fragments digested with *NotI* and *ApaI* were cloned into similar sites into a modified pKK202 that contains unique restriction sites for *NotI*, *XhoI* and *SfiI* (Tempel *et al.*, *Infect Immun* 74:5095-5105, 2006).

LPS gel analysis

10 The LPS from *F. novicida* wild-type and mutant strains was isolated from whole cells after growth in TSBC for 24 hours. Bacteria (1 ml) were pelleted, resuspended in 100 µl lysis buffer (187 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 4% 2-mercaptoethanol, 0.03% bromophenol blue), heated to 100°C for 10 minutes, and cooled to room temperature. Subsequently, 25 µg proteinase K was
 15 added to each sample and incubated at 60°C for one hour. Finally, samples were incubated at 100°C for 10 minutes, cooled briefly on ice and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using Bio-Rad Ready Gel precast 10-20% gradient Tris-Tricine/Peptide polyacrylamide gels (Hercules, CA). After electrophoresis, the LPS was stained with Pro-Q Emerald 300
 20 LPS stain kits (Invitrogen, Carlsbad, CA), according to the manufacturer's recommendation, visualized, and photographed using the AlphaImagerTM digital imaging system (Alpha Innotech Corp., San Leandro, CA).

Statistics

Statistical significance of data was determined by using an unpaired analysis
 25 of variance and the Tukey-Kramer multiple-comparisons test (GraphPad Prism 4, San Diego, CA).

Example 2: Identification of hypercytopathogenic *Francisella* mutants

This example describes a comprehensive screen of a *Francisella* transposon
 30 library to identify *Francisella* virulence factors. Thirteen mutants were identified that exhibit a hypercytopathogenic phenotype in macrophages, but are attenuated *in vivo*.

One class of mutants kills J774 cells faster than the parental strain U112

Following infection, cells were examined for morphological changes at several time points in comparison to mock infected and parental U112 infected cells.

- 5 Some mutants killed J774 cells faster than their parental strain U112. Under the current experimental settings, U112 caused obvious cell damage at 24 hours, whereas a class of mutants (scattered among the 32 plates) killed J774 cells at 12 hours post infection or even earlier and with a lower input MOI. Those 29 mutants (listed in Table 2) were re-examined for their cytotoxic phenotype after carefully
- 10 normalizing the input number of bacteria for each well. This screen was carried out to identify mutations that showed the strongest phenotype (*i.e.* cytotoxicity). Only one of the four mutants (FTN0757 (FTT0584)) that have been previously identified met this criteria (Weiss *et al.*, *Proc Natl Acad Sci USA* 104:6037-42, 2007).

15 **Table 2: List of novel hypercytopathogenic mutants in the 2-allele library**

FTN #	Name	LDH% (12h) ^a	Mice survival ^b	IVGI ^c	Polar ^d
0% survival group					
FTN0946	<i>pilF</i>	73.4	0/3	1	yes
FTN1138	<i>pilP</i>	68.7	0/3	0.9	yes
FTN1139	<i>pilO</i>	75.2	0/3	0.98	yes
FTN1140	<i>pilN</i>	80.3	0/3	0.94	yes
Partial survival group					
FTN0070	<i>pilE</i>	95.6	1/3	0.86	yes
FTN0415	<i>pilA</i>	89.9	1/3	0.94	no
FTN0429	unknown	64.9	1/3	1.01	yes
FTN0642	<i>cydD</i>	80.5	1/3	0.9	yes
FTN0672	<i>secA</i>	100	2/3	0.84	no
FTN0756	<i>fopA</i>	78.3	2/3	0.89	no
FTN1000	<i>pilD</i>	14.3	1/3	1.07	yes
FTN1115	<i>pilB</i>	71.2	1/3	0.9	yes
FTN1116	<i>pilC</i>	71.1	2/3	0.9	no
FTN1137	<i>pilQ</i>	78.2	2/3	0.95	no
FTN1141	<i>pilM</i>	73.9	2/3	1	yes
FTN1681	<i>fur</i>	19.4	1/3	0.95	no
U112	wild type	7.7	1/3	1	n/a

FTN #	Name	LDH% (12h) ^a	Mice survival ^b	IVGI ^c	Polar ^d
100% survival group					
FTN0071	<i>htrB</i>	100	3/3	0.87	yes
FTN0355	<i>tolB</i>	58.9	3/3	0.78	yes
FTN0408	unknown	16.6	3/3	0.89	yes
FTN0528	<i>lpxH</i>	100	3/3	0.97	no
FTN0558	<i>ostA1</i>	100	3/3	1	yes
FTN0664	<i>fimT</i>	66.6	3/3	1.32	yes
FTN0757	unknown	25	3/3	0.87	yes
FTN1253	<i>lpcC</i>	76.2	3/3	0.93	no
FTN1254	unknown	35.6	3/3	0.76	yes
FTN1417	<i>manB</i>	87.7	3/3	0.9	no
FTN1418	<i>manC</i>	71.9	3/3	0.86	yes
FTN1420	<i>wzx</i>	ND	3/3	0.91	no
FTN1661	<i>nusA</i>	26.5	3/3	0.92	yes

^a J774 cells were infected and LDH release assay was performed as described in Example 1 and 50 µl of supernatant was taken at 12 hours PI and used for LDH assay; values are representative results from one of two similar experiments with standard deviations less than 10%; ND, not done.

5 ^b Mice were infected with one LD₅₀ dose and survival was monitored for 28 days.

^c *In vitro* growth index (IVGI) was calculated at 18 hours as described in Example 1; values are representative results from one of two similar experiments.

10 ^d Whether Tn insertion of a gene has possible polar effect is predicted as described in Example 1; n/a = not applicable.

FIG. 1 is an example of direct visualization of cytopathology caused by *F. novicida* infection. At 10 hours PI, cells infected with an outer membrane porin mutant (*fopA*; FTN 0756) showed obvious morphological changes (FIG. 1C; rounded up and detached, indication of cell death). In contrast, most of the U112-infected cells (FIG. 1B) were almost the same in morphology as the uninfected (mock) control cells at this time (FIG. 1A). At 24 hours PI, U112 killed most of the J774 cells (FIG. 1E) as previously observed (Tempel *et al.*, *Infect Immun* 74:5095-105, 2006), while cells infected with *fopA* at 1/10 of this MOI exhibited a similar degree of cell death (FIG. 1F). The rest of the hypertoxic mutants, like the *fopA*

mutant, killed J774 cells faster and with a lower MOI than wild-type strain U112 did.

- The treatment of J774 cells with cytochalasin D completely blocked all but four of the hypertoxic mutants for the induction of cell death. Infection with
- 5 mutants of FTN1253, FTN1254, FTN1417 or FTN1418 still killed J774 cells in the presence of cytochalasin D.

Mutants resulting in a decrease in cytopathology in J774 cells

- Several groups have identified *F. tularensis* mutants that are less
- 10 cytopathogenic to cultured cells and less virulent in mice by screening a library for mutants that are attenuated for intramacrophage growth/survival (Maier *et al.*, *Infect Immun* 75:5376-5389, 2007; Qin and Mann, *BMC Microbiol* 6:69, 2006; Su *et al.*, *Infect Immun* 75:3089-3101, 2007; Tempel *et al.*, *Infect Immun* 74:5095-5105, 2006; Weiss *et al.*, *Proc Natl Acad Sci USA* 104:6037-6042, 2007). Screening of the two-
- 15 allele mutant library identified 17 genes in which mutations confer greatly reduced cytopathology that have not been previously reported (see Table 3).

Table 3: List of novel hypocytopathogenic mutants in the 2-allele library

FTN #	Name	LDH% (24h) ^a	Mice survival ^b	IVGI ^c	Polar ^d
0% survival group					
FTN0036	<i>pyrD</i>	4.5	0/3	0.68	no
FTN1586	unknown	8.6	0/3	1.07	yes
FTN1630	<i>secG</i>	17.5	0/3	0.98	no
FTN1682	<i>frgA</i>	18.5	0/3	0.96	yes
U112	wild type	72.8	0/3	1	n/a
Partial survival group					
FTN0019	<i>pyrB</i>	5.8	2/3	0.69	no
FTN0021	<i>carA</i>	11.8	2/3	0.97	yes
FTN0024	<i>pyrC</i>	7.5	1/3	0.63	no
FTN0035	<i>pyrF</i>	11.5	1/3	0.88	yes
FTN0107	<i>lepA</i>	10.7	2/3	0.8	yes
FTN1063	unknown	15.5	1/3	0.95	yes

FTN #	Name	LDH % (24h) ^a	Mice survival ^b	IVGI ^c	Polar ^d
100% survival group					
FTN0108	<i>trmU</i>	3.1	3/3	0.65	no
FTN0124	<i>ssb</i>	0.2	3/3	0.87	no
FTN0422	<i>purE</i>	9.5	3/3	0.55	yes
FTN0897	<i>guaA</i>	5.1	3/3	0.34	yes
FTN1185	<i>rpiA</i>	11.3	3/3	0.59	no
FTN1286	<i>mltA</i>	15.6	4/4	1.35	yes
FTN1640	<i>gltA</i>	7.6	3/3	0.5	yes

^a J774 cells were infected and LDH release assay was performed as described in Example 1 and 50 µl of supernatant was taken at 24 hours PI and used for LDH assay; values are representative results from one of two similar experiments with standard deviations less than 10%.

5 ^b Mice were infected with a 100 LD₅₀ dose and survival was monitored for 28 days.

^c *In vitro* growth index (IVGI) was calculated at 18 hours as described in Example 1; values are representative results from one of two similar experiments.

10 ^d Whether Tn insertion of a gene has possible polar effect is predicted as described in Example 1; n/a = not applicable.

Quantitative measurement of cytopathogenicity by LDH release assay

LDH is a cytosolic enzyme of eukaryotic cells that upon cell membrane permeabilization is released to the media and is thus used as an indicator of cell damage. The LDH release assay has been used for many years to quantify cell lysis by *F. tularensis* following infection of cultured cells (Bonquist *et al.*, *Infect Immun* 76(8):3502-3510, 2008; Lai *et al.*, *Infect Immun* 69:4691-4694, 2001; Maier *et al.*, *Infect Immun* 75:5376-5389, 2007; Tempel *et al.*, *Infect Immun* 74:5095-5105, 2006; Weiss *et al.*, *Proc Natl Acad Sci USA* 104:6037-4602, 2007). To quantitate cell lysis, J774 cells were seeded in 96-well plates and infected with each of the mutant strains at the same input MOI (50:1 and 500:1). Cell culture supernatants were taken at specified time points and assayed for LDH release.

In comparison with the wild-type strain U112, the class of hypercytopathogenic mutants (Table 2) killed J774 cells faster and released more

LDH at an early time point (12 hours) whereas the class of hypocytotoxic mutants (Table 2) kill J774 cells slower or not at all with much less LDH release even at later time point (24 hours).

Some mutants have a growth defect in vitro

5 The intracellular growth conditions are likely to be completely different than conditions encountered by the bacteria in the usual laboratory culture media. Nevertheless, mutations with a growth defect in culture may also have a decrease in intracellular growth rate and therefore result in less cytopathology following infection. An *in vitro* growth index for each mutant was calculated as described in

10 Example 1. Based on sampling all the less grown mutants observed in the first round screen, 45 (about 3%) of the complete library (1484 genes) has a longer division time than the parent. However, among the 17 newly identified hypocytotoxic mutants, 8 grew significantly more slowly than the parent in culture (Table 2), suggesting that a general growth defect may account for their phenotype.

15 *Possibility of polar effect*

 The possibility of polar effects on downstream gene expression within operons is a concern for most insertional mutagenesis techniques. Some of the genes inactivated are located within operons, suggesting that they could be polar on downstream gene expression and that the phenotype observed probably results not

20 from the inactivation of the mutated gene but rather of a gene downstream in the same operon. Possible polar effects are indicated in Tables 2 and 3. This may explain why the mutation in *manC* (FTN1418) is hypercytotoxic because it is located upstream of *manB*. A *manB* mutation has this same phenotype when inactivated. Likewise, the phenotype for an insertion in *tolB* (FTN0355) may be a

25 consequence of a polar effect on FTN0356 (unknown function) because this mutation is not in the library. However, in many cases the phenotype is associated with insertion in a single gene within an operon and all other genes, either upstream or downstream, do not have the same cytotoxic phenotype. Of the mutations that are hypercytotoxic, avirulent, and located in an operon, the phenotype can be positively

30 associated with the following genes: FTN0071 (a fatty acid acyltransferase), FTN0408 (mannose-6-phosphate isomerase), FTN0558 (*ostA*), FTN0664 (*fimT*), FTN0757 (unknown membrane protein), FTN1254 (a type 6 glycosyl transferase),

and FTN1661 (*nusA*). Thus, it appears that polar effects can be ruled out for all but two of these mutations.

Virulence studies of hypercytotoxic mutants

Virulence test in a suitable animal model is the gold standard to determine if a bacterial mutant is attenuated. For each of the identified hypercytotoxic mutants (see Table 2), 60 bacteria were administered i.p., which is equal to the LD₅₀ of the parental strain. Indeed, mutations in 16 genes did not significantly affect the LD₅₀ from that observed for the parental strain, with the possible exception of a mutation in *pilP* (FTN1138). This strain killed all 3 mice within 2 days - faster than observed for the parent and all of the strains tested. Of particular note was the result that 13 of 29 mutants (*tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, FTN0408, FTN0757 and FTN1254) did not kill mice that were administered with a dose equal to one wild-type LD₅₀ even after 28 days (see Table 2, %100 survival group).

It was anticipated that many of the 17 hypocytotoxic mutants would be attenuated and therefore animals were administered $100 \times \text{LD}_{50}$ i.p. As shown in Table 3, many of these mutants were attenuated although there was considerable variation. Seven mutants were attenuated at this dosage suggesting that their LD₅₀ is higher than 6×10^3 . Six mutants resulted in some surviving mice when administered at $100 \times \text{LD}_{50}$ of the parent dose suggesting that this dose is close to LD₅₀. There was no survival in mice that were infected with 4 of the 17 mutants (*pyrD*, *secG*, *frgA*, and FTN1586.).

The LD₅₀ of 12 mutant strains showing attenuation in the initial screen was determined by infecting mice with increasing doses of each mutant strain. All 12 of the mutants had a LD₅₀ that was at least one order of magnitude higher than the parental strain U112 (Table 4). Mutants identified to be hypercytotoxic *in vitro* were more likely to be avirulent (12 of 28 strains, 43%) as compared to transposon insertion mutations screened at random for virulence in which 4-6% of insertion mutations were avirulent (Su *et al.*, *Infect Immun* 75:3089-3101, 2007).

Table 4: Estimated LD₅₀ of the attenuated mutants^a

FTN#	Gene	Estimated LD ₅₀	Corresponding FTT#
U112 (wt)		$\leq 6 \times 10^1$	NA
FTN0071	<i>htrB</i>	6.25×10^7	FTT0231c
FTN0355	<i>tolB</i>	6.5×10^3	FTT0840
FTN0408	unknown	6.5×10^3	(FTT0882)
FTN0528	<i>lpxH</i>	6.0×10^2	FTT0436c
FTN0558	<i>ostA1</i>	6.25×10^6	FTT0467
FTN0664	<i>fimT</i>	6.5×10^2	FTT1314c
FTN0757	unknown	7.5×10^4	(FTT0584)
FTN1253	<i>lpcC</i>	6.75×10^3	FTT1235c
FTN1254	unknown	6.25×10^2	FTT1236
FTN1417	<i>manB</i>	6.75×10^3	FTT1447c
FTN1418	<i>manC</i>	6.75×10^2	FTT1448c
FTN1661	<i>nusA</i>	6.25×10^6	FTT0049

^a3-5 mice were used for each dilution and survival was monitored for 28 days.

Vaccination and challenge studies

To test the capability of each of the 13 attenuated mutants to confer protection to naïve mice, vaccination and challenge studies were performed essentially as described previously (Tempel *et al.*, *Infect Immun* 74(9):5095-5105, 2006). Six- to 8-week old female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals were fed autoclaved food and water *ad libitum*. Mice were inoculated intraperitoneally with approximately 6×10^3 cfu of bacteria in 150 μ l (total volume) of PBS. Surviving mice were challenged 28 days later by intraperitoneal inoculation of 6×10^3 cfu wild-type U112. The results are shown in Table 5 below. Protection (%) is calculated as the number of mice that survived the vaccination divided by the number of mice that survived challenge with wild-type *Francisella*. The 50% lethal doses (LD₅₀) were calculated by the method of Reed and Muench (*The American Journal of Hygiene* 27:493-497, 1938). Mice were checked for signs of illness or death twice each day following infection.

Table 5: Vaccination with attenuated *Francisella* mutants

FTN#	Gene	% Survival	% Protection	FTT#
FTN0071	<i>htrB</i>	100	67	FTT0231c
FTN0355	<i>tolB</i>	100	100	FTT0840
FTN0408	hypothetical	100	100	(FTT0882)
FTN0528	<i>lpxH</i>	0	NA	FTT0436c
FTN0558	<i>ostA1</i>	100	0	FTT0467
FTN0664	<i>fimT</i>	0	NA	FTT1314c
FTN0757	hypothetical	100	100	(FTT0584)
FTN1253	<i>lpcC</i>	100	100	FTT1235c
FTN1254	hypothetical	33	100	FTT1236
FTN1417	<i>manB</i>	100	100	FTT1447c
FTN1418	<i>manC</i>	67	100	FTT1448c
FTN1420	<i>wzx</i>	100	100	No FTT
FTN1661	<i>nusA</i>	100	67	FTT0049

NA = not applicable

Candidate genes

- The studies described above identified 13 genes for which genetic
- 5 inactivation resulted in a hypercytotoxic phenotype in macrophages and attenuation in mice. Based on these findings, the 13 genes are candidate genes for the development of live *Francisella* vaccines. Table 6 lists the name and function (if known) of each candidate gene.

10

Table 6: Candidate genes for live *Francisella* vaccines

Gene	Function
<i>tolB</i>	Group A colicin translocase
manC	Mannose-1-phosphate guanylttransferase
manB	Phosphomannomutase
lpcC	Glycosyl transferase group 1 family protein
htrB	LPS fatty acid acyltransferase
FTN1254 (FTT1236)	Hypothetical protein
<i>fimT</i>	Type IV pili fiber building block protein
<i>ostA1</i>	Organic solvent tolerance protein
<i>nusA</i>	Transcription elongation factor
lpxH	UDP,-2,3- diacylglucosamine hydrolase
FTN0757 (FTT0584)	Membrane protein of unknown function

Gene	Function
FTN0408	mannose-6-phosphate isomerase
wzx	O-antigen flippase

Several of the candidate genes (show in bold in Table 6) are involved in polysaccharide synthesis, specifically mannose metabolism. For example, *manC* encodes a mannose-1-phosphate guanyltransferase and *manB* encodes a phosphomannomutase. These proteins are likely to be required for the LPS modifications that prevent *Francisella* lipid A from stimulating TLR4. In addition, *lpxH* encodes a UDP-2,3-diacylglucosamine hydrolase, which plays a role in lipid A biosynthesis, *lpcC* encodes a glycosyl transferase, *htrB* encodes an LPS fatty acid acyltransferase and *wzx* encodes an O-antigen flippase. It is known that structural modification of the LPS in *Francisella* prevents it from being recognized by toll-like receptors present on macrophages, preventing initiation of an innate immune response. Thus, mutations in genes that modulate LPS modification may result in a stronger inflammatory response, which results in clearing the bacteria before disease symptoms occur.

15

Role of cell contact in cytotoxicity

Francisella is less inflammatory than many pathogens in part because its LPS is not recognized by TLR4. An alternative scenario is that *Francisella* secretes proteins that function to inhibit cell death. For example, as hypothesized in Hager *et al.* (*Mol Microbiol* 62:227-237, 2006), secreted proteases could remove cell surface proteins (*e.g.* Fas and TNF α R) that are involved in signaling the cell to undergo apoptosis. Therefore, to determine if cell contact between the bacteria and macrophages is necessary for cell death, a transwell (Nunc 137044) was divided with a membrane containing 0.22 μ m pores (too small for bacterial passage); on one side macrophages were grown, and on the other side bacteria were inoculated. An increase in cell death was not observed when macrophages were separated from the wild-type U112 or mutant bacteria, indicating that contact with bacteria was necessary for cell death.

25

Next, in order to determine whether internalization of the mutant *Francisella* strain is required for macrophage cell death, actin polymerization was inhibited by adding cytochalasin D (cytD) before and during infection with the 28 transposon mutants and U112 in J774 macrophages. CytD has been previously shown to be an inhibitor of phagocytosis of *Francisella* (Clemens *et al.*, *Infect Immun* 73:5892-5902, 2005; Lai *et al.*, *Infect Immun* 69:4691-4694, 2001; Lindemann *et al.*, *Infect Immun* 75:3178-3182, 2007; Gavrilin *et al.*, *Proc Natl Acad Sci USA* 103:141-146, 2006). Three of the 28 transposon mutant strains (*lpcC*, *manB* and *manC*) were highly cytotoxic when infection took place in the presence of cytD (FIG. 4A). For the remaining mutants, the cell toxicity was greatly reduced following treatment with cytD (FIG. 4A). In order to observe differences in U112-infected macrophages, LDH release was quantified after 24 hours. As shown in FIG. 4B, cytD significantly reduced the cell toxicity of the parent strain 24 hours p.i.

Internalization of three mutant strains occurs independently of actin polymerization

The three transposon mutant strains that remained hypercytotoxic in the presence of cytD contained transposon insertions in genes necessary for biosynthesis of LPS core (*lpcC*, *manB*, and *manC*). Transposon insertions can have polar effects on downstream genes, and *manB* and *manC* are within an operon; therefore, deletions of *lpcC*, *manB*, and *manC* were constructed in the U112-based restriction-deficient strain MFN245 (see Example 1). In this background, it was confirmed that individual deletions of these genes still result in a hypercytotoxic phenotype that is independent of actin polymerization. LDH release from J774 macrophages infected with the deletions of FTN1253 ($\Delta lpcC$), FTN1417 ($\Delta manB$), and FTN1418 ($\Delta manC$) with a multiplicity of infection (MOI) of 100 or 1000 was significantly ($p < 0.01$) higher than macrophages infected with the same MOI of the parent strain and similar to the LDH release observed after infection with the transposon mutants (see below). LDH release from macrophages infected with MFN245 was comparable to LDH release from macrophages infected with wild-type U112. Furthermore, the presence of cytD did not affect LDH release in J774 macrophages infected with $\Delta lpcC$, $\Delta manB$, and $\Delta manC$. To confirm that the hypercytotoxicity was not specific to a macrophage cell line, the experiment was repeated in bone marrow derived

macrophages prepared from BALB/c mice. As shown in FIG. 5, $\Delta lpcC$, $\Delta manB$, and $\Delta manC$ were more toxic to primary macrophages than parent strain MFN245.

Because the hypercytotoxicity of the *lpcC*, *manB*, and *manC* mutant strains is not dependent upon actin polymerization, it was next determined if the bacteria were being internalized by the macrophages. Bacteria were visualized in macrophages infected with either one of the mutant strains or MFN245 using a fluorescently conjugated anti-*Francisella* antibody. It was observed that approximately one hundred times more $\Delta lpcC$, $\Delta manB$, and $\Delta manC$ bacteria were in macrophages two hours after infection as compared to the parent strain (FIG. 6A). In the presence of cytD, very few MFN245 bacteria were visualized internally. In contrast, a similar number of mutant bacteria were visualized inside macrophages in the presence of cytD as were visualized in the absence of cytD (FIG. 6B). Z sections prepared using the API Deltavision deconvolution microscope confirmed that the bacteria were inside the cell and not simply associated with the cell externally (FIG. 6). Increasing the concentration of cytD to 50 μ m did not inhibit the entry or cytotoxicity of the $\Delta lpcC$, $\Delta manB$ and $\Delta manC$ bacteria. The localization of wild-type U112 bacteria in infected macrophages was indistinguishable from MFN245 under similar conditions.

As a way to quantitate the above results, the number of intracellular bacteria in J774 macrophages was determined after infection with $\Delta lpcC$, $\Delta manB$, $\Delta manC$ and MFN245 for two hours in the presence or absence of cytD. After two hours, the macrophages were washed and treated with gentamicin to remove extracellular bacteria and then lysed with saponin to determine the number of intracellular bacteria. As shown in FIG. 7, the addition of cytD significantly lowered the uptake of the parent ($p < 0.01$) but not the number of internal mutant bacteria (FIG. 7). These data combined with the microscopy above suggest that some bacteria may be killed upon phagocytosis. Furthermore, at 8 hours p.i., the number of intracellular MFN245 bacteria was greater than the number of $\Delta manB$ and $\Delta manC$ suggesting that either the mutant strains grow more slowly than the wild-type strain or they are being killed inside macrophages.

Increased cell uptake alone does not result in hypercytotoxicity

It was reasoned that the hypercytotoxic phenotype of $\Delta lpcC$, $\Delta manB$ and $\Delta manC$ might be solely due to the increased number of bacteria internalized by the macrophages. To test this hypothesis, the MOI was varied for the wild-type parent and mutant strains and assayed for LDH release. Increasing the MOI of MFN245 did not increase the LDH released from infected J774 macrophages 10 hours p.i. (FIG. 8A). LDH released from macrophages infected with the mutants increased with increasing MOI (FIG. 8A) and was always higher than the LDH released following MFN245 infection at the same MOI. The increased LDH release in the mutants was abolished when the wild-type gene was expressed *in trans* indicating that the phenotype is specific to the deleted gene. The number of parent bacteria internalized at an input MOI of 10,000 was confirmed visually to be comparable to the mutant strains infected at a MOI of 100 (FIG. 8B vs. FIG. 6) and internalization of MFN245 at 10,000 MOI remained dependent upon actin polymerization (FIG. 8B). These data indicate that the parental strain is remarkably non-toxic to cells even if there are very high numbers of intracellular bacteria.

A bacterial surface structure is sufficient for increased macrophage invasions

In the case of *Salmonella* and many other intracellular pathogens, cell invasion requires synthesis of specific proteins. To determine if the exposed LPS core polysaccharide or some other exposed structure on the surface of the bacteria was sufficient to promote uptake, it was determined whether bacteria treated with 4% formaldehyde, a cross-linking reagent, were internalized by J774 macrophages. Internalization of the mutant strains was compared to the parent strain MFN245 at the same input MOI by direct microscopic observations using polyclonal anti-*Francisella* antibodies to visualize the bacteria. As shown in FIG. 9A, the number of internalized *lpcC* mutant bacteria is at least 10-fold higher than the parent at all concentrations of fixed bacteria. Similar results were observed for $\Delta manB$ and $\Delta manC$ strains. These results indicate that the mutant bacteria possess a structure that promotes their uptake by macrophages, which is not exposed on the parent strain. Even though the dead parent and mutant strains were internalized by the macrophages, LDH release was low for all macrophages (FIG. 9B). These results

demonstrate that viable *Francisella* bacteria are necessary to promote macrophage cell death.

Internalization of live Francisella bacteria is essential for macrophage killing

5 Because fixed mutant strains failed to induce macrophage cell death, it was next determined how long the intracellular bacteria had to be viable for cell death to occur. Ciprofloxacin, a cell permeable bactericidal antibiotic, was added at different times following infection of J774 cells with $\Delta lpcC$, $\Delta manB$, or $\Delta manC$ strains. The macrophages were infected with the bacterial strains for two hours and then washed
10 and treated with gentamicin to remove extracellular bacteria. Ciprofloxacin was added to infected macrophages at six separate time points after the initial treatment with gentamicin (hour 0) and then LDH release was measured 10 hours later (12 hours after initial infection). Without addition of ciprofloxacin, the $\Delta lpcC$, $\Delta manB$, and $\Delta manC$ bacteria killed >95% of J774 within 12 hours of infection at an input
15 MOI of 100 (FIG. 10). LDH released from cells infected with the parent at the same input MOI showed <10% LDH release at 12 hours p.i. The addition of ciprofloxacin simultaneously with gentamicin reduced LDH release by 90%. Ciprofloxacin added at one or two hours post-gentamicin treatment reduced LDH release by 80% or 70%, respectively (FIG. 10). These results show that four to five hours after infection
20 with $\Delta lpcC$, $\Delta manB$, or $\Delta manC$, the majority of J774 macrophages are committed to cell death.

 Once internalized, the number of mutant bacteria ($\Delta lpcC$) did not significantly increase eight hours p.i. (FIG. 13), which indicates that either the mutant bacteria replicate more slowly than the parental strain or are killed by the
25 host cell at a rate that is approximately equal to their rate of replication. Therefore, the cytotoxicity of these strains is not the result of an increase in bacterial uptake or by an accelerated rate of replication.

lpcC, manB, and manC mutants have a shortened LPS structure

30 Because *lpcC*, *manB*, and *manC* may be involved in LPS synthesis, it was analyzed whether deleting these genes alters the LPS. The structure of the LPS can be partially deduced by its size following electrophoresis because it is assembled

sequentially and as a consequence, mutants missing the core or O-antigens will migrate faster. The size of LPS synthesized in the deletion mutants was compared to wild-type strain U112, a strain that synthesizes a LPS without the core and O-antigen ($\Delta kdtA$), two strains that synthesize a LPS lacking the O-antigen ($\Delta wbtA$, *wzx*), and hypercytotoxic transposon mutant *htrB*, which is predicted to alter the acylation of lipid A (McLendon *et al.*, *Infect Immun* 75:5518-5531, 2007). As shown in FIG. 11, *lpcC*, *manB*, and *manC* mutant strains expressed a shortened LPS that resembled the LPS from the $\Delta kdtA$ mutant strain (FIG. 11). These strains lack the O-antigen and likely contain a defect in the core. The O-antigen is also absent in $\Delta wbtA$ and *wzx* but these two strains seem to synthesize a complete core. The core and O-antigen were present in both wild-type U112 and transposon mutated *htrB*.

These data suggest that the LPS of the *lpcC*, *manB*, and *manC* deletion strains lack an O-antigen and have an absent or altered core. LPS is synthesized starting with lipid A, followed by the core, followed by the O-antigen; therefore, these three genes are involved in the synthesis of the core or of the O-antigens. As *lpcC*, *manB*, and *manC* are all involved in mannose biosynthesis, it is very likely that these enzymes are required for complete synthesis of the core (FIG. 3). The exact changes in LPS structure after deleting *lpcC*, *manB*, and *manC* remain to be determined because altering LPS at one position may influence other modifications (Raetz *et al.*, *J Lipid Res* 47:1097-1111, 2006; Stead *et al.*, *J Bacteriol* 187:3374-3383, 2005).

Other LPS biosynthesis mutants

In the initial screening of the two-allele library, the other transposon mutants of LPS biosynthesis genes behaved like U112 or were hypocytotoxic, as judged by direct microscopic observation. However, as shown above, the LPS structure of $\Delta lpcC$, $\Delta manB$, and $\Delta manC$ is similar to $\Delta kdtA$ and both $\Delta wbtA$ and *wzx* synthesize a LPS lacking an O-antigen; therefore, the cytotoxicity of these mutants was determined by assaying LDH release from infected macrophages 10 hours after infection. As shown in FIG. 12A, $\Delta kdtA$ killed infected macrophages as quickly as the three LPS mutants, $\Delta lpcC$, $\Delta manB$, and $\Delta manC$. In contrast, $\Delta wbtA$ and *wzx* had a similar release of LDH as the parental strain MFN245. When a full-length copy of

the *kdtA* gene was expressed *in trans* in $\Delta kdtA$, LDH released from infected macrophages was not significantly different from the LDH released from MFN245-infected macrophages (FIG. 12A). *KdtA* was probably not identified in the initial library screen because of polar effects on expression of downstream genes.

5 To determine whether internalization of $\Delta wbtA$, $\Delta kdtA$, and *wzx* was independent of actin polymerization, LDH release and the number of intracellular bacteria were determined in infected J774 macrophages in the presence of cytoD. High LDH release was observed for $\Delta kdtA$ at 10 hours, which was unchanged in the presence of cytoD (FIG. 12A). As shown by microscopy in FIG. 12B, the number of
10 intracellular mutant *wzx* and *wbtA* bacteria was similar to what was observed for MFN245 (FIG. 7A) and reduced by the presence of cytoD. In contrast, the number of intracellular $\Delta kdtA$ bacteria was greater than *wzx* and $\Delta wbtA$ and comparable to what was observed for $\Delta lpcC$, $\Delta manB$, and $\Delta manC$ (FIG. 6 and FIG. 12B). Furthermore, the number of internal $\Delta kdtA$ bacteria was not affected by the presence of cytoD
15 (FIG. 12B). These results show that modifications to the O-antigen, as observed with *wzx* and Δwbt , are not sufficient to result in a hypercytotoxic phenotype and that alterations to the core were required.

Example 3: Pharmaceutical compositions

20 The disclosed attenuated *Francisella* mutants can be incorporated into pharmaceutical compositions (such as immunogenic compositions or vaccines). Any pharmaceutical composition provided herein can be prepared using well-known methods.

Pharmaceutical compositions can include one or more *Francisella* bacteria
25 containing genetic inactivations in one or more of *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB*, and one or more of *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, FTT0408, FTT0757 and FTT1254 (for example see Table 2). Pharmaceutical compositions within the scope of the disclosure can include one or more other compounds, which can be either biologically active or inactive. Particular examples
30 for other compounds include, but are not limited to, physiologically acceptable carriers, excipients, immunostimulants, or combinations thereof. The pharmaceutical compositions can also include preservatives, carbohydrates (such as

glucose, mannose, sucrose or dextrans), mannitol, antioxidants, and chelating agents. In some examples, an immunostimulatory composition includes one or more adjuvants and one or more pharmaceutically acceptable carriers.

5 *Immunostimulants*

In particular examples, pharmaceutical compositions include an immunostimulant. An immunostimulant is any substance that enhances or potentiates an immune response to an exogenous antigen. Examples of immunostimulants include adjuvants, biodegradable microspheres (such as
10 polylactic galactide microspheres) and liposomes (see, for example, U.S. Patent No. 4,235,877). Any of a variety of immunostimulants can be employed in the pharmaceutical compositions that include an immunogenically effective amount of attenuated *Francisella*.

Adjuvants are non-specific stimulators of the immune system that can
15 enhance the immune response of the host to the immunogenic composition. Some adjuvants contain a substance designed to protect the antigen from rapid catabolism, for example, aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable adjuvants are commercially available as, for example,
20 Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.), TiterMax Gold (TiterMax, Norcross, GA), ISA-720 (Seppic, France), ASO-2 (SmithKlineGlaxo, Rixensart, Belgium); aluminum salts such as aluminum hydroxide (for example, Amphogel, Wyeth Laboratories, Madison, NJ) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars;
25 cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and saponins such as quil A and QS-21 (Antigenics, Framingham, MA). Cytokines, such as GM-CSF or interleukin-2, -7, or -12, can be used as adjuvants.

The adjuvant composition can be designed to induce an immune response
30 predominantly of the Th1 type. High levels of Th1-type cytokines (such as IFN- γ , TNF- α , IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines

(such as IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following administration of a pharmaceutical composition as provided herein, a subject may support an immune response that includes Th1- and Th2-type responses. However, in examples where the response is predominantly a Th1-type, the level of Th1-type cytokines increases to a greater extent than the level of Th2-type cytokines. The levels of these cytokines can be readily assessed using standard assays.

Adjuvants for use in eliciting a predominantly Th1-type response include, but are not limited to, a combination of monophosphoryl lipid A, such as 3-de-O-acylated monophosphoryl lipid A (3D-MPL) (Corixa, Hamilton IN), together with an aluminum salt. MPL adjuvants are available from Corixa (Seattle, WA; see also U.S. Pat. Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CG-containing oligonucleotides (in which the CG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in PCT publications WO 96/02555 and WO 99/33488. Immunostimulatory DNA sequences are also described, for example, by Sato *et al.*, *Science* 273:352, 1996. Another adjuvant is a saponin such as QS21 (Antigenics, Framingham, MA), which can be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a monophosphoryl lipid A and saponin derivative, such as the combination of QS21 and 3D-MPL as described in PCT Publication No. WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in PCT Publication No. WO 96/33739. Other formulations include an oil-in-water emulsion and tocopherol. An adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in PCT Publication No. WO 95/17210.

Still further adjuvants include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the ASO-2 series of adjuvants (SmithKlineGlaxo, Rixensart, Belgium), Detox (Corixa, Seattle, WA), RC-529 (Corixa, Seattle, WA), Aminoalkyl glucosaminide 4-phosphates (AGPs), copolymer adjuvants, CG oligonucleotide motifs and combinations of CG oligonucleotide motifs, bacterial extracts (such as mycobacterial extracts), detoxified endotoxins, and membrane lipids. Still other adjuvants include polymers and co-

polymers. For example, copolymers such as polyoxyethylene-polyoxypropylene copolymers and block co-polymers can be used. A particular example of a polymeric adjuvant is polymer P1005. Combinations of two or more adjuvants can be used in the pharmaceutical compositions provided herein.

5 Adjuvants are utilized in an adjuvant amount, which can vary with the adjuvant, subject, and immunogen. Typical amounts of non-emulsion adjuvants can vary from about 1 ng to about 500 mg per administration, for example, 10 µg to 800 µg, such as 50 µg to 500 µg per administration. For emulsion adjuvants (oil-in-water and water-in-oil emulsions) the amount of the oil phase can vary from about
10 0.1% to about 70%, for example about 0.5% to 5% oil in an oil-in-water emulsion and about 30% to 70% oil in a water-in-oil emulsion. Those skilled in the art will appreciate appropriate concentrations of adjuvants, and such amounts can be readily determined.

15 *Pharmaceutically acceptable carriers*

 While any suitable carrier known to those of ordinary skill in the art can be employed in the pharmaceutical compositions, the type of carrier can vary depending on the mode of administration. Pharmaceutical compositions can be formulated for any appropriate manner of administration, including for example,
20 oral (including buccal or sublingual), nasal, rectal, aerosol, topical, intravenous, intraperitoneal, intradermal, intraocular, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, exemplary carriers include water, saline, alcohol, glycerol, fat, wax, buffer (such as neutral buffered saline or phosphate buffered saline), or combinations thereof. For
25 oral administration, any of the above carriers or a solid carrier can be employed. Biodegradable microspheres (such as polylactate polyglycolate) can also be employed as carriers for the pharmaceutical compositions. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

30 Carriers for use with the disclosed compositions are biocompatible, and can also be biodegradable, and the formulation can provide a relatively constant level of active component release. Suitable carriers include, but are not limited to,

microparticles of poly(lactide-co-glycolide), as well as polyacrylate, latex, starch, cellulose and dextran. Other delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (such as a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (see, for example, U.S. Pat. No. 5,151,254 and PCT publication Nos. WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Any of a variety of delivery vehicles can be employed with the disclosed pharmaceutical compositions to facilitate production of an antigen-specific immune response to *Francisella*. Exemplary vehicles include, but are not limited to, hydrophilic compounds having a capacity to disperse the attenuated *Francisella* bacteria and any additives. The attenuated bacteria can be combined with the vehicle according to methods known in the art. The vehicle can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), and suitable mixtures thereof. Other exemplary vehicles include, but are not limited to, copolymers of polycarboxylic acids or salts thereof, carboxylic anhydrides (for example, maleic anhydride) with other monomers (for example, methyl (meth)acrylate, acrylic acid and the like), hydrophilic vinyl polymers, such as polyvinyl acetate, polyvinyl alcohol, polyvinylpyrrolidone, cellulose derivatives, such as hydroxymethylcellulose, hydroxypropylcellulose and the like, and natural polymers, such as chitosan, collagen, sodium alginate, gelatin, hyaluronic acid, and nontoxic metal salts thereof.

A biodegradable polymer can be used as a base or vehicle, such as polyglycolic acids and polylactic acids, poly(lactic acid-glycolic acid) copolymer, polyhydroxybutyric acid, poly(hydroxybutyric acid-glycolic acid) copolymer, and mixtures thereof. Other biodegradable or bioerodable polymers include, but are not limited to, such polymers as poly(epsilon-caprolactone), poly(epsilon-aprolactone-CO-lactic acid), poly(epsilon.-aprolactone-CO-glycolic acid), poly(beta-hydroxy butyric acid), poly(alkyl-2-cyanoacrilate), hydrogels, such as poly(hydroxyethyl

methacrylate), polyamides, poly(amino acids) (for example, L-leucine, glutamic acid, L-aspartic acid and the like), poly(ester urea), poly(2-hydroxyethyl DL-aspartamide), polyacetal polymers, polyorthoesters, polycarbonate, polymaleamides, polysaccharides, and copolymers thereof. In some examples, vehicles include
5 synthetic fatty acid esters such as polyglycerin fatty acid esters and sucrose fatty acid esters. Hydrophilic polymers and other vehicles can be used alone or in combination, and enhanced structural integrity can be imparted to the vehicle by partial crystallization, ionic bonding, cross-linking and the like.

The vehicle can be provided in a variety of forms, including, fluid or viscous
10 solutions, gels, pastes, powders, microspheres and films. In one example, pharmaceutical compositions for administering attenuated *Francisella* bacteria are formulated as a solution, microemulsion, or other ordered structure suitable for high concentration of active ingredients. Proper fluidity for solutions can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of a desired
15 particle size in the case of dispersible formulations, and by the use of surfactants.

Time release formulations

The disclosed compositions can be administered as part of a sustained release formulation (such as a capsule, sponge or gel that includes the attenuated
20 *Francisella* bacteria) that provides a slow release of the composition following administration. These compositions can be prepared with vehicles that protect against rapid release, and are metabolized slowly under physiological conditions following their delivery (for example in the presence of bodily fluids). Many methods for preparing such formulations are well known to those skilled in the art
25 (see, for example, Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978). Examples include, but are not limited to, a polymer, controlled-release microcapsules, and bioadhesive gels. For example, sustained-release formulations can contain attenuated *Francisella* bacteria dispersed in a carrier matrix or contained within a reservoir surrounded by a
30 rate controlling membrane. In one example, a controlled-release formulation can be administered by, for example, subcutaneous implantation at the desired target site.

Packaging

Pharmaceutical compositions can be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are typically
5 hermetically sealed to preserve sterility of the formulation until use. In general, formulations can be stored as suspensions, solutions or as emulsions in oily or aqueous vehicles. In particular examples, the disclosed compositions are stored at temperatures from about 4°C to -100°C until use.

The pharmaceutical compositions of the disclosure typically are sterile and
10 stable under conditions of manufacture, storage and use. Sterile solutions can be prepared by incorporating the disclosed attenuated *Francisella* bacteria (alone or in the presence of a pharmaceutically acceptable carrier or an adjuvant (or other biologically active agent) in the desired amount in an appropriate solvent followed by sterilization, such as by filtration, radiation, or heat. Generally, dispersions are
15 prepared by incorporating the attenuated *Francisella* bacteria into a sterile vehicle that contains a dispersion medium and other desired ingredients. In the case of sterile powders, methods of preparation include vacuum drying and freeze-drying which yields a powder of the attenuated *Francisella* bacteria plus any additional desired ingredient from a previously sterile-filtered solution thereof. For vaccine
20 use, the attenuated *Francisella* bacteria of the disclosure can be used directly in vaccine formulations, or lyophilized, as desired, using lyophilization protocols well known in the art. Lyophilized attenuated *Francisella* bacteria are typically maintained at about 4°C. When ready for use the lyophilized attenuated *Francisella* bacteria can be reconstituted in a stabilizing solution (such as saline).

25

Example 4: Methods of stimulating an immune response

This example describes methods using the disclosed immunogenic compositions to stimulate an immune response in a subject, such as a mammal, for example a human or veterinary subject.

30 Methods for inoculation are routine in the art. In some examples, a determination is made as to whether the subject would benefit from administration of the disclosed immunogenic compositions, prior to administering the

immunogenic composition. For example, subjects who have been exposed or are likely to be exposed to a virulent form of *Francisella* can be selected to receive the immunogenic composition. Administration can be achieved by any method known in the art, such as oral administration, inhalation, or inoculation (such as

5 intramuscular, i.p., or subcutaneous). In some examples, the immunogenic composition includes live attenuated *Francisella* bacteria containing a genetic inactivation in one or more of the *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB* genes, and one or more of the *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, FTN0408, FTN0757 and FTN1254 genes (such as those combinations listed in

10 Table 2). In particular examples, attenuated *Francisella* bacteria are administered in the presence of other agents, such as an adjuvant or pharmaceutical carrier (or both).

The amount of live attenuated *Francisella* bacteria containing a genetic inactivation in one or more of the *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB* genes and one or more of the *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*,

15 FTN0408, FTN0757 and FTN1254 genes administered is sufficient to induce in the host an effective immune response against virulent forms of *Francisella*. An effective amount can be readily determined by one skilled in the art, for example using routine trials establishing dose response curves. The immunogenic compositions disclosed herein can be administered to the subject as needed to confer

20 immunity against *Francisella* to the subject. For example, the composition can be administered in a single bolus delivery (which can be followed by one or more booster administrations as needed), via continuous delivery over an extended time period, in a repeated administration protocol (for example, by an hourly, daily, weekly, or monthly repeated administration protocol).

25 In some examples, live attenuated *Francisella* bacteria containing a genetic inactivation in one or more of *dsbB*, FTT0742, *pdpB*, *fumA*, or *carB* and one or more of *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, FTN0408, FTN0757 and FTN1254 are administered to a subject. In particular examples, the inactivated whole-cell vaccine is administered to the subject (for example orally, nasally, or via

30 injection). Exemplary doses of bacteria (as measured by colony-forming units), include, but are not limited to, 10^3 - 10^{10} bacteria per dose, for example at least 10^3

bacteria, at least 10^4 bacteria, at least 10^5 bacteria, at least 10^8 bacteria, or at least 10^9 bacteria per dose.

Provided below are particular examples of methods that can be used to stimulate an immune response in a mammalian subject. However, the disclosure is
5 not limited to these particular examples.

Calculation of LD₅₀

The LD₅₀ for the desired attenuated *Francisella tularensis* bacterium containing two more functionally deleted genes can be determined using methods
10 known in the art. For example, increasing amounts of attenuated *Francisella tularensis* bacteria are administered to a laboratory animal (such as a mouse, rat, rabbit, or non-human primate), and the animal monitored for survival for up to 30 days. The mean time to death can be calculated by dividing the sum of the survival times of all animal by the total number of animals examined.

15 The dose of attenuated *Francisella tularensis* bacteria used to stimulate an immune response in a mammal (such as a human) is generally about 100- to 1000-fold lower than the calculated LD₅₀.

Administration of live bacteria

In one example, attenuated *Francisella tularensis* bacteria that include at
20 least two functionally deleted genes are administered to a mammal, such as a veterinary subject or human, via scarification. For example, the bacteria can be administered as a single dose in about 0.1 ml by scarification to the forearms of a human. In particular examples, the dose of bacteria is about 10^6 - 10^8 bacteria.

In another example, attenuated *Francisella tularensis* bacteria that include at
25 least two functionally deleted genes are administered to a mammal, such as a veterinary subject or human, via aerosol. For example, the bacteria can be administered intranasally as a single dose in about 50-500 μ l physiological saline. In particular examples, the dose of bacteria is about 10^3 to 10^{10} bacteria.

In yet another example, attenuated *Francisella tularensis* bacteria that
30 include at least two functionally deleted genes are administered to a mammal, such as a veterinary subject or human, via intradermal or subcutaneous injection. For example, the bacteria can be administered as a single dose in about 50 μ l – 1 ml

physiological saline. In particular examples, the dose of bacteria is about 10^3 to 10^7 bacteria. In one example, mice are injected subcutaneously with 50 μ l-100 μ l of an inoculum containing about 10^3 to 10^5 bacterium in the flank or at the base of the tail.

5 Exemplary assessment in mice

In a particular example, wild-type mice (such as pathogen-free female BALB/c 8 to 12 week old, mice (Jackson Laboratory, Bar Harbor, ME)) are used to demonstrate the efficacy of attenuated *Francisella tularensis* bacteria. Mice are intranasally administered an immunogenic composition containing live attenuated
 10 *Francisella tularensis* that have at least one functionally deleted gene selected from the *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB* genes, and at least one functionally deleted gene selected from the *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, FTN0408, FTN0757 and FTN1254 genes (50 μ l of immunogenic composition). Alternatively, the immunogenic composition can be administered
 15 intradermally into a fold of skin in the mid-belly utilizing a 26.5 gauge needle. If desired, mice can be anesthetized with isofluorane prior to administration of the immunogenic composition. Mice each are administered approximately 10^{10} – 10^{11} TCID₅₀ (amount of bacteria required for 50% infectivity of susceptible cells in tissue culture) of live attenuated *Francisella tularensis* that have at least two functionally
 20 deleted genes, or with phosphate-buffered saline (PBS) as a negative control.

Subsequently, mice are administered wild-type virulent *F. tularensis* (such as type A or type B *F. tularensis*, for example type A strain FSC033). For example, 4-12 weeks following administration of the immunogenic composition, mice are challenged intradermally (for example administered into the base of the tail or into a
 25 fold of skin in the mid-belly) with about 10 cfu of virulent type A or type B strain of *F. tularensis* in phosphate-buffered saline and survival monitored. Alternatively, 4-12 weeks following administration of the immunogenic composition, mice are challenged intranasally (for example via a Lovelace nebulizer) with about 20 cfu of virulent type A or type B strain of *F. tularensis* and survival monitored.

30 All inoculated animals are observed daily for signs of tularemia (ruffled fur, inertia, or death). Blood can be collected from mice 15-30 days after infection (such as 21 days post infection). Serum samples are analyzed for the presence of

neutralizing antibody to *F. tularensis*, using any standard immunoassay known to those skilled in the art. Blood will be collected before euthanasia when necessary.

Assessment in a non-human primate model

5 As an alternative to using mice to assess the efficacy of an immunogenic composition that includes live attenuated *Francisella* bacteria, the ability of such bacteria to be used as an immunogen can be determined in rhesus monkeys. The live attenuated *Francisella* bacteria disclosed herein can be administered to monkeys and the immune response assayed, for example using the methods described above
10 for mice. Briefly, juvenile rhesus monkeys are administered 10^3 - 10^{11} cfu of attenuated bacteria orally, intraperitoneally, or by aerosol. The ability of the attenuated *Francisella* bacteria to stimulate an immune response in the treated monkeys can be determined as described above.

 Monkeys can be subsequently challenged with 1000 x LD₅₀ of a virulent
15 strain of a native *Francisella tularensis*.

Measurement of immune response

 The following methods can be used to assess immunogenicity of the live attenuated *Francisella tularensis* that have at least two functionally deleted genes as described herein. The presence of neutralizing antibodies can be assessed by testing
20 serum samples obtained from the subject for the presence of antibodies to *F. tularensis*. For example, the microagglutination method of Bevanger *et al.* (*J. Clin. Microbiol.* 26:433-437, 1988, herein incorporated by reference) can be used to determine the antibody titer in the serum. In particular examples, antibody titers of $\geq 1:80$ are considered responsive, while nonresponders have a titer of $\leq 1:20$.

25 In another example, following immunization, sera is obtained from immunized and non-immunized subjects. For example, sera can be analyzed for the presence of specific neutralizing antibodies to *F. tularensis*, for example using an agglutination assay.

 Production of specific neutralizing antibodies when inoculated with live
30 attenuated *F. tularensis* that have at least two functionally deleted genes (as disclosed herein) would give evidence of protective immunity.

Further evidence that attenuated *F. tularensis* bacteria provide protection from illness or death resulting from infection with *F. tularensis*, can be obtained from challenge studies. For example, following administration of the attenuated *F. tularensis* bacteria, animals are challenged with dosages of virulent *F. tularensis* sufficient to cause illness or death in unprotected laboratory animals (such as mice or monkeys), for example a dose equivalent to 100-1000 times the LD₅₀. The absence of signs of tularemia (or a decrease in the severity of such signs) or absence of death when challenged indicates that the laboratory animals are protected by their prior exposure to attenuated *F. tularensis* bacteria.

Example 5: $\Delta lpcC$ in Schu S4 is hypercytotoxic in macrophages

J774 macrophages were seeded to confluency in flat-bottom 96-well plates in DMEM without phenol red. Half of the cells were pre-treated with 5 μ g/ml cytochalasin D (cytD) for 30 minutes prior to infection to inhibit actin polymerization. Wild-type Schu S4 or $\Delta lpcC$ (in-frame deletion in *lpcC* in Schu S4) were used to infect the macrophages at an input MOI of 10 or 100. Cells were incubated with bacteria at 37°C with 5% CO₂. After two hours, the cells were washed three times with PBS and overlaid with DMEM (without phenol red) containing 10 μ g/ml gentamycin to kill any extracellular bacteria. The infections proceeded for 6 or 21 hours (8 and 23 hours total infection time). Thirty minutes prior to analysis, uninfected control wells were lysed to completion with 0.5% saponin.

For analysis of cell lysis, 50 μ l of supernatant from each well was transferred to a new 96-well plate. Fifty μ l of cytotox 96 substrate (Promega) was added to each well, and the plate was covered with foil and incubated at room temperature for 10-30 minutes. At the end of incubation, 50 μ l of stop solution was added to each well, and the reaction was analyzed on a spectrophotometer at 490 nm. As shown in FIGS. 14A and 14B, the Schu S4 $\Delta lpcC$ mutant killed the J774 macrophages more quickly than wild-type Schu S4 (as indicated by the greater LDH release).

Furthermore, in the presence of cytD, the cytotoxicity was reduced for wild-type Schu S4 but not for the $\Delta lpcC$ mutant. Similar to what was observed in the U112

$\Delta lpcC$ mutant (Lai *et al. PLoS One* 5(7):e11857, 2010), deletion of $lpcC$ in Schu S4 results in a hypercytotoxic phenotype that is independent of actin polymerization.

Example 6: $\Delta lpcC$ is attenuated and protective in mice and augments $\Delta pdpB$ in a vaccine strain

An in-frame deletion of $lpcC$ was created in wild-type Schu S4 and $\Delta pdpB$ background strains. Mice were vaccinated subcutaneous with the strains and at the doses shown in Table 7 (200 μ l total volume). Four weeks after vaccination, the mice were challenged with an intranasal administration of either 10 or 100 CFU of wild-type Schu S4 (10 μ l total volume). Survival was monitored for 28 days post-challenge. The $\Delta lpcC$ mutant exhibited attenuation in mice, protection to low levels of challenge and improved $\Delta pdpB$ vaccine efficacy.

Table 7: Results of vaccination with $\Delta lpcC$, $\Delta pdpB$, and $\Delta lpcC\Delta pdpB$ mutants

Strain	Vaccine dose (CFU)	% Survival (5 mice)	Challenge dose (CFU)	% Survival at day 28 (5 mice)	Median Time to Death*
$\Delta lpcC\Delta pdpB$	10^9	100	10	80*	>28
			100	40	13
	10^8	100	10	100	>28
			100	20	6
$\Delta pdpB$	10^9	100	10	80	>28
			100	20	6
	10^8	100	10	75**	>28
			100	40	12
$\Delta lpcC$	10^3	100	10	80	>28
			100	20	5
	10^2	100	10	100	>28

Strain	Vaccine dose (CFU)	% Survival (5 mice)	Challenge dose (CFU)	% Survival at day 28 (5 mice)	Median Time to Death*
			100	40	7

Vaccination: groups of 10 BALB/c mice, subcutaneous, 200 μ l

Challenge: groups of 5 BALB/c mice, intranasal, 5 μ l/nare (10 μ l total)

*one mouse was eliminated from the study due to complications from administering the challenge dose.

5

In view of the many possible embodiments to which the principles of the disclosed invention may be applied, it should be recognized that the illustrated embodiments are only examples of the invention and should not be taken as limiting the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

10

CLAIMS

1. An isolated *Francisella* bacterium comprising a genetic inactivation of two or more genes that attenuate the *Francisella* bacterium, wherein at least one of the genes is selected from the group consisting of *dsbB*, FTT0742, *pdpB*, *fumA* and *carB*, and at least one of the genes is selected from the group consisting of *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254.
2. The isolated *Francisella* bacterium of claim 1, wherein the *Francisella* bacterium comprises a genetic inactivation of at least two genes selected from the group consisting of *dsbB*, FTT0742, *pdpB*, *fumA* and *carB*.
3. The isolated *Francisella* bacterium of claim 1, wherein the *dsbB* gene is genetically inactivated.
4. The isolated *Francisella* bacterium of claim 1, wherein the FTT0742 gene is genetically inactivated.
5. The isolated *Francisella* bacterium of claim 1, wherein the *pdpB* gene is genetically inactivated.
6. The isolated *Francisella* bacterium of claim 1, wherein the *fumA* gene is genetically inactivated.
7. The isolated *Francisella* bacterium of claim 1, wherein the *carB* gene is genetically inactivated.
8. The isolated *Francisella* bacterium of claim 1, wherein the *Francisella* bacterium comprises a genetic inactivation of at least two genes selected from the group consisting of *tolB*, *htrB*, *lpxH*, *ostA1*, *fimT*, *lpcC*, *manB*, *manC*, *nusA*, *wzx*, *kdtA*, FTN0408, FTN0757 and FTN1254.

9. The isolated *Francisella* bacterium of claim 1, wherein the *Francisella* bacterium comprises a genetic inactivation of at least *lpcC*, *manB*, *manC* or *kdtA*.

5

10. The isolated *Francisella* bacterium of claim 9, wherein the *Francisella* bacterium further comprises a genetic inactivation of *pdpB*.

11. The isolated *Francisella* bacterium of claim 1, wherein the *Francisella* bacterium comprises a genetic inactivation of *lpcC* and *pdpB*.

10

12. The isolated *Francisella* bacterium of claim 1, wherein the *Francisella* bacterium is a strain of *Francisella tularensis*.

13. The isolated *Francisella* bacterium of claim 12, wherein the *Francisella* bacterium is a strain of *Francisella tularensis* subspecies *tularensis*.

15

14. The isolated *Francisella* bacterium of claim 12, wherein the *Francisella* bacterium is a strain of *Francisella tularensis* subspecies *novicida*.

20

15. The isolated *Francisella* bacterium of claim 1, wherein the *Francisella* bacterium is live.

16. The isolated *Francisella* bacterium of claim 1, wherein the two or more genes are genetically inactivated by complete or partial deletion mutation or by insertional mutation.

25

17. An immunogenic composition comprising the isolated *Francisella* bacterium of any one of claims 1-16.

30

18. The immunogenic composition of claim 17, further comprising an adjuvant.

19. The immunogenic composition of claim 17, further comprising a pharmaceutically acceptable carrier.

5 20. Use of the immunogenic composition of claim 17 to induce an immune response against *Francisella* in a subject.

21. Use of claim 20, wherein the subject is a human subject.

10 22. Use of claim 20, wherein administering comprises intranasal administration.

23. Use of claim 20, wherein the therapeutically effective amount comprises 100 to 1000 colony forming units of the isolated *Francisella* bacterium.

15 24. Use of the immunogenic composition of claim 17 for treating tularemia in a subject.

25. Use of the immunogenic composition of claim 17 for treating
20 infection by a *Francisella* species in a subject.

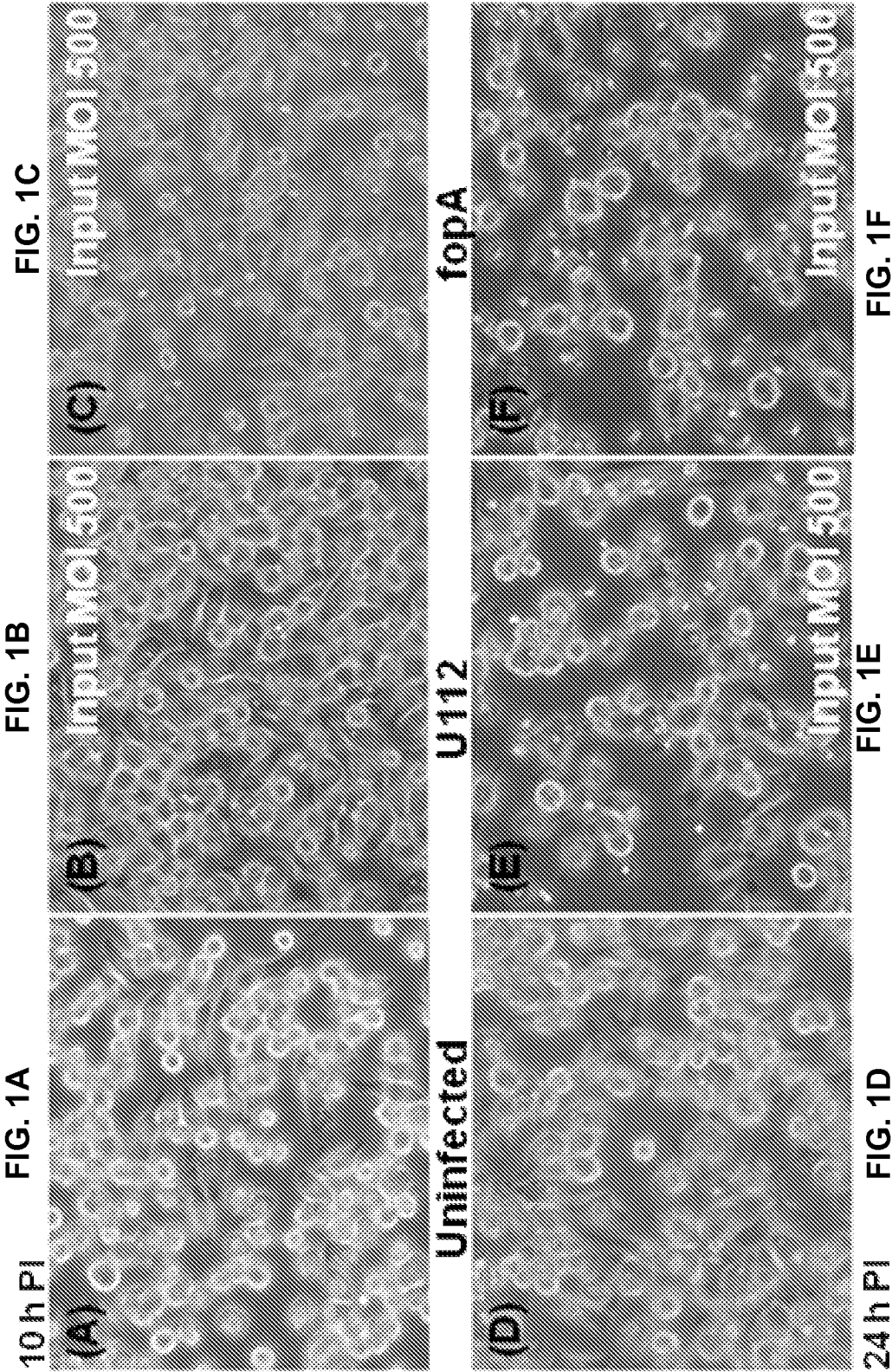


FIG. 2 - Exemplary combinations of functional deletions in *Francisella*. *

	<i>dsbB</i>	FTI0742	<i>pdpB</i>	<i>fumA</i>	<i>carB</i>	<i>hrrB</i>	<i>tolB</i>	<i>lpcC</i>	<i>manB</i>	<i>manC</i>	<i>masA</i>	<i>wzx</i>	FTN0408	FTN0757	FTN1254	<i>ostA1</i>
1	x															
2		x														
3			x													
4				x												
5					x											
6						x										
7							x									
8								x								
9									x							
10										x						
11											x					
12												x				
13													x			
14														x		
15															x	
16																x
17	x		x													
18	x	x														
19		x	x													
20	x		x													
21	x	x	x	x												
22	x	x	x	x	x											
23	x	x	x		x											
24	x	x		x												
25		x	x	x												
26	x		x	x												
27			x	x												
28			x			x										
29			x				x									
30			x					x								
31			x						x							
32			x							x						
33			x								x					
34			x									x				
35			x										x			
36			x											x		

*“X” in the box indicates gene is functionally deleted

FIG. 3

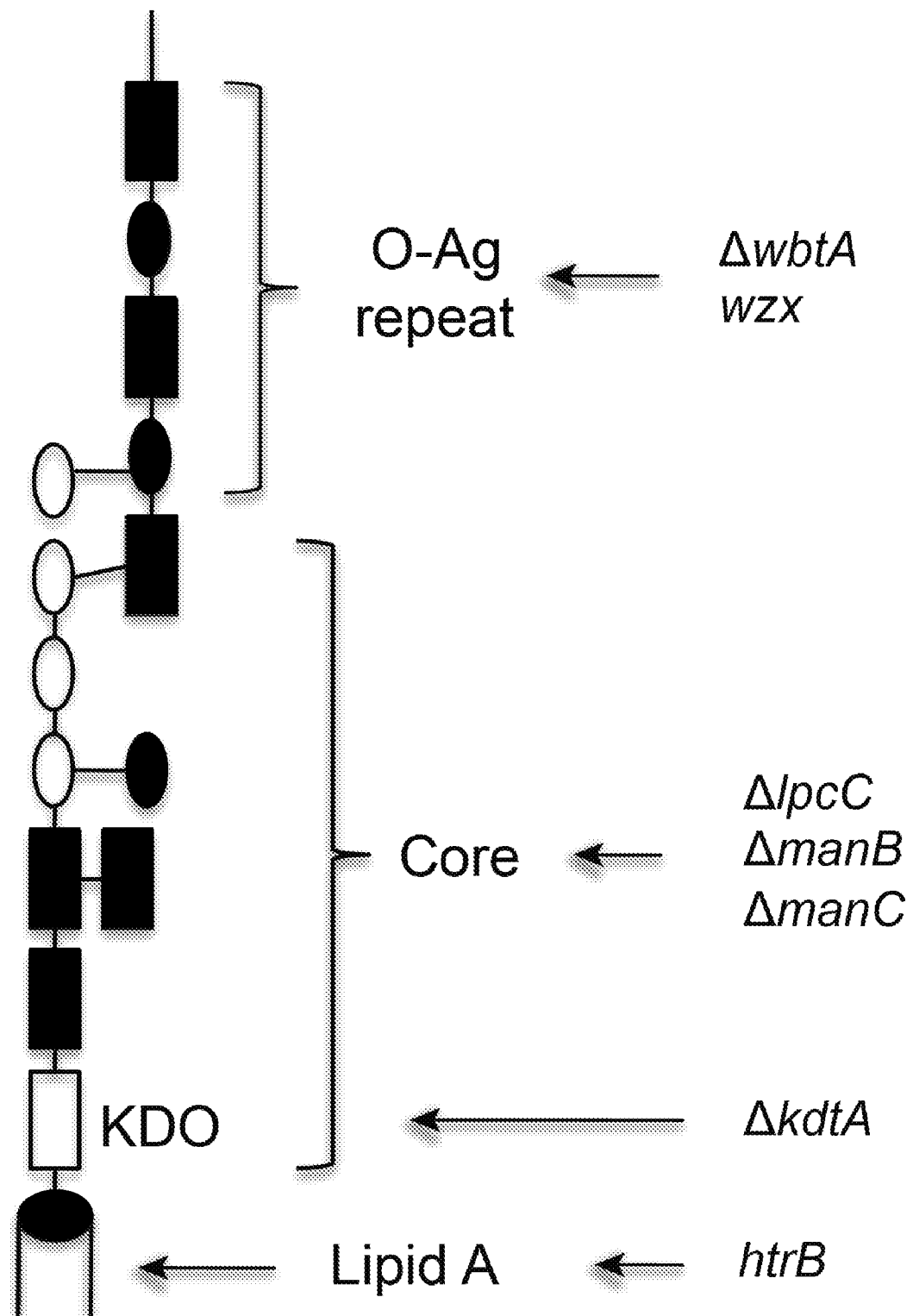


FIG. 4A

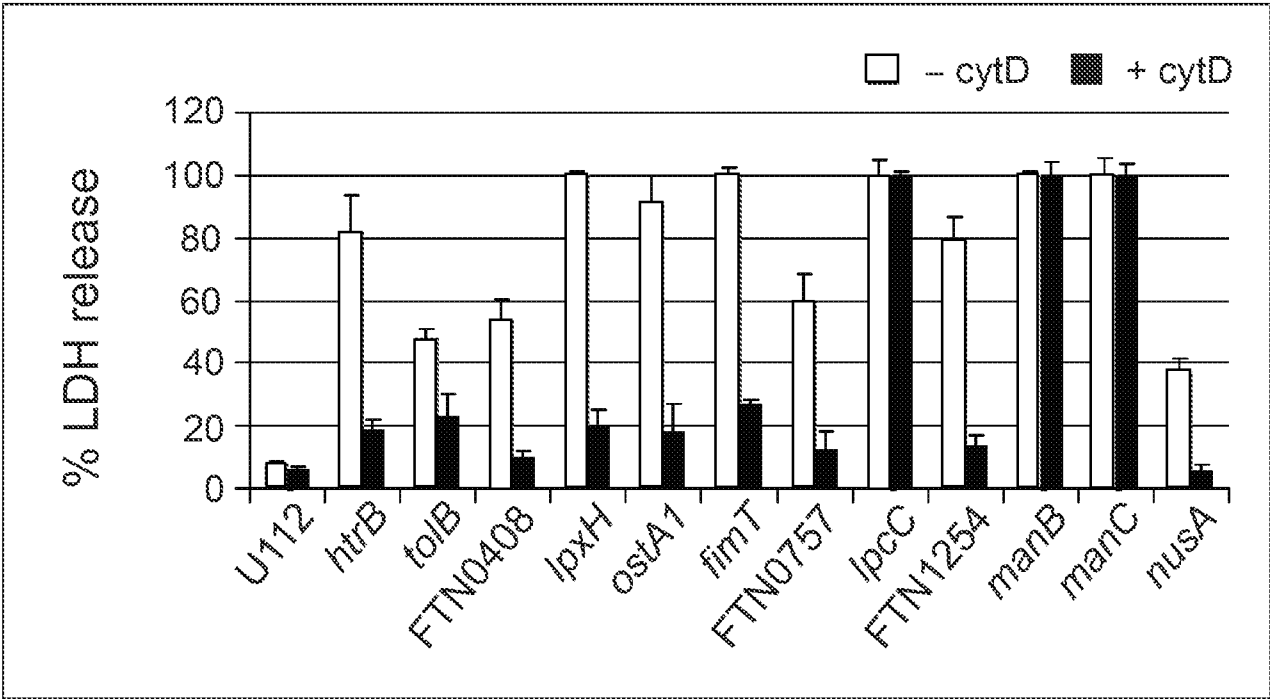


FIG. 4B

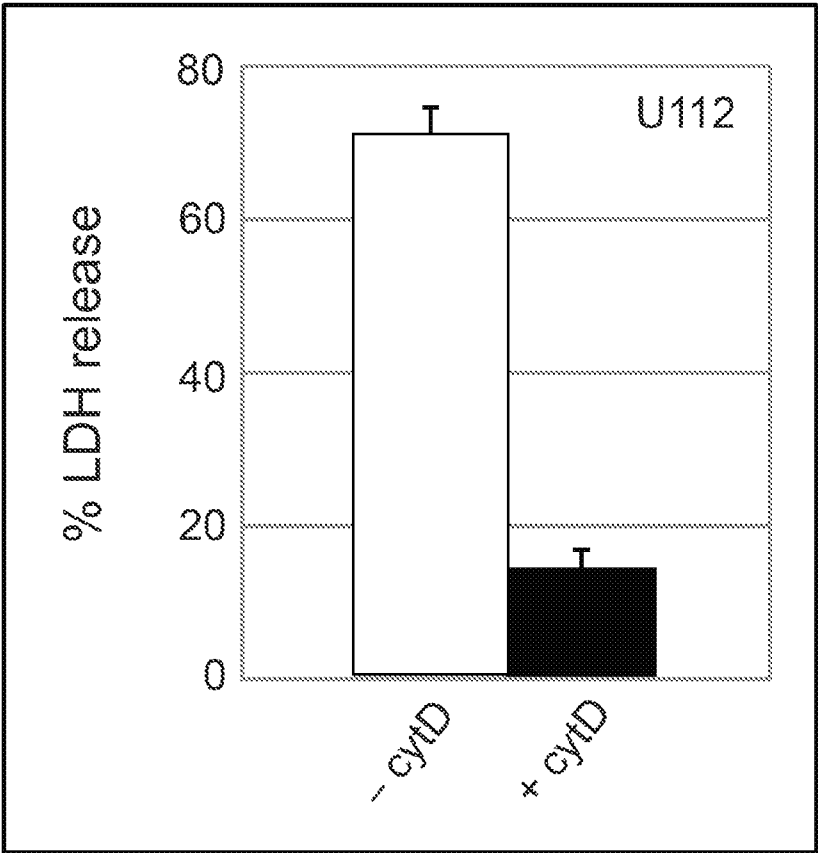


FIG. 5

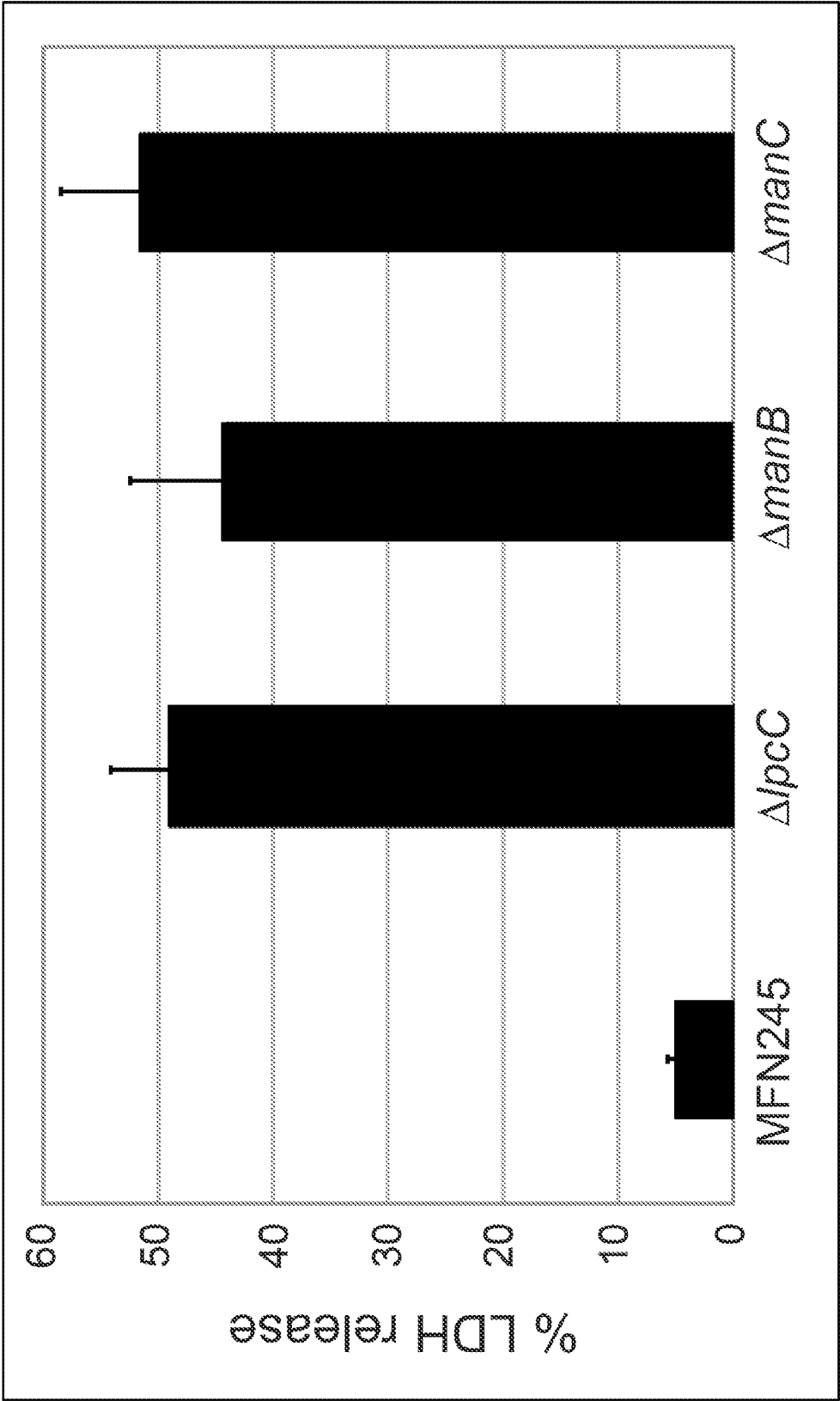


FIG. 6A

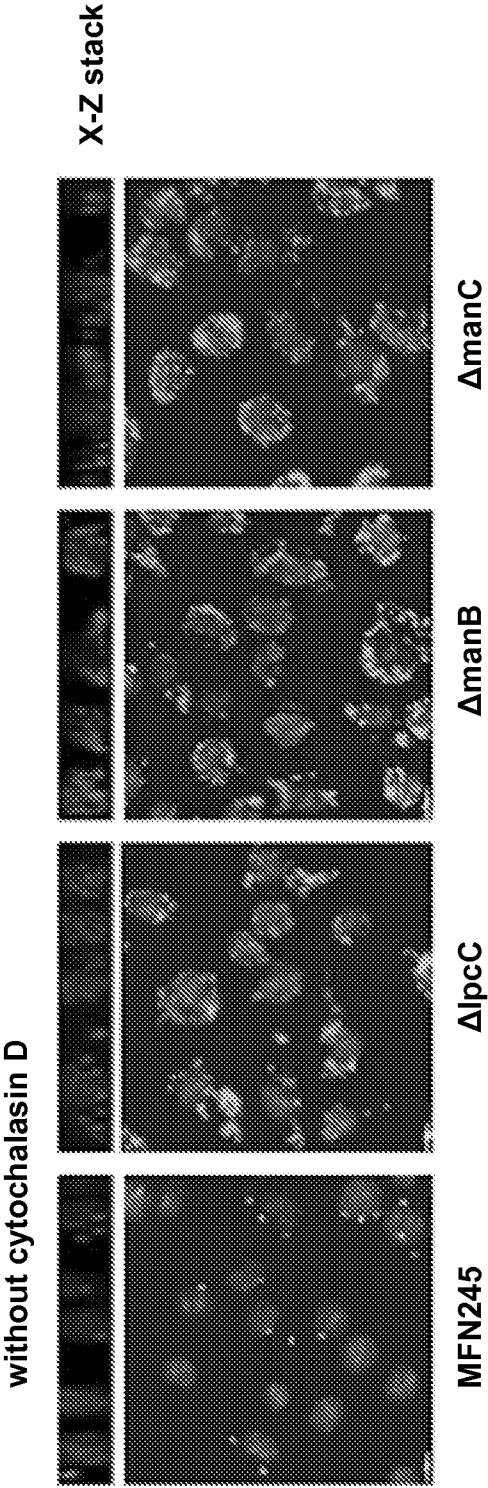


FIG. 6B

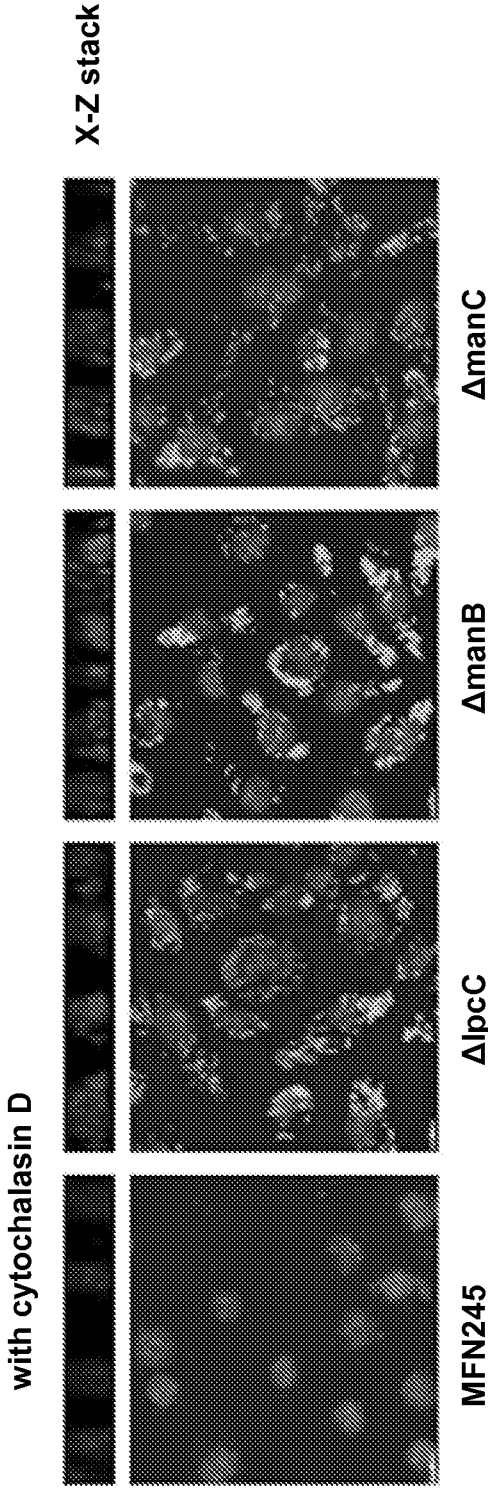


FIG. 7

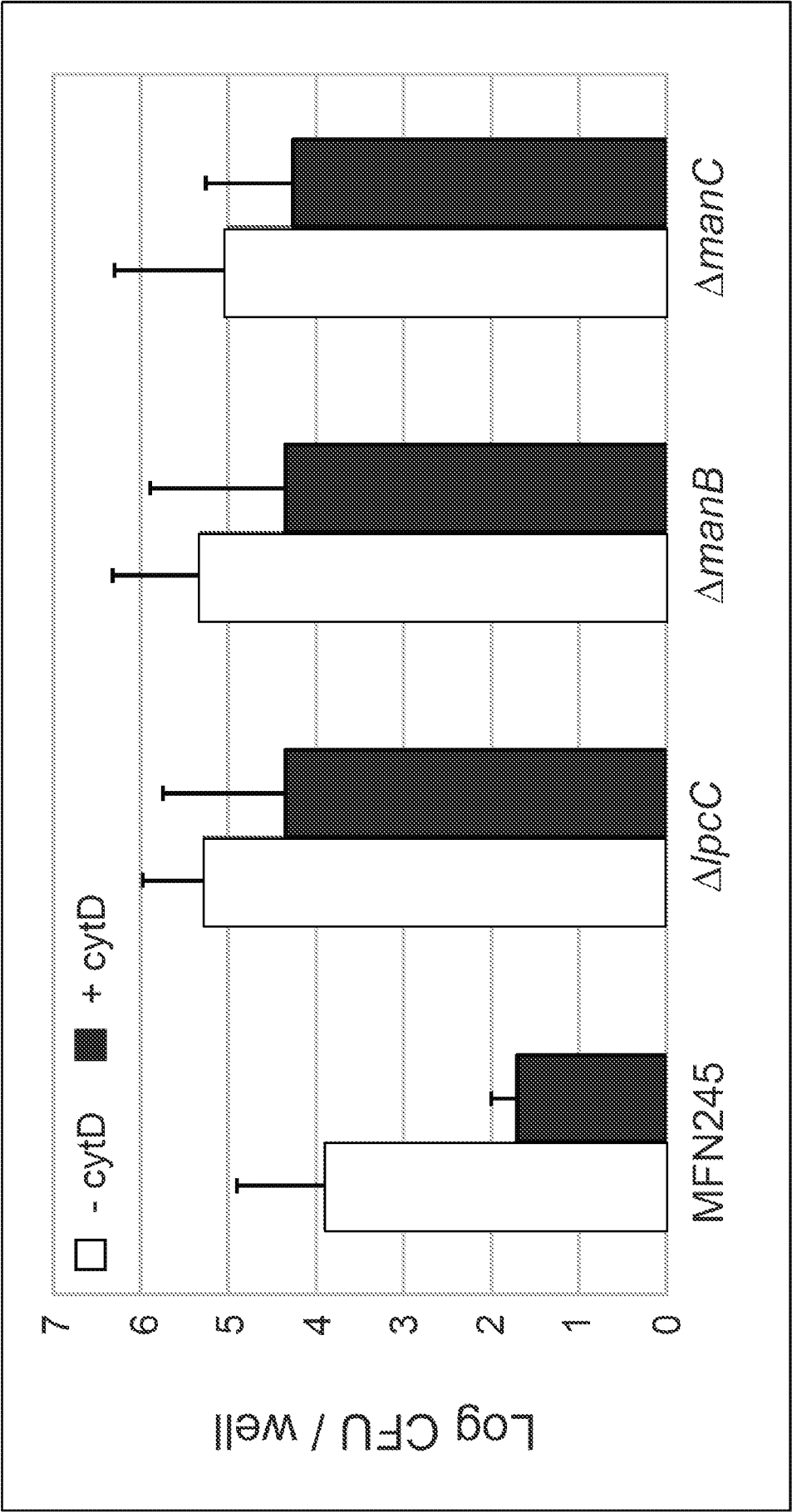


FIG. 8A

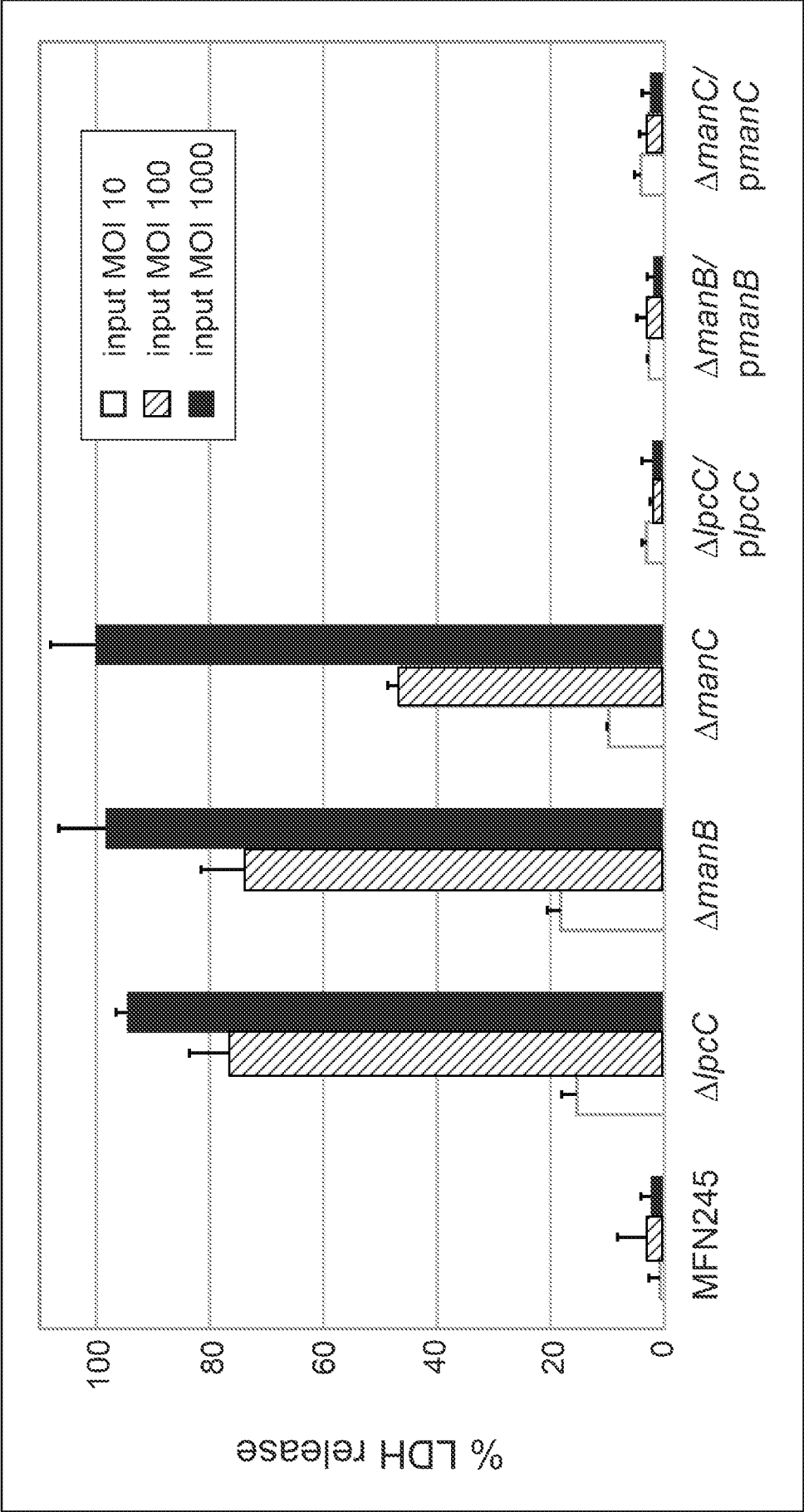


FIG. 8B

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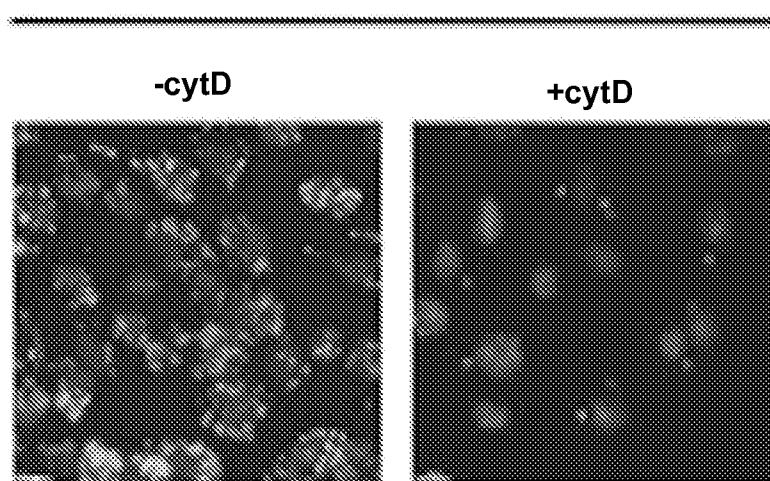


FIG. 9A

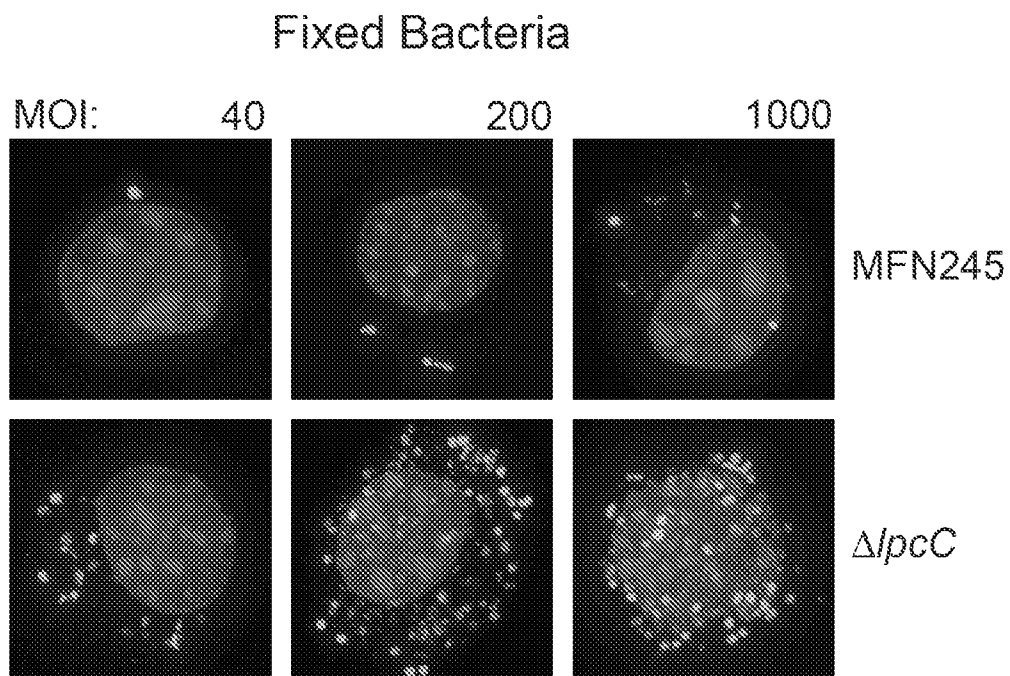


FIG. 9B

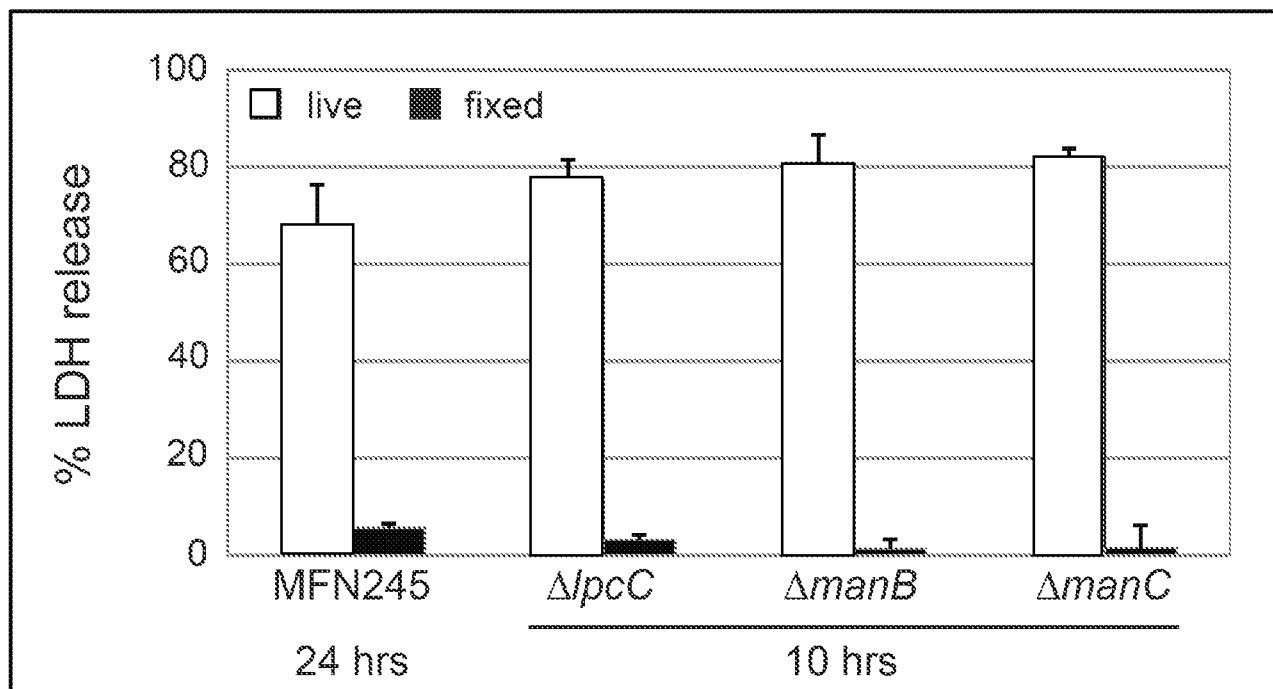


FIG. 10

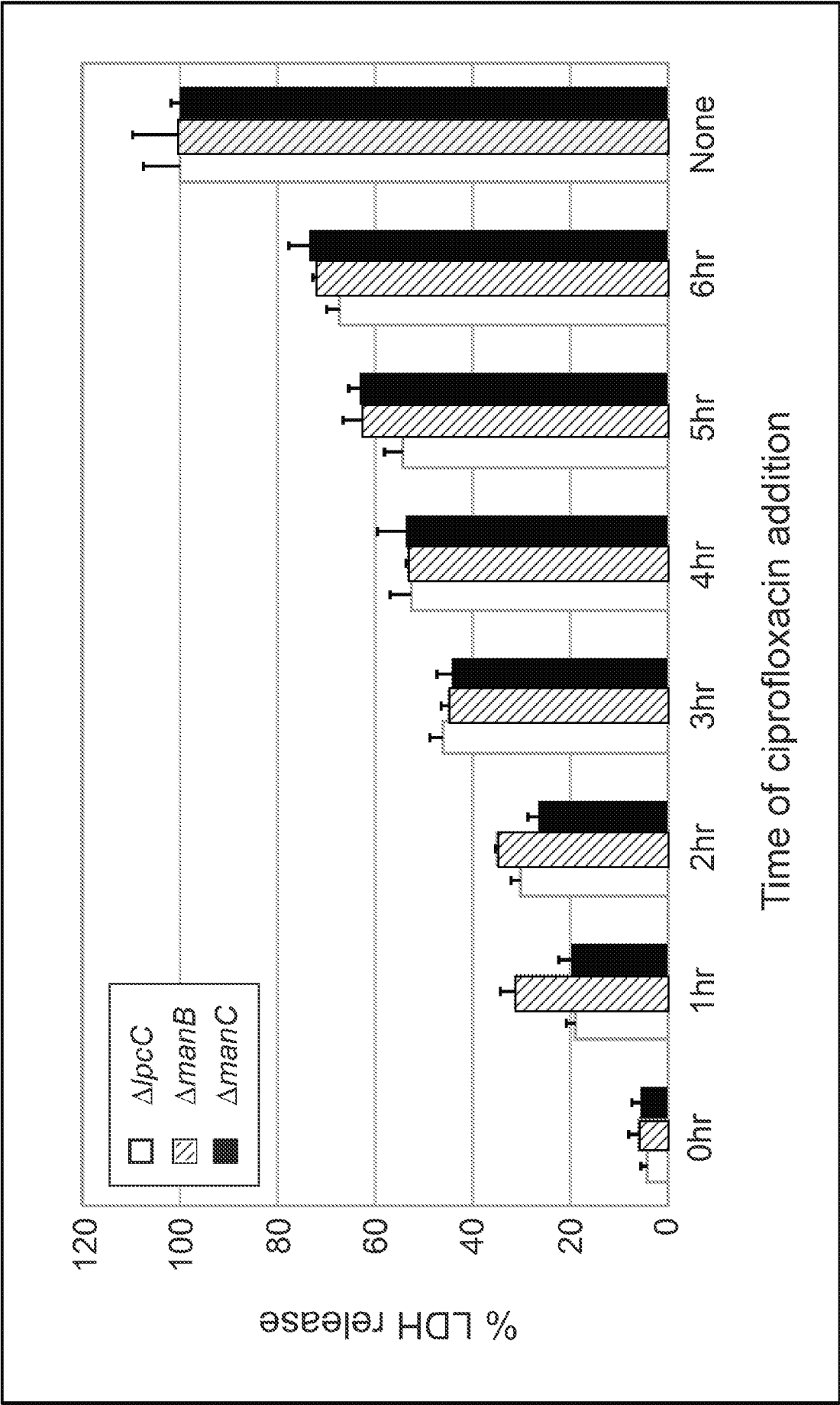


FIG. 11

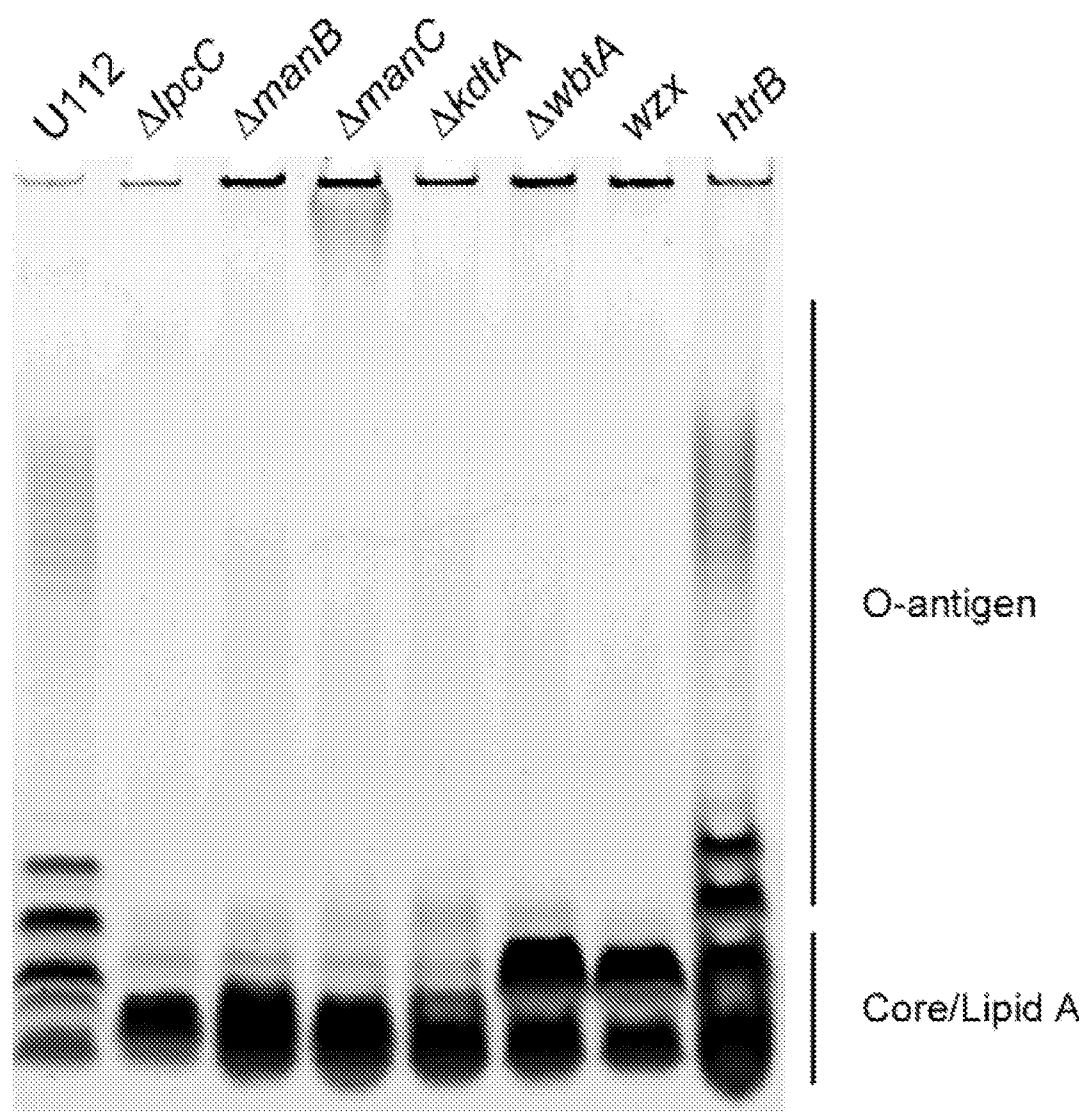


FIG. 12A

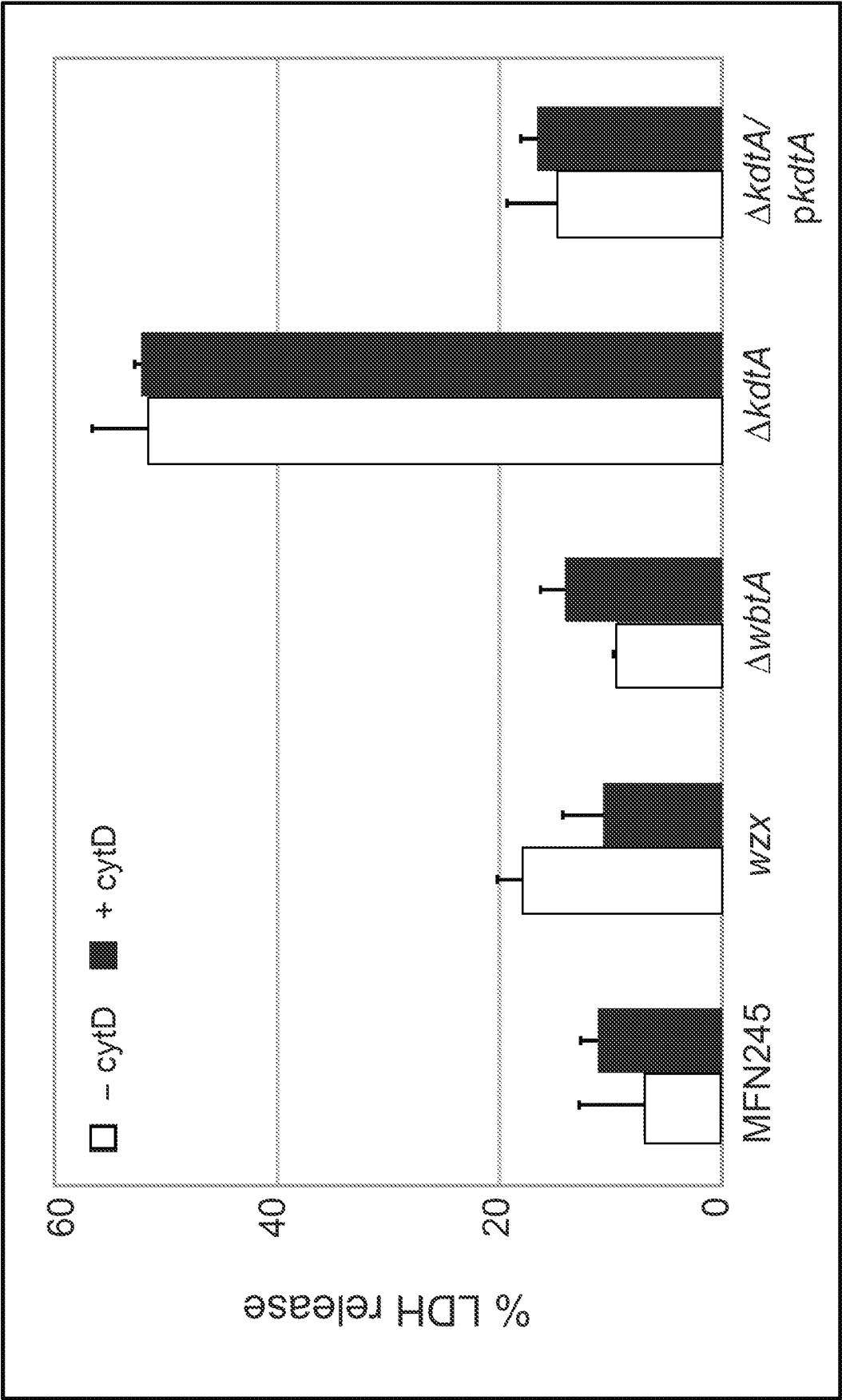


FIG. 12B

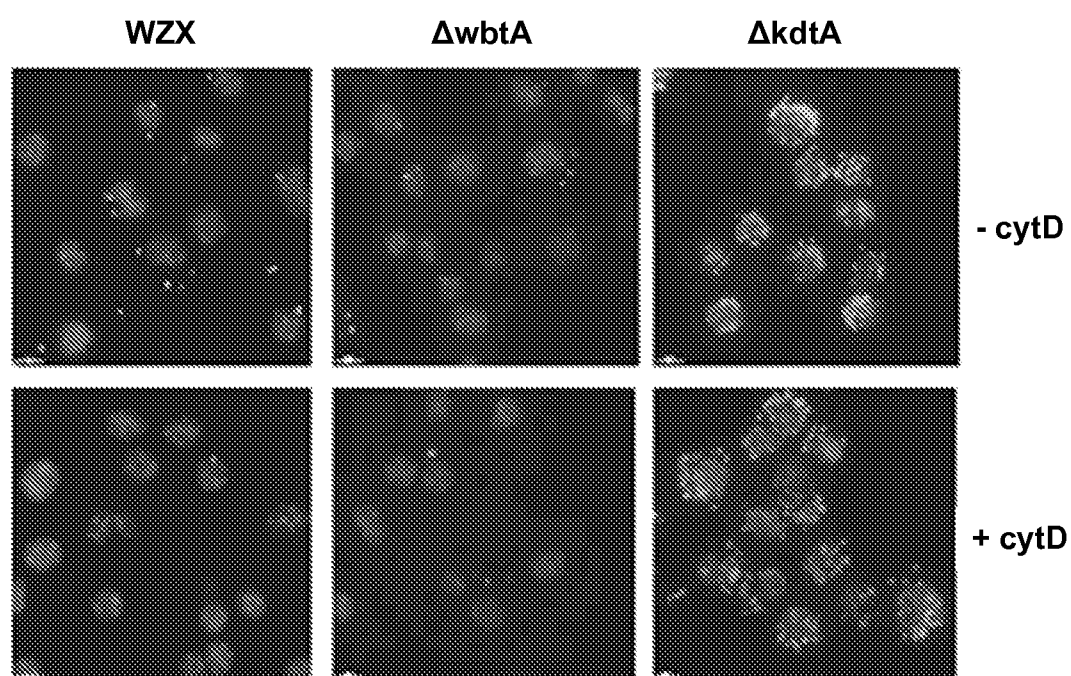


FIG. 13

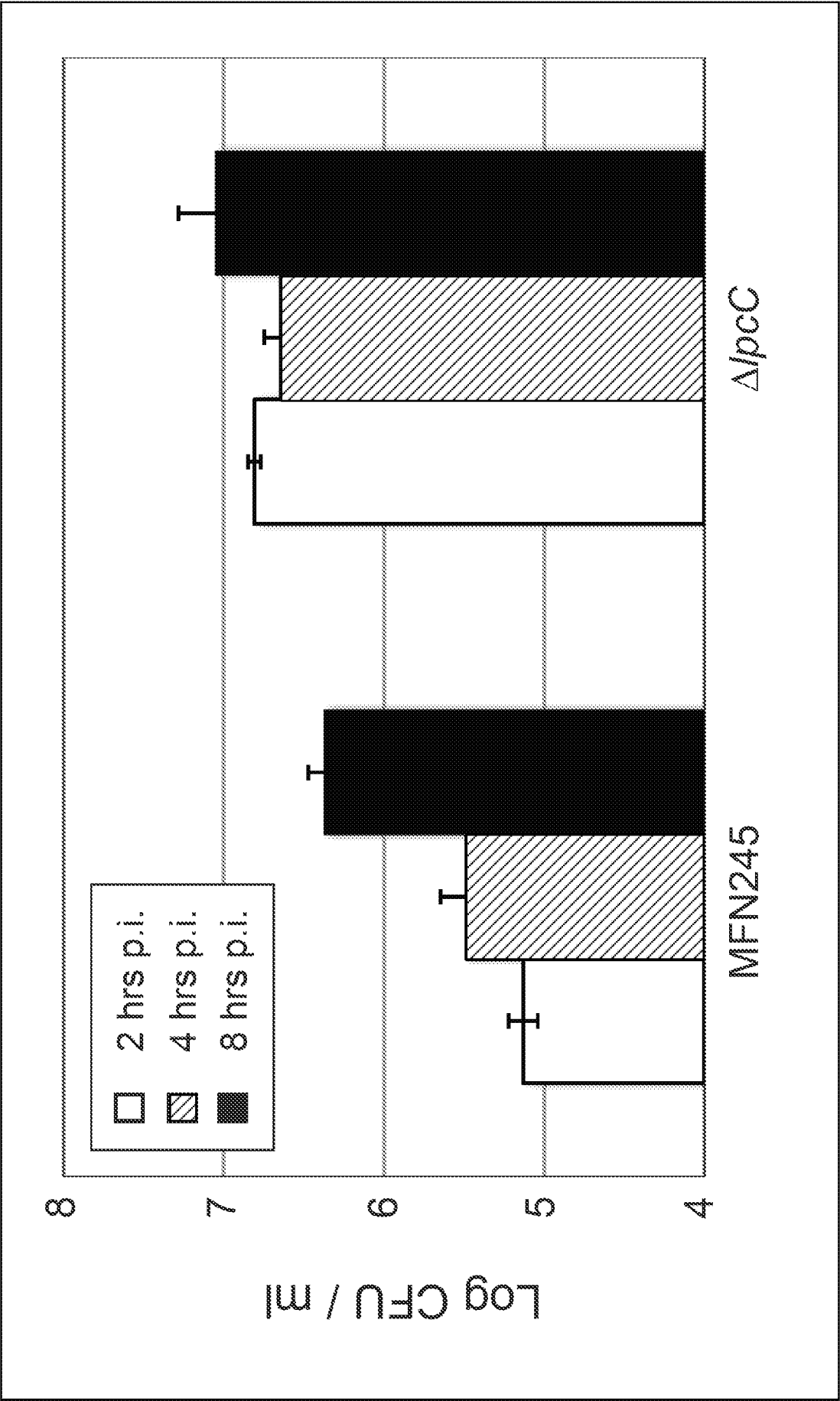


FIG. 14A

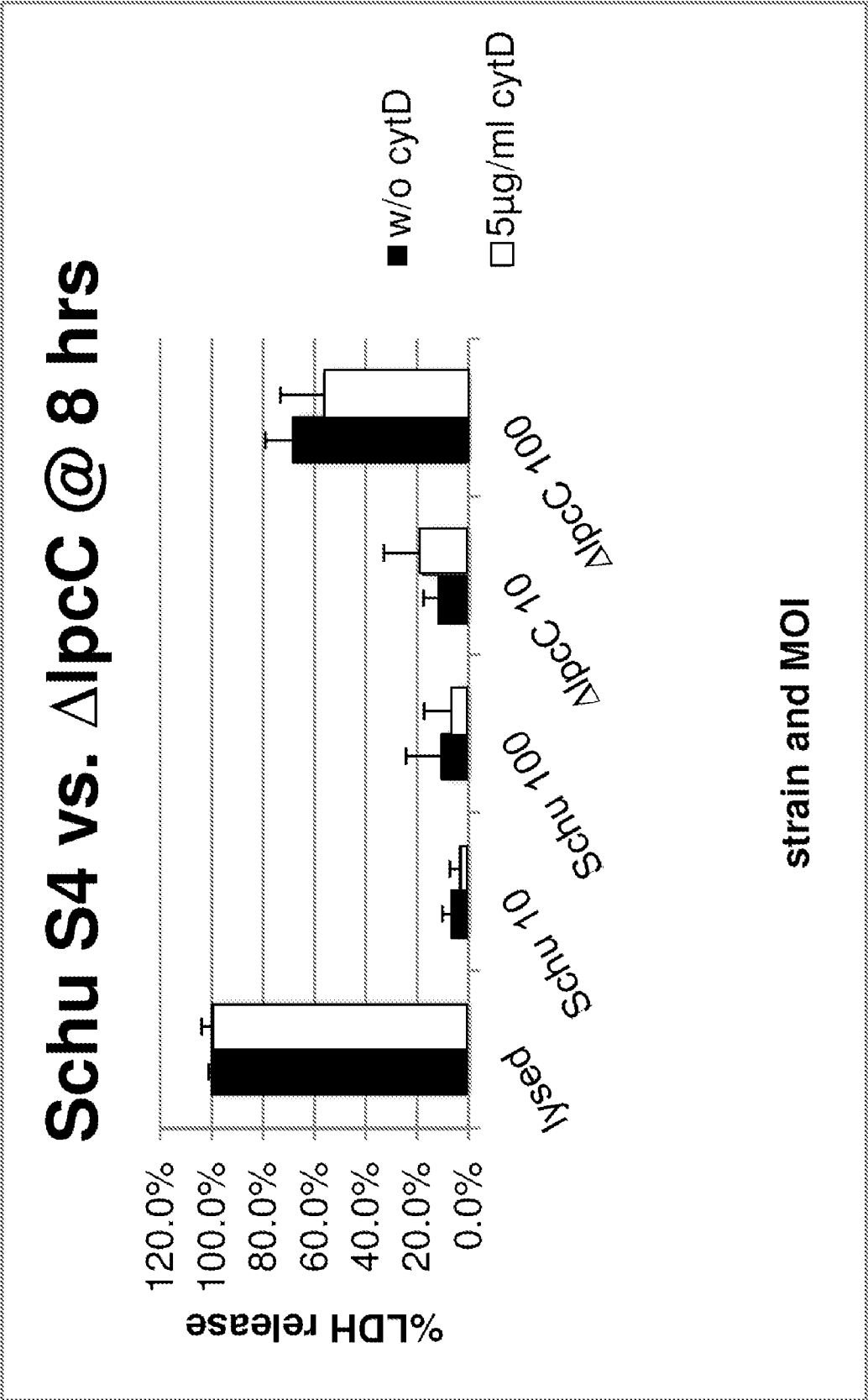


FIG. 14B

