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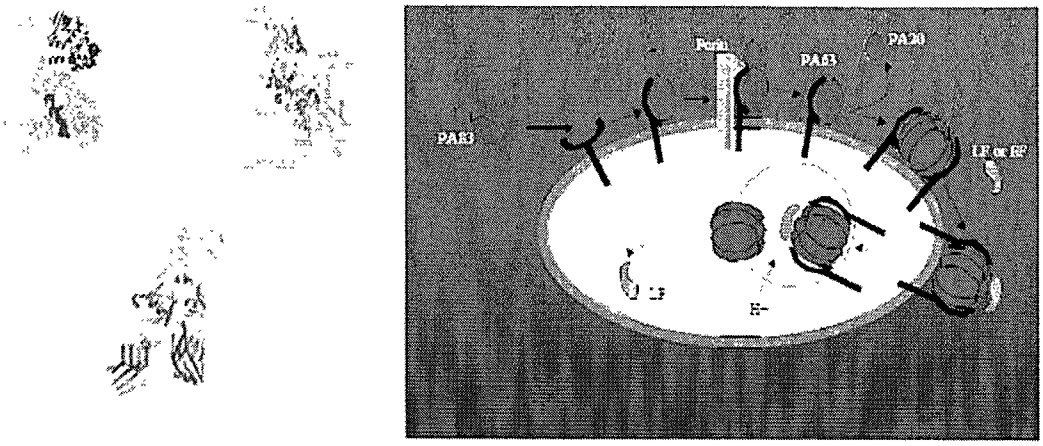
(54) **Title:** SALMONELLA BASED ORAL VACCINES FOR ANTHRAX

The Anthrax toxin cell uptake model



(57) **Abstract:** A vaccine for the prevention of anthrax, including a live, attenuated Salmonella and at least one nucleotide sequence encoding anthrax protective antigen (PA) or a fragment thereof and a nonlethal mutated form of anthrax lethal factor (LF) or a fragment thereof. In another implementation, the vaccine is constituted for the prevention of anthrax and at least one additional pathogen, as including a live, attenuated Salmonella and at least one nucleotide sequence encoding at least a fragment of a nonlethal mutated form of anthrax lethal factor (LF) and at least one nucleotide sequence encoding at least a fragment of an antigen of an additional pathogen. Vaccines of such types can be administered to stimulate antibody response in a subject, whereby the antibody response confers immunity to the subject.

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SALMONELLA BASED ORAL VACCINES FOR ANTHRAX

RELATED APPLICATION DATA

[0001] This application claims the benefit of priority under 35 USC 119(e) of U.S. Provisional Patent Application Serial No. 60/736,457, filed November 14, 2005, the disclosure of which is hereby incorporated herein by reference in its entirety, for all purposes.

GOVERNMENTAL INTERESTS

[0002] The invention was made with government support under Grant No. U19A1058578-01, awarded by the National Institutes of Health's (NIH) National Institute of Allergy and Infectious Diseases (NIAID). The United States government has certain rights in this invention.

FIELD OF THE INVENTION

[0003] The invention relates generally to *Salmonella* based oral vaccines for the treatment or prevention of anthrax, alone or in addition to another pathogen, and more specifically to live oral vaccines for inducing an immune response in a subject.

BACKGROUND OF THE INVENTION

[0004] Anthrax is an infection caused by the spore-forming bacterium *Bacillus anthracis*. Anthrax may enter the body and cause infection by means of inhalation, ingestion or subcutaneous exposure. While animals are most at risk for anthrax exposure, humans working with such animals may also be at risk. Additionally, recent heightened awareness of the possibility of bioterrorism has raised concerns about the use of *B. anthracis* or related strains, both newly emerging or genetically engineered, as bio-weapons.

[0005] There is therefore a need to develop vaccines for widespread use in the event of a bioterrorist attack, in order to minimize the exposure of a population to the bacteria. In particular, the ability to confer protection following oral dosing is particularly attractive in the context of a bioterrorism event as it would greatly simplify the process of mass vaccinations. Additionally, such a vaccine may be used in situations where a population may be at high risk of exposure to the bacteria, whether through bioterroristic activity or natural exposure, due to proximity to infected animals or to the spores.

[0006] The infective process of anthrax occurs when the spores are taken up by the body, through inhalation, ingestion or subcutaneous exposure. The spores become active toxic bacteria and express anthrax toxin, which will ultimately halt the host's immune response and cause cell death. Anthrax toxin has three components: anthrax protective antigen (PA), anthrax edema factor (EF) and anthrax lethal factor (LF). PA binds an anthrax toxin receptor (ATR) on the surface of the host cell. The PA is then cleaved by a host protease, activating the PA, which then binds to other active PAs to form a heptamer. The heptamer then binds EF or LF and the entire complex is drawn into the cell via endocytosis, forming an endosome within the host cell. The EF or LF is ejected from the endosome, into the cytosol of the cell. Once in the cytosol, LF and EF exert their enzymatic activities, interrupt cell signaling and damage the cells. EF ultimately causes edema and LF ultimately causes cell lysis.

[0007] The current FDA approved vaccine is a sterile product made from an avirulent, nonencapsulated strain of *B. anthracis*. The vaccine was approved by the FDA in 1970 and is generally administered to those considered at high risk, especially those in the United States who work in close contact with potentially infected animals or animal products, such as hides, hairs or bones, *e.g.* veterinarians and laboratory workers. The vaccine, BioThrax® (Anthrax Vaccine Adsorbed or "AVA"), is manufactured by one company, Emergent Biosolutions of Gaithersburg, Maryland (formerly Bioport Corporation, Lansing, Michigan). Possible reactions to the vaccine include local reactions, and very rare systemic reactions, causing flu-like symptoms. This vaccine requires six vaccinations over eighteen months (at 0, 2 and 4 weeks and at 6, 12 and 18 months), followed by yearly boosters; *see* BioThrax® AVA, prescription information, dosage instructions.

[0008] Various additional vaccines have been developed against anthrax. PA, as a potent immunogen is generally the target for such vaccines. PA is non-toxic and has been shown in numerous animal studies to be capable of stimulating the production of protective antibodies when given as a vaccine. It is thought that these antibodies protect by inhibiting the binding of PA to the host cell and/or binding to EF and/or LF. However, current vaccines suffer from problems such as poor levels of expression and the need for multiple dosing.

[0009] Thus, while certain prevention and treatment approaches may prove useful in modulating the effects of anthrax toxin, there remains a need for an effective and safe vaccine that would effectively produce immunity to anthrax with fewer doses.

[0010] Various vaccines have been discussed that target the natural mechanism of PA, LF and/or EF. For example, U.S. Patent No. 5,591,631 and U.S. Patent No. 5,677,274 describe fusion proteins including domains of PA and/or LF.

[0011] In another approach, U.S. Patent Application No. 2004/0166120 has described a composition which contains PA and a truncated, non-functional *B. anthracis* LFn for eliciting a *B. anthracis* immune response.

[0012] Additionally, U.S. Patent Application No. 2003/0003109 discusses vaccines that administer a polynucleotide with a coding sequence for a mutated LF protein or an immunogenic fragment of an LF protein and a polynucleotide with a coding sequence for PA or an immunogenic fragment of PA to a subject.

[0013] U.S. Patent Application No. 2005/0063986 discusses recombinant DNA constructs containing wild type or mutant type PA, LF or EF.

[0014] Additional approaches have focused on live vaccines as expression systems for PA, LF or EF, but have not been able to develop these vaccines for human use. Specific attempts focused on use of live *Salmonella* (Coulson, *et al. Vaccine*, vol. 12, No. 15, 1395-1401 (1994); Garmory, *et al. Infect. Immun.*, 71(7): 3831-6 (2003)) and *B. anthracis* (Aloni-Grinstein, *et al. Infect. Immun.*, 73(7): 4043-53 (2005)) have met with limited success, but have not been developed for human use. Additional work has focused on the possibilities of development of live vaccines (U.S. Application No. 2004/0197343), but have not identified a specific vaccine for use in humans utilizing a live virus containing genetic material from *B. anthracis*.

[0015] The utility of attenuated strains of *Salmonella* as a live oral vaccine for typhoid has resulted in the development of a licensed, FDA approved vaccine. There is considerable interest in building on this approach to develop *Salmonella* based vaccines capable of conferring protection against a range of infectious agents and cancer. In particular, development of a live oral anthrax vaccine would be desirable. Additionally, development of a live oral anthrax vaccine with additional activity against one or more additional pathogens would be desirable. However, attempts to use these methods with regard to anthrax have not succeeded to date. Investigations of the use of a live vaccine for expression and delivery of PA, LF and/or EF, have met with limited success, suffering the problem of low levels of expression. Live vaccines evoke the most effective immunity and are the least expensive to

produce but in practice are very difficult to make. Additionally, there is a concern that such vaccines would not be effective against genetically modified strains of *B. anthracis* or against other strains such as *Bacillus cereus* G9241, which has acquired the *B. anthracis* toxins and causes an anthrax-like infection in humans.

[0016] Therefore, there remains a need in the art for development of a vaccine using specific antigens from anthrax that are expressed in high quantity and do not require excessive dosing. A live vaccine would be preferred. Such a vaccine would preferably be effective against *B. anthracis*, genetically modified *B. anthracis*, anthrax-like strains, and/or additional pathogens, such as plague. In particular, an oral vaccine would be desirable for ease of administration. Such a vaccine is desirable for use in humans.

SUMMARY OF THE INVENTION

[0017] The present invention relates to live oral vaccines for the prevention of infection by anthrax, genetically modified *B. anthracis* or anthrax-like strains. The present invention also relates to live oral vaccines for the prevention of infection by anthrax, genetically modified *B. anthracis* or anthrax-like strains, and additional pathogen(s), e.g., plague.

[0018] Thus in one aspect the invention provides a vaccine for the prevention of anthrax comprising a live, attenuated *Salmonella*, where the *Salmonella* comprises at least one nucleotide sequence encoding PA and at least one nucleotide sequence encoding a non lethal mutated form of LF.

[0019] In another embodiment, the invention provides a vaccine for the prevention of anthrax, as above, but where the live, attenuated *Salmonella* comprises at least one nucleotide sequence encoding at least a fragment of PA and at least one nucleotide sequence encoding at least a fragment of a non lethal mutated form of LF.

[0020] In a preferred embodiment, the invention provides a vaccine for the prevention of anthrax, as above, but where the live, attenuated *Salmonella* comprises at least one nucleotide sequence encoding at least a fragment of PA and at least one nucleotide sequence encoding at least a fragment of a non lethal mutated form of LF, wherein the fragments of the PA and LF include at least one active epitope.

[0021] In still another embodiment the invention provides a method for inducing a cellular

immune response in a subject comprising administering a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding PA and at least one nucleotide sequence encoding a non lethal mutated form of LF.

[0022] The invention also provides a vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella*, where the *Salmonella* comprises at least one nucleotide sequence encoding PA or a fragment thereof and at least one nucleotide sequence encoding a non lethal mutated form of LF or a fragment thereof.

[0023] In yet another embodiment, the invention provides a vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella,* where the *Salmonella* comprises at least one nucleotide sequence encoding PA or a fragment thereof and at least one nucleotide sequence encoding a non lethal mutated form of LF or a fragment thereof, wherein the wherein the PA or fragment thereof and LF or fragment thereof include at least one active epitope.

[0024] In still a further embodiment, the invention provides a vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) Domain 4 or a fragment thereof.

[0025] In yet another embodiment, the invention provides a vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) Domain 1 or a fragment thereof.

[0026] In still another embodiment, the invention provides a vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof and at least one nucleotide sequence encoding an antigen of an additional pathogen, or fragment thereof. In one aspect the sequence encoding the LF and the sequence encoding the antigen of the additional pathogen are fused. In a further aspect the LF or fragment thereof is Domain 1. In still another aspect, the additional pathogen is plague.

[0027] In still another embodiment, the invention provides a method of stimulating antibody response in a subject through administration of a live, attenuated *Salmonella,* where the *Salmonella* comprises at least one nucleotide sequence encoding PA or a fragment thereof

and at least one nucleotide sequence encoding a non lethal mutated form of LF or a fragment thereof, wherein the LF contains at least one active epitope, wherein at least one antibody to the epitope is stimulated.

[0028] In a still further embodiment, the invention provides a method of stimulating antibody response in a subject, comprising administration of a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof, fused to a nucleotide sequence encoding an antigen of an additional pathogen, or fragment thereof, wherein the LF contains at least one active epitope, and wherein at least one antibody to the epitope is stimulated and at least one antibody to the antigen of the additional pathogen is generated. In another aspect the antibody response to the LF and the antigen of an additional pathogen confers immunity to the subject against a subsequent exposure to anthrax and the additional pathogen.

[0029] These and other aspects of the present invention are described with respect to particular preferred embodiments and will be apparent to those skilled in the art from the teachings herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] Figure 1 is a model of cellular uptake of the anthrax toxin. The illustration shows how, upon release PA molecules will selectively bind to host cell receptors. The PA is then cleaved by a protease and forms an activated PA that binds to other active PAs to form a heptamer. LF or EF will bind to the heptamer and the entire complex is drawn into the cell via endocytosis. Once in the cytosol, LF and EF exert their enzymatic activities and damage the cells. EF causes edema and LF causes cell lysis.

[0031] Figure 2 is a graph showing human serum samples obtained from vaccinated (U.S. and U.K. licensed anthrax vaccines) and infected individuals who had been treated for cutaneous anthrax. Samples were analyzed for the presence of antibodies to PA, LF and EF by ELISA.

[0032] Figure 3 is two graphs, A and B, showing titers of antibodies to LF Domain 1 protein (A) or PA (B) in the sera of BALB/c mice immunized with DNA plasmids encoding PA (pCPA), LF Domain 1 (pCLF4) or a combination of pCPA and pCLF4.

[0033] Figure 4 is an illustration showing the known toxin neutralizing antibody domains of PA.

[0034] Figure 5 is an illustration showing the LF domains expressed from *E. coli*.

[0035] Figure 6 is an illustration showing a LFn epitope delivery construct.

[0036] Figure 7 is a model of antigen uptake utilizing a *Salmonella* model.

[0037] Figure 8 is a graph of the immunogenicity of the various LF domains expressed from *E. coli* and purified.

[0038] Figure 9 is a graph of the results of an assay performed in mice of the ability of antibodies specific to individual LF domains to neutralize anthrax toxin activity. The mice were immunized with biologically inactive LF, an LF domain or domains or PA, as set forth in Example 2.

[0039] Figure 10 is two graphs of the immunogenicity in mice of LF domains in the absence (A) and presence (B) of PA, as determined by the process set forth in Example 3.

[0040] Figure 11 is a bar graph of the titer results of a toxin neutralization titer from mice immunized with LF domains and PA, as set forth in Example 3.

[0041] Figure 12 is a bar graph summarizing the titer results for LF Domain 1 and full length biologically inactive LF in the presence of PA, as determined by Example 3.

[0042] Figure 13 is a graph showing the percent survival of mice immunized by rLF protein and subsequently challenged with *B. anthracis* STI spores by the i.p. route, as set forth in Example 4.

[0043] Figure 14 is a graph showing anti-spore activity over time by macrophages and macrophages facilitated by antibodies.

[0044] Figure 15 is a Western blot of PA and LF domains with serum from a rabbit exposed to *B. cereus* G9241, where 1 is the MW ladder, 2 is PA, 3 is LF, 4 is MW ladder, 5 is LF Domain 1, 6 is LF Domain 2, 7 is LF Domain 3, 8 is LF Domain 4 and 9 is LF Domains 2-4.

[0045] Figure 16 is a comparison of the gene sequence of *B. anthracis* lethal factor (LF) as filed with Genebank (accession number M29081) (SEQ ID NO: 1) and the gene sequence of

biologically inactive LF as used herein (SEQ ID NO: 2), where the only significant change is replacement of amino acid 687 glutamic acid (GAA) with cysteine (TGC). The codon of the change is shown in lower case (tgc).

[0046] Figure 17 is the optimized sequence of biologically inactive *B. anthracis* LF (SEQ ID NO: 3).

[0047] Figure 18 is a comparison of the protein sequences of the translated gene sequences of *B. anthracis* LF (SEQ ID NO: 4) and biologically inactive LF set forth in Figure 16 (SEQ ID NO: 5).

[0048] Figure 19 shows two Western blots of LF Domain recognition of IQNLF (SEQ ID NO: 26), where a) is a native blot and b) is an SDS-denaturing blot.

[0049] Figure 20 shows a Western blot of LF Domain 1 expression in *S. enterica serovar Typhimurium* SL3261, where lane 1 is Bio-Rad low range standards, lane 2 is SL3261/pSEC10-LFnN#26, lane 3 is SL3261/pSEC10-LFnN#29, lane 4 is SL3261/pSEC10-LFnN#31, lane 5 is SL3261/pSEC10-LFoN#26, lane 6 is SL3261/pSEC10-LFoN#27, lane 7 is SL3261/pSEC10-LFoN#33), lane 8 is Recombinant LF dom1, lane 9 is *E. coli* TOP10/pSEC10, and lane 10 is Bio-Rad low range standards. Probed with mouse monoclonal antibody to LF Domain 1.

[0050] Figure 21 is a comparison of the immunogenicity of *S. Typhi* Ty21a (pSEC/PA) and Ty21a (pVDL9-3pA83ec), as determined by a) presence of IgG a-PA antibodies and b) presence of IgG a-LPS antibodies.

[0051] Figure 22 shows the results of a heterologous prime boost in mice immunized with *S. Typhi* Ty21a expressing PA, where the boost was a single dose of 1 ug recombinant PA. Naïve mice boosted with 1 ug recombinant PA were provided as a control.

[0052] Figure 23 shows the results of protection studies of mice challenged with *B. anthracis* after oral inoculation with *S. Typhimurium* expressing PA plasmids.

[0053] Figure 24 is an alignment of the original (SEQ ID NO: 11) and codon optimized (SEQ ID NO: 12) nucleotide sequences coding for the optimized region of the LF Domain 1-PA Domain 4 fusion protein, as described in Example 10.

[0054] Figure 25 is a protein alignment of the optimized region of the LF Domain 1-PA Domain 4 fusion protein, aligning the original (SEQ ID NO: 13) and codon optimized (SEQ ID NO: 14) sequences, as set forth in Example 10.

[0055] Figure 26 is the full length optimized LF Domain 1-PA Domain 4 fusion protein (SEQ ID NO: 15), as described in Example 10.

[0056] Figure 27 is a Western blot of the immunogenicity of the LF Domain 1-PA Domain 4 fusion protein, as determined by recognition by rabbit polyclonal anti-PA and rabbit polyclonal anti-LF specific antisera, as set forth in Example 10.

[0057] Figure 28 shows the IgG specific antibody response of BALB/c mice immunized with the LF Domain 1-PA Domain 4 fusion protein, as set forth in Example 11.

[0058] Figure 29 shows the immunogenicity of LFn/V and V/LFn fusion proteins in BALB/c mice, as set forth in Example 12.

[0059] Figure 30 shows a multi-agent delivery construct of the invention.

[0060] Figure 31 is the complete amino acid sequence (SEQ ID NO: 24) of the LcrV-MCS-LFR4-IQLF-PA2D3-PAD4loop/DR1-F1- L fusion protein, as set forth in Example 13.

[0061] Figure 32 is the *Salmonella* codon optimized gene sequence (SEQ ID NO: 25) of the LcrV-MCS-LFR4-IQLF-PA2D3-PAD4loop/DR1-F1- L fusion protein, as set forth in Example 13.

[0062] Figure 33 is a Western blot showing the immunogenicity of the LcrV-MCS-LFR4-IQLF-PA2D3-PAD4loop/DR1-F1- L fusion protein, as set forth in Example 13.

[0063] Figure 34 shows the IgG specific antibody response of BALB/c mice immunized with proteins LFD1, PA and LcrV-MCS-LFR4-IQLF-PA2D3-PAD4loop/DR1-F1- L.

DETAILED DESCRIPTION OF THE INVENTION

[0064] A new generation of vaccine against anthrax would ideally contain PA and LF in combination, would be a live vaccine, safe and non-toxic to humans and require minimal dosage in an easily administered manner. The present invention provides such a new generation of vaccine. As set forth below in detail, the vaccines of the invention offer the following advantages:

[0065] i) stimulate an LF specific immune response and contribute to protection;

[0066] ii) confer sterile immunity against newly emerging anthrax causing organisms and genetically engineered spore formers;

[0067] iii) enhance the antibody response to both PA and LF;

[0068] iv) stimulate strong T cell memory responses in humans;

[0069] v) as an LFn fusion system, facilitate the uptake of additional protective epitopes for anthrax ; and

[0070] vi) provide a platform for the delivery of vaccine targets against a range of targets such as the F1 and LcrV antigens of plague.

[0071] "Vaccine" as used herein is a preparation that stimulates an immune response that produces immunity. Vaccines may be used to prevent infection, to create resistance to an infection or to ameliorate the effects of infection. Vaccines may contain, but are not limited to, live, attenuated infectious material such as viruses or bacteria, and dead or inactivated organisms or purified products derived therefrom. A vaccine can be administered by injection, orally or by inhalation. Injection may be, but are not limited to, subcutaneous (sc), intramuscular (im), intraperitoneal (ip), intradermal (id) or intravenous (iv).

[0072] "Immunogen" or "antigen" as used herein is a substance that that is foreign to the body that stimulates an immune response, such as the production of antibodies when introduced into the body. Immunogens or antigens are also capable of reacting with the products of an immune response. Immunogens or antigens may include, but are not limited to proteins or polypeptides, enzymes, toxins, bacteria, viruses, foreign tissues, foreign blood cells, and the cells of transplanted organs. Correspondingly, "immunogenicity" is the ability of an immunogen or antigen to stimulate an immune response.

[0073] "Antibody" or "immunoglobulin," as used herein is a protein produced by the immune system that helps destroy disease-causing organisms. Antibodies are made and secreted by B lymphocytes in response to stimulation by antigens, which may include vaccines. Antibodies are generally specific, binding only to the specific antigen that stimulated its production. A given antigen can have many epitopes, each one reacting with the immune system to create antibodies specific for each of the epitopes. Antibodies can be

effective defenders against both bacteria and viruses, in addition to toxins. Antibodies can be polyclonal or monoclonal.

[0074] "Pathogen" as used herein refers to a biological agent, for example, a microbe such as a virus or bacteria that infects its host, causing disease, illness or other deleterious effect on the host. Such pathogens may be introduced through bio-terrorism actions or may be naturally encountered. The vaccine of the claimed invention is useful against pathogens such as, but not limited to, *B. anthracis*, *B. cereus*, *S. typhi*, *F. tularensis*, *B. abortis*, *B. pseudomallei*, *Y. pestis* and *C. botulinum* neurotroxins.

[0075] As used herein, a "fusion gene" is a gene created by removing the stop protein from the sequence of a gene and attaching the DNA sequence of a second gene to the first. By fusing one nucleotide sequence to another, the host cell will express the sequences together, as a single fused protein. Fusion genes may contain two or more fused genes. Accordingly, "fusion protein" as used herein is a protein produced by expression of a fusion gene.

[0076] PA is the key protective immunogen of the currently licensed human anthrax vaccines. Traditionally, PA based vaccines have suffered from poor levels of expression of PA, requiring multiple dosages of the vaccine. Attempts have been made to develop live *Salmonella* vaccines against anthrax, but attempts to date have been unsuccessful. It is believed that this poor immunogenicity of PA expression in *Salmonella* strains may be due to a number of factors. The codon usage of the PA gene is profoundly different from that on the majority of *Salmonella* genes such that it could cause translation problems. Additionally, high level expression of large foreign genes can place an enormous burden on the *Salmonella*, compromising the biological fitness of the *Salmonella* itself and encouraging the emergence of mutations which inactivate gene expression. The protein itself may be toxic for *Salmonella* resulting in suppression of further protein production. Once expressed in the cytoplasm, the protein is liable to proteolytic degradation by *Salmonella* proteases such that little remains to be exported out of the bacterium and finally the bacterial export system may not be able to efficiently secrete the protein resulting in further degradation of exported protein due to it being unable to assume a stable tertiary configuration.

[0077] However, the current invention provides live vaccines, such that they are administered in live, attenuated strains of *Salmonella*.

[0078] PA is a powerful immunogen and has generally been the focus of vaccines against

anthrax. However, there is also animal protection data to support the inclusion of detoxified LF in a combined PA/LF vaccine. Studies in mice have demonstrated that LF, when expressed from a DNA vaccine, in the absence of PA, is capable of conferring some protection against injected toxin challenge (Price, *et al. Infect. Immun.*, 69(7):4509-15 (2001)). Studies with DNA vaccines expressing human codon optimized LF Domain 1-3 demonstrated partial protection in rabbits against aerosol challenge with spores of the highly lethal Ames strain (Galloway, *et al. Vaccine* 22(13-14):1604-8 (2004); Hermanson *et al. Proc. Natl. Acad. Sci. USA* 14;101(37):13601-6(2004)).

[0079] The inventor of the present invention has shown that in addition to conferring protection, LF appears to be a more potent human immunogen than PA. The inventor has found that individuals who contract cutaneous anthrax respond much earlier to LF than PA and mount a more robust antibody response. (Figure 2.)

[0080] The inventor has combined his findings with the fact that LF co-administered with PA enhanced the PA specific antibody response of immunized mice. (Pezard *et al. Infect. Immun.* 63(4):1369-72 (1995); Price *et al. Infect. Immun.* 69(7):4509-15 (2001)). This is also illustrated in Figure 3.

[0081] The individual regions of the PA and LF proteins have been determined. The domains of PA have been identified as set forth in Figure 4. Individual regions that bind protective mouse and human antibodies have been identified. The present inventor has previously identified two such regions, one located within PA Domain 3 which binds the mouse protective monoclonal antibody 2D3 and is recognized by the serum from anthrax vaccinated humans (Baille et al., 2004). An additional binding region within the PA domains is the host binding cell domain of PA (Domain 4) which has also been shown to bind mouse (Flick-Smith et al., 2002a, 2002b) and human toxin neutralizing antibodies (unpublished data, Figure 4) and be capable of conferring complete protection against lethal spore challenge.

[0082] A toxin neutralizing PA Domain 4 specific monoclonal antibody was isolated from an individual who had been vaccinated with a licensed human anthrax vaccine. The ability of the antibody to recognize PA was determined by ELISA and Western blot. The ability of the antibody to neutralize toxin activity was demonstrated *in vitro* and the ability of the antibody to passively protect mice against a lethal *B. anthracis* spore challenge was confirmed by experiment.

[0083] Similarly, the regions of LF have been identified. The domains of LF have been identified as set forth in Figure 5. The adjuvant effect of LF in combination with PA appears to lie in the N-terminal 254 amino acids of LF (LF Domain 1), the region of the protein which binds to PA and has been exploited by researchers as a means of delivering antigens ranging in size from CTL epitopes of 9 amino acids to HIV proteins of 550 amino acids into the cytosol of antigen presenting cells, leading to the stimulation of CD8 and CD4 T cell responses. (Ballard *et al. Infect. Immun.* 66(10):4696-9 (1998); Ballard *et al. Infect. Immun.* 66(2):615-9 (1998); Lu, *et al. Proc Natl Acad Sci USA* 97(14):8027-32 (2000); Price *et al. Infect. Immun.* 69(7):4509-15 (2001)).

[0084] In addition to isolating PA specific human monoclonals, a protective human monoclonal was also isolated which recognized LF Domain 1. The amino acid recognition sequence of this antibody is discussed more fully herein and illustrated in Figure 19.

[0085] Once identified, the individual domains were expressed and assessed for immunogenicity and protective efficacy in mice.

[0086] In particular, Example 1 as set forth below determines the IgG specific antibody responses to each of the individual LF domains. The LF gene used for Domain 1 was optimized as set forth in Figure 16. The codon usage of a gene is known to have a profound effect on its ability to express in different bacterial backgrounds. Previously it was demonstrated that altering the codon usage of the PA gene to that common to *E.coli* markedly increased the level of protein expression from *E.coli* and *Salmonella spp.* (Garmory *et al. Infect. Immun.* 71(7):3831-6 (2003)). Interestingly other researchers have also attempted to optimize the codon use of PA for *Salmonella* with little success. However, alteration of the LF gene in the present invention, as set forth in Figure 16, reflecting the codon usage of *Salmonella typhi* vaccine strain TY21a will optimize expression of LF Domain 1.

[0087] The level of anthrax toxin neutralizing antibodies has been demonstrated to be a correlate of protection against anthrax in mice, guinea pigs and rabbits. Therefore, Example 2 sets forth a determination of the level of anthrax toxin neutralizing antibodies specific to the individual LF domains.

[0088] Example 3 then determines the level of immunogenicity of each of the domains, as co-delivered with PA. It is noted that the results of Example 3 show that only LF Domain 1, when given with PA stimulates a toxin neutralizing titer higher than that seen for PA alone.

This may be due to the presence of a B cell epitope described herein which is recognized by a human toxin neutralizing antibody. It should be noted that the co-administration of a full length LF with PA reduced the toxin neutralizing titer to a level lower than that seen for PA alone. These results suggest that LF Domain 1 is a stronger vaccine candidate than full length LF in terms of its ability to stimulate toxin neutralizing antibodies in the presence of PA.

[0089] Example 4 provides the first demonstration that Domain 1 of LF, when delivered as a purified protein, is able to confer complete protection against lethal spore challenge with *B. anthracis*. This result is supported by fact that Domain 1 contains a protective B cell linear epitope recognized by a human monoclonal antibody which is able to passively protect mice from a lethal spore challenge. Example 5 shows that both this hmAb and a similar protective hmAb against PA are able to bind to spores and facilitate the subsequent killing of the organism by macrophages.

[0090] In one embodiment the invention provides a vaccine for the prevention of anthrax comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF). In a preferred aspect, the codons in the nucleotide sequences are preferred by the *Salmonella*.

[0091] In another embodiment, the invention provides a method for enhancing antibody response for anthrax protective antigen (PA) in a subject. The method comprises administering a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF). In a preferred aspect, the codons in the nucleotide sequences are preferred by the *Salmonella*.

[0092] In still another embodiment, the invention provides a vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding at least a fragment of anthrax protective antigen (PA) and at least one nucleotide sequence encoding at least a fragment of a non lethal mutated form of anthrax lethal factor (LF). In a preferred aspect, the sequence encoding the fragment of anthrax PA is fused to the sequence encoding the fragment of anthrax LF, forming a fusion gene construct. In a further preferred aspect, the vaccine induces a CTL response in a subject. The mutated

form of LF may be wild type LF with replacement of amino acid 687 glutamic acid encoded by the codon GAA with cysteine encoded by the codon TGC, among other mutations. In a preferred aspect, the codons in the nucleotide sequences are preferred by the *Salmonella*, such that expression is maximized.

[0093] In another embodiment, the invention provides a method for inducing a cellular immune response in a subject comprising administering a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF). In a preferred aspect, the cellular immune response comprises increased secreting T lymphocytes. In another preferred aspect, the subject is human.

[0094] In still another embodiment, the invention provides a vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) or a fragment thereof and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof. In a preferred aspect, the sequence encoding the PA or fragment of PA is fused to the sequence encoding the LF or fragment of LF, forming a fusion gene construct. In a further preferred aspect, the mutated form of LF may be wild type LF with replacement of amino acid 687 glutamic acid encoded by the codon GAA with cysteine encoded by the codon TGC, among other mutations. In one aspect, the fragment of LF is Domain 1. In another aspect, the PA is full length and the LF is a fragment, where the fragment is Domain 1. In yet another aspect the vaccine induces a CTL response in a subject. In another preferred aspect, the vaccine induces an anthrax toxin neutralizing activity. In still another preferred aspect, the codons in the nucleotide sequences are preferred by the *Salmonella*, such that expression is maximized.

[0095] Additionally, the invention provides a vaccine that delivers epitopes to the immune system. In one aspect a construct comprising LF Domain 1 will be utilized where the LF contains at least one epitope of interest. Such epitopes may include, but are not limited to key protective T (CD4) and B cell epitopes.

[0096] The inventor has characterized the inherent immunogenicity of the LF Domain 1 for humans. Using serum and cells from human volunteers immunized with the U.K. licensed anthrax vaccine, which contains PA and low levels of LF, it has been shown that LF Domain

1 contains both protective B cell (SDVLEMYKAIGGKIYIVDGDITKHISLEAL (SEQ ID NO: 6)) and immunodominant human CD4 T cell epitopes (Baillie *et al.*, 2004; manuscript in preparation). The inventor has also generated a human monoclonal antibody from a previously immunized donor which binds LF Domain 1 and protects laboratory animals from a lethal anthrax spore challenge.

[0097] In addition to neutralizing toxin activity the inventor has also identified certain PA and LF specific antibodies with opsonic activity. It has been previously found that polyclonal human PA specific IgG antibodies bind to the anthrax spore surface and promote uptake by macrophages in such away that the germinating bacterium is subsequently killed before it has the chance to initiate significant gene expression thus achieving sterile immunity (Kang *et al.*, *Infect. Immun.* 73(11):7495-501 (2005)). The inventor has demonstrated a similar phenomenon with human monoclonal antibodies which recognize PA and LF Domain 1 (unpublished data).

[0098] The ability to kill the organism before it has the change to express virulence genes, be they naturally present or acquired as the result of genetic engineering, is of considerable advantage in the context of emerging bio-threats. In recent years the feasibility of introducing additional virulence genes in to *B. anthracis* was demonstrated by researchers in the former Soviet Union who generated a strain which defeated the Russian human anthrax vaccine (Pomerantsev *et al.*, *Vaccine* 15(17-18):1846-50 (1997)). It is also possible to transfer the major toxin genes from *B. anthracis* and have them expressed in the genetic close relative *B. cereus* (Hoffmaster *et al.*, *Proc. Natl. Acad. Sci. USA* 101(22):8449-54 (2004)).

[0099] Therefore, the present invention provides a vaccine containing relevant PA and LF specific epitopes, as described above, which stimulate antibodies which confer sterile immunity and thus confer protection against these strains of anthrax.

[00100] Thus in one embodiment, the invention provides vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding at least a fragment of anthrax protective antigen (PA) and at least one nucleotide sequence encoding at least a fragment of a non lethal mutated form of anthrax lethal factor (LF), wherein the fragments of the protective antigen and lethal factor include at least one active epitope. In various aspects of the invention, the active epitope(s) may include, but are not limited to a furin cleavage site of the protective antigen, a host cell

binding domain of the protective antigen, the N-terminal region of the lethal factor, T and B cell epitopes. In an additional aspect of the invention, the nucleotide sequence further encodes for C3D at the C-terminal end of the fusion construct. In a preferred aspect, the codons in the nucleotide sequences are preferred by the *Salmonella.*

[00101] In another embodiment, the invention provides a vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) or a fragment thereof and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof, wherein the wherein the PA or fragment thereof and LF or fragment thereof include at least one active epitope.

[00102] In a still another embodiment, the mutated form of LF may be wild type LF with replacement of amino acid 687 glutamic acid encoded by the codon GAA with cysteine encoded by the codon TGC, among other mutations. In one aspect, the fragment of LF is Domain 1. In another aspect, the PA is full length and the LF is a fragment, where the fragment is Domain 1. In still another aspect, the epitope is a T cell epitope. In still a further aspect, the epitope is a B cell epitope. In still another preferred aspect, the codons in the nucleotide sequences are preferred by the *Salmonella*, such that expression is maximized.

[00103] In still another embodiment, the invention provides a method of stimulating antibody response in a subject, comprising administration of a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) or a fragment thereof and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof, wherein the LF contains at least one active epitope, wherein at least one antibody to the epitope is stimulated. In a further preferred aspect, the mutated form of LF may be wild type LF with replacement of amino acid 687 glutamic acid encoded by the codon GAA with cysteine encoded by the codon TGC, among other mutations. In one aspect, the fragment of LF is Domain 1. In another aspect, the PA is full length and the LF is a fragment, where the fragment is Domain 1. In still another aspect, the epitope is a T cell epitope. In still a further aspect, the epitope is a B cell epitope. In one aspect the antibody stimulated is a monoclonal antibody. In another aspect the antibody is a human monoclonal antibody. In still another aspect, the antibody confers immunity to the subject such that the subject is protected against a strain of anthrax subsequently introduced to the subject. In a further aspect of this embodiment, the strain of anthrax is *B. anthracis* or

B. cereus G9241. In still another preferred aspect, the codons in the nucleotide sequences are preferred by the *Salmonella*, such that expression is maximized. In an additional aspect the subject is human.

[00104] Including epitopes to both LF in addition to PA would be useful in the case of deliberate circumvention of epitope binding sites within PA such that they are no longer recognized by previously protective antibodies but still retain their biological function (Rosovitz *et al.*, 2003). For example, *B. cereus* G9241 possess two homologs of the PA gene, the first encodes a protein (PA1) identical to that of *B. anthracis* while a second homolog (PA2) which shows 60% amino acid identity and is thought to be biologically functional and is not recognized by antibodies raised against PA1 (Hoffmaster *et al.*, 2004; unpublished data). Since PA1 is the major protective immunogen of both the current licensed human anthrax vaccine and its second generation replacement, there are concerns that these vaccines will be unable to stimulate a protective response against G9241.

[00105] While G9241 expresses two copies of PA it has only one complete copy of LF. The expression of this gene was confirmed by Western blotting individual LF domains with serum from a rabbit which had been exposed to G9241 (Figure 12).

[00106] Therefore in another embodiment, the invention provides vaccines including individual epitopes. In one embodiment the invention provides a vaccine for the prevention of anthrax comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) Domain 4 or a fragment thereof. In a further aspect the PA Domain 4 or a fragment thereof contains at least one active epitope.

[00107] In another embodiment the invention provides a vaccine for the prevention of anthrax comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) Domain 1 or a fragment thereof. In a further aspect the LF Domain 1 or a fragment thereof contains at least one active epitope.

[00108] In addition to contribution directly to protection, the LF protein can also be employed to present fusion proteins to the immune system. One example of a fusion construct of the invention is set forth in Figure 6. There appear to be at least two mechanisms involved in LF mediated uptake. The first is PA dependent and makes use of the ability of the PA to transport LF into the cytosol of the cell (Ballard *et al. Infect. Immun.* 66(10):4696-9

(1998); Ballard *et al. Infect. Immun.* 66(2):615-9 (1998); Lu, *et al. Proc Natl Acad Sci USA* 97(14):8027-32 (2000)). It is capable of delivering LF Domain 1 fusion proteins as large as 500 amino acids into antigen presenting cells and stimulating both CTL and CD4 responses. This system is extremely potent requiring as little as 300 fmol of fusion to stimulate a response in mice and does not require the presence of an external adjuvant. Using this approach, researchers were able to prime a CTL response without invoking an antibody response to either PA or LF Domain 1 (Ballard *et al.*, 1998). The second mechanism is PA independent and is enhanced by the presence of the adjuvant Alum (Cao *et al.*, J. Infect. Dis. 185(2):244-51 (2002), Kushner *et al.*, *Proc Natl Acad Sci USA* 100(11):6652-7 (2003)). The fusion protein were found to localize with the 20s subunit, which degrades proteins into peptides for presentation to CD8 T cells by the MHC class 1 pathway. These results suggest that LF Domain 1 may be used as a carrier to deliver antigens into the cytosol of antigen presenting cells for efficient induction of T cell responses.

[00109] Utilization of LF Domain 1 as such a carrier can be effected by fusing LF Domain 1 to the additional vaccine targets from other infectious agents for delivery to the immune system. The ability of LF Domain 1 to deliver vaccine epitopes to the immune system has been previously reported by Ballard. An example of this utility is set forth below in Example 10, where a fusion protein of LF Domain 1 and PA Domain 4 has been generated and delivered.

[00110] Additionally, the invention provides an LFn-fusion construct which includes epitopes for an additional pathogen. Such epitopes may include, but are not limited to, protective epitopes or regions of PA, protective regions or epitopes of anthrax, LcrV and/or F1, as set forth in the examples below. Such a fusion construct may consist of one or more additional epitopes for one or more additional pathogens.

[00111] In still another aspect, the additional pathogen is plague. In the case of plague, two vaccine target antigens have been identified, F1 and LcrV, which are currently undergoing phase II human trials in the U.S. and Europe. The F1 protein is expressed optimally at 37°C and is thought to inhibit phagocytosis through the formation of a capsule-like structure on the bacterial surface. LcrV plays a key role in type III secretion by *Yersinia spp*, a process that allows the injection of a set of effector proteins (YOPS) directly into the cytosol of eukaryotic target cells which promote the killing of phagocytic cells. Evidence has accumulated from a number of studies that antibody plays a key role in protection against

plague. Circulating antibodies specific for F1 and LcrV antigens are thought to access the bacterium in its predominantly extracellular state and bind to surface exposed protein. While F1 and LcrV antigens have been shown to induce protective immunity when administered individually, in combination or as a fusion, these proteins have been shown to have an additive protective effect when used to immunize mice against plague. As set forth in Example 12, LFn/V and V/LFn fusion proteins are immunogenic.

[00112] Based on the above findings, a further aspect of the invention is a multivalent vaccine platform base in an attenuated *Salmonella* strain which is able to deliver LFn linked to fusion proteins such as LcrV and F1 or specific protective B and T cell epitopes, as set forth in Figure 30. Still another aspect of the invention is a fusion protein as set forth above, but omitting PA. This latter aspect is based on the protective character of LFn, *e.g.*, when administered as a protein. This protection can be achieved by incorporating protective regions from PA into the LFn fusion construct.

[00113] Accordingly, in one embodiment the invention provides vaccine for the prevention of anthrax comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof and at least one nucleotide sequence encoding an antigen of an additional pathogen or fragment thereof. In one aspect the sequence encoding the LF or fragment thereof is fused to the sequence encoding the antigen of the additional pathogen. In still another aspect the LF or fragment thereof includes at least one active epitope. In another aspect the LF is LF Domain 1. In a further aspect the additional pathogen is plague. In still another aspect the antigen includes at least one active epitope. In a still further aspect, the active epitope may be, but is not limited to F1 or LcrV.

[00114] A still further aspect of the invention provides a fusion protein with only those B and T cell epitopes key to protection against a certain pathogen fused to LF Domain 1. The resulting protein will be considerably smaller than a protein containing large numbers of fused epitopes and thus entails a lower likelihood of physiological stress when expressed from Salmonella, as well as a lower likelihood of toxicity issues.

[00115] In yet another embodiment, the invention provides a method of stimulating antibody response in a subject comprising administration of a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax

lethal factor (LF) or a fragment thereof fused to at least one nucleotide sequence encoding an antigen of an additional pathogen or fragment thereof. In the embodiment, the LF contains at least one epitope and at least one antibody is generated to the epitope. Furthermore, at least one antibody is generated to the antigen of the additional pathogen. In another aspect the stimulated antibody response to the LF epitope confers immunity to the subject against anthrax, genetically modified *B.* anthracis or anthrax-like strains subsequently introduced to the subject. In another aspect the stimulated antibody response to the antigen of the additional pathogen confers immunity to the subject against a strain to the pathogen subsequently introduced to the subject. In one aspect the subject is human. In another aspect the additional pathogen is plague.

[00116] In addition, heterologous primer-boosting may be performed to increase the immune response to an antigen. In particular, heterologous primer-boosting may be described as the administration of two different vaccines that encode the same antigen at various times points, by the same or alternative routes. The implementation of such a strategy has been shown to improve the immune response to PA in a range of animal species including primates. An example of such heterologous primer-boosting can be seen in Example 8 set forth below. Therefore, in one aspect, the invention provides vaccines generating an increased immune response due to heterologous primer-boosting, as compared to administration of the vaccine without such boosting.

[00117] Finally, the invention provides a fusion construct with the molecular adjuvant C3D incorporated into the fusion construct at the C terminus. C33 is utilized due to its inability to enhance the level and affinity of the antibody response. C3D is a fragment of the third component of the compliment activation pathway. (Figure 7) It is thought to exert its effect in 2 ways, 1) acting directly on memory B cells via the CD21 receptor amplifying the activation of the cell following binding of the antigen to its immunoglobulin receptor, which in turn stimulates antibody production and 2) enhancing binding of the fusion protein to follicular dendritic cells resulting in the generation of high affinity antibodies thus enhancing the quality of the immune response. This approach has been previously used to enhance antibody responses and protective immunity to influenza virus by generating high affinity neutralizing antibodies to hemagglutinin (Ross et al 2000). Accordingly, inclusion of C3D will similarly enhance the antibody response to PA and LF and protective immunity to anthrax.

[00118] A vaccine of the invention, comprising PA and Domain 1 of LF, affords considerable advantages over the current vaccine, particularly against newly emerging and genetically engineered anthrax strains. Such strains may include, but are not limited to, *B. cereus* G9241. The ability to target both PA and LF provides a broad spectrum utility of this vaccine. In addition the PA/LF Domain 1 system provides a basis for a multi-agent platform capable of conferring protection against a range of infectious agents.

[00119] The vaccines of the invention may also be used to confer protection against infectious agents in addition to anthrax. In this embodiment, a single oral dose would confer protection against all included threats of infection. Such additional agents may include, but are not limited to, plague, such as that caused by *S. typhi*. As such, the vaccines will protect the subject against a range of public health pathogens. Such a multi-agent platform can be utilized to confer protection against any one or a combination of the following infectious agents: *B. anthracis*, *B. cereus*, *S. typhi*, *F. tularensis*, *B. abortis*, *B. pseudomallei*, *Y. pestis* and *C. botulinum* neurotroxins. Other such agents will be known to those in the art.

[00120] The vaccines of the invention may be used in methods of prophylaxis and treatment. As utilized in treatment, the vaccines may be administered to a subject previously exposed to an infectious agent. Administration of the vaccines of the invention stimulates an immune response in a subject, generating antibodies against the infectious agent. As such, the vaccines of the invention may be administered before or after exposure to an infectious agent in order to stimulate such an immune response.

[00121] The following examples are intended to illustrate but not limit the invention.

EXAMPLE 1
DETERMINATION OF IMMUNOGENICITY OF INDIVIDUAL LF DOMAINS

[00122] This example was performed to determine the regions of LF capable of stimulating a protective immune response when given as a vaccine. In order to maximize LF expression in *E. coli* and *Salmonella*, the codon usage of the LF gene was optimized, as shown in Figure 16. The resynthesized gene comprising the mature protein was detoxified, by replacing the glutamic acid at position 687 within the catalytic center with a cysteins (Klimpel, Arora and Leppla, *Mol. Microbiol.*, 1994, 13(6); 1093.). Individual LF domains (see Figure 5) were cloned and expressed from *E. coli* M15 (pREP4) using a pQE-30/pQE-

80L, an N-terminal Hist tag expression system (QIAgen, Inc., Valencia, CA).

[00123] The immunogenicity of the expressed and purified LF domains was determined in BALB/c mice. Each group, comprising 10 animals, received i.m. 10ug of domain protein together with the adjuvant alum on days 0 and 28. Animals were bled on days 1,13,27,56 and 72. The LF specific IgG antibody responses were determined by ELISA. The results are shown in Figure 8, showing the extreme immunogenicity of domains 1, 2, 4 and 2-4, as compared to a negative control and phosphate buffered saline (PBS).

EXAMPLE 2
TOXIN NEUTRALIZING ACTIVITY BY ANTIBODIES SPECIFIC TO INDIVIDUAL LF DOMAINS

[00124] The ability of the individual expressed and purified LF domains to stimulate toxin neutralizing antibodies was determined in BALB/c mice. Each group, comprising 10 animals, received i.m. 10ug of domain protein together with the adjuvant alum on days 0 and 28. Animals were bled on days 1,13,27,56 and 72. The LF specific IgG antibody responses were determined by ELISA.

[00125] The ability of antibodies specific to individual LF domains to neutralize toxin activity was determined. The results are set forth in Figure 9. As can be seen in the figure, full length PA was the most effective at stimulating toxin neutralizing antibodies. Domains 1, 2, 4 and 2-4 showed moderate levels of stimulation of toxin neutralizing antibodies, as did biologically inactive full length LF. Domains 3 and 4 showed no detectable toxin neutralization.

EXAMPLE 3
DETERMINATION OF IMMUNOGENICITY OF INDIVIDUAL LF DOMAINS CO-DELIVERED WITH PA

[00126] Groups of 10 BALB/c mice were immunized with 10ug of each protein with PA with alum i.m. on days 0 and 28. Animals were bled on days 1, 13, 27, 56 and 72 and the IgG response to PA and each LF domain was determined by ELISA. Figure 10 shows the results. Only Domain 3 showed an increase in LF specific IgG antibody response when co-administered with PA. The same was true for the PA specific IgG response (data not shown).

[00127] It should be noted that the individual domain specific antibody responses stimulated during this study were extremely high and as a consequence any adjuvant effects

may have been swamped. The toxin neutralizing titer (Example 2) is a more precise measure of protection against anthrax. The mean toxin neutralizing antibody titers for the animals immunized with PA and LF domains is shown in Figures 11 and 12.

[00128] This data suggests that with the exception of LF Domain 1, that co-administration of full length LF with PA actually inhibits the quality of the toxin neutralizing response.

EXAMPLE 4
PROTECTION AGAINST ANTHRAX BY ADMINISTRATION OF BIOLOGICALLY INACTIVE FULL LENGTH LF OR LF DOMAIN 1

[00129] The ability of biologically inactive LF (replacement of amino acid 687 glutamic acid (GAA) with a cysteine (TGC) (Klimpel *et al.,* 2004); *See* Figure 16) and LF Domain 1 to protect A/J mice against a lethal anthrax spore challenge was determined.

[00130] Each group, comprising 8 animals, received *i.m.* 10ug of protein together with the adjuvant Alhydrogel on days 0, 4 and 28. Animals were given a lethal i.p. spore challenge (100 MLDs) on day 80. Results are shown in Figure 13 and it can be seen that Domain 1 of LF conferred complete protection against a lethal spore challenge with *B. anthracis.*

EXAMPLE 5
GENERATION OF ANTIBODIES TO LF DOMAIN 1 AND PA

[00131] Domain 1 contains a protective B cell linear epitope recognized by human monoclonal antibody which is able to passively naive protect mice from a lethal spore challenge.

[00132] As previously noted this hmAb, and a similar protective hmAb against PA, is able to bind to spores and facilitate the subsequent killing of the organism by macrophages (Figure 14).

EXAMPLE 6
EXPRESSION OF LF DOMAIN 1 IN *S. ENTERICA* SEROVAR TYPHIMURIUM SL3261

[00133] As LF Domain 1 is protective as a protein against *B. anthracis,* both codon optimized (LFoN) and native (LFnN) versions of LF Domain 1 were generated and

individually fused to *S. enterica* serovar Typhimurium cytolysin A (ClyA) as a ClyA fusions in pSEC10. Expression of the protein from *Salmonella* is shown by Western blot in Figure 20, as probed with mouse monoclonal antibody to LF Domain 1. Individual lanes in Figure 20 show the following: lanes: 1) Bio-Rad low range standards; 2) SL3261/pSEC10-LFnN#26; 3) SL3261/pSEC10-LFnN#29; 4) SL3261/pSEC10-LFnN#31; 5) SL3261/pSEC10-LFoN#26; 6) SL3261/pSEC10-LFoN#27; 7) SL3261/pSEC10-LFoN#33); 8) Recombinant LF dom1; 9) *E. coli* TOP10/pSEC10; 10) Bio-Rad low range standards.

EXAMPLE 7
EXPRESSION OF PA IN *SALMONELLA*

[00134] Two plasmid constructs were made expressing an identical, codon optimized version of the PA gene. (Williamson et al., WO/2002/04646.)

[00135] The first construct, pVDL-9.3PAsec is a low copy number plasmid which has been used to express PA as a fusion to the *Escherichia coli* haemolysin (Hly) export system, in order to enable export of the expressed PA protein from the *Salmonella* (Garmory et al 2003). The second construct, pSEC10PA, is based on a recently described *S. enterica* serovar Typhi cytolysin A (ClyA) export system which has been used to express a domain of PA from *Salmonella* (Galen et al., 2004).

[00136] To determine the immunogenicity of the constructs, the plasmids described above were transformed into the human vaccine strain *S. typhi* Ty21a and assessed for immunogenicity in BALB/c mice when given via the intranasal route. The results are seen in Figure 21.

[00137] The results show that Ty21a pSEC/PA stimulated an extremely robust PA specific IgG antibody response (Figure 21a). This marked difference in immune response was not due to any failure in the mice in recognizing the *Salmonella* construct as all animals immunized with *Salmonella* generated strong LPS specific immune responses (Figure 21b).

EXAMPLE 8
HETEROLOGOUS PRIME BOOST

[00138] Recently it was demonstrated that the attenuated vaccine strain *Salmonella enterica* serovar Typhi strain CVD 908-*htrA* expressing fragment C of tetanus toxin was able

to stimulate significantly higher serum antitoxin responses in mice when the animals received an initial intranasal priming dose of *Salmonella* followed by an intramuscular boost with tetanus toxoid (Vindurampulle et al., 2004).

[00139] This example provides animals immunized with *S.Typhi* Ty21a expressing PA and boosted with a single i.m. dose of 1ug recombinant PA (rPA) protein with the adjuvant alum 56 days after the last *Salmonella* dose. A group of naïve mice received 1ug of rPA as a reference control. The results are summarized in Figure 22.

[00140] Figure 22 shows that boosting with rPA further enhances the magnitude of the PA specific immune response in all of the animals. While naïve mice demonstrated a three fold increase in PA specific titer the response of the mice immunized with Ty21a expressing PA varied depending on the vector.

[00141] Those animals given Ty21apVDL9.3pA83ec showed a much greater increase in PA titer, >3 logs, than the mice previously immunized with Ty21apSECPA/10 who only managed an increase in the 1-2 log range, comparable to that seen in the naïve animals which received rPA alone. This may be due to the fact that the animals given Ty21a pSEC/PA10 already had high levels of PA specific IgG at the time of the protein boost and as a consequence the antibody response of the animals was overloaded.

[00142] These results suggest that while Ty21a pVDL9.3pA83ec may not be as effective as Ty21a pSEC/PA10 at directly stimulating PA specific IgG response it can confer a robust memory response.

EXAMPLE 9
PROTECTION STUDIES OF MICE CHALLENGED WITH *B. ANTHRACIS* AFTER INOCULATION WITH *S. TYPHIMURIUM* EXPRESSING PA PLASMIDS

[00143] To determine the ability of *Salmonella* constructs expressing PA to protect against exposure to a fatal aerosol challenge with spore of *B. anthracis*, groups of A/J mice were immunized 3 times at two week intervals with approx 1 x 10^9 cfu/ml of *S. typhimurium* SL3261 by the oral route. Positive control mice were immunized by the i.p. route with 3 x 10 μg of purified recombinant protein adjuvanted with alhydrogel at two week intervals.

[00144] Following immunization, mice were challenged with approximately 1 x 10^5 CFU of aerosolized *B. anthracis* STI (Tox^+ Cap^-) spores and monitored for survival. Naïve

mice and those given the SL3261 parental control strain succumbed to infection within 6 days, while the mice receiving SL3261/pSEC10 all died by day 8.

[00145] However, 2 of 8 mice immunized with SL3261/pVDL-9.3PAsec were protected against challenge, and 5 of the 6 mice inoculated with SL3261/pSEC10PA also survived. Thus, SL3261/pSEC10PA expressing the full-length PA protein as a fusion with ClyA, afforded significant protection against aerosolized *B. anthracis* spore challenge ($p<0.01$ compared to SL3261/pSEC10).

EXAMPLE 10
FUSION PROTEIN OF LF DOMAIN 1 AND PA DOMAIN 4

As PA Domain 4 is the immunodominant region of PA and is capable of conferring protection against anthrax when administered as a protein (Flick-Smith et al., 2002), and as LF Domain 1 is also able to confer protection (Example 4), the following fusion protein comprising Domain 1 of LF linked to Domain 4 of PA via a multiple cloning site has been generated:

LF Domain 1
AGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVK
KEAAEKLLEKVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDAL
LHEHYVYAKEGYEPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSKINQPYQKFL
DVLNTIKNASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQ
HRDVLQLYAPEAFNYMDKFNEQEINL (SEQ ID NO: 7)

Mutiple cloning site
GAG CTC GGT ACC (SEQ ID NO: 8)

E L G T (SEQ ID NO: 27)

PA Domain 4
TNIYTVLDKIKLNAKMNILIRDKRFHYDRNNIAVGADESVVKEAHREVINSSTEGLLL
NIDKDIRKILSGYIVEIEDTEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYI
SNPNYKVNVYAVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG (SEQ ID NO: 9)

The amino acid sequence representing Domain 4 was extended at the N-terminal region to include a region of PA thought to bind a protective human toxin neutralizing monoclonal antibody called 2D3 (unpublished).

IKLNAKMNILIRDKRFHYDRN (SEQ ID NO: 10)

[00146] The original (SEQ ID NO: 11) and codon optimized (SEQ ID NO: 12) nucleotide sequences of the optimized region of the corresponding fusion protein, optimized for *Salmonella*, are shown in alignment in Figure 24. Figure 25 is a protein alignment of the optimized region of the LF Domain 1-PA Domain 4 fusion protein, aligning the original

(SEQ ID NO: 13) and codon optimized (SEQ ID NO: 14) sequences. Figure 26 is the full length optimized LF Domain 1-PA Domain 4 fusion protein (SEQ ID NO: 15).

[00147] The fusion protein was expressed using the QIAgen expression system and purified as described in the manual using a Nikel affinity column. The ability of the fusion protein to be recognized by rabbit polyclonal anti-PA and rabbit polyclonal anti-LF specific antisera was determined by Western blot (Figure 27).

EXAMPLE 11
IMMUNOGENICITY OF LF DOMAIN 1 -PA DOMAIN 4 FUSION PROTEIN

[00148] The immunogenicity of the LF DOMAIN 1 -PA DOMAIN 4 fusion protein was determined in mice as follows. Groups of 10 BALB/c mice were immunized i.m. on days 0 and 28 with 10ug of each components (LFD1-PAD4 fusion, LFD1, PAD4, Control (PBS)) in combination with alum. Animals were tail bled on days –1, 13, 27, 56 and 72 and PA and F1 specific antibody responses were determined by ELISA. Results are set forth in Figure 28.

[00149] As set forth in Figure 28, the results show that the mice that received the LF1PAD4 fusion mount a comparable LF specific immune response to that seen in the mice which received only LFD1. This suggests that the addition of PAD4 as a 3' fusion has no adverse effect on the LF specific antibody response. The same appears to be true for the PA specific response.

EXAMPLE 12
FUSION PROTEIN OF LF DOMAIN 1 AND PLAGUE VACCINE TARGETS

[00150] Preliminary animal studies have confirmed the utility of the PA/LFn protein fusion approach set forth above for LcrV. Mice immunized with rPA in combination with rLFn-LcrV demonstrated a significantly higher antibody response to LcrV. It was also found that the orientation of the fusion protein was important, linking LFn to the C terminal of LcrV resulted in a significantly higher antibody response.

[00151] Figure 29 demonstrates that LFn/V and V/LFn fusion proteins are immunogenic and elicit anti-LF and anti V serum IgG responses in BALB/c mice. Animals were immunized on days 0, 14 and 28. Data shown represented pooled samples at day 40.

EXAMPLE 13
LcrV-MCS-LFR4-IQLF-PA2D3-PAD4loop/DR1-F1- L FUSION PROTEIN

[00152] A LcrV-MCS-LFR4-IQLF-PA2D3-PAD4loop/DR1-F1- L fusion protein has been generated from the following component sequences:

LcrV- A vaccine candidate for plague

MIRAYEQNPQHFIEDLEKVRVEQLTGHGSSVLEELVQLVKDKNIDISIKYDPRKDSEV
FANRVITDDIELLKKILAYFLPEDAILKGGHYDNQLQNGIKRVKEFLESSPNTQWELR
AFMAVMHFSLTADRIDDDILKVIVDSMNHHGDARSKLREELAELTAELKIYSVIQAEI
NKHLSSSGTINIHDKSINLMDKNLYGYTDEEIFKASAEYKILEKMPQTTIQVDGSEKKI
VSIKDFLGSENKRTGALGNLKNSYSYNKDNNELSHFATTCSDKSRPLNDLVSQKTTQ
LSDITSRFNSAIEALNRFIQKYDSVMQRLLDDTSGK (SEQ ID NO: 16)

MCS- multiple cloning site to enable the incorporation of additional protective epitopes

GCA TGC GAG CTC GGT ACC (SEQ ID NO: 17)

LFR4- a region of LF thought to contain a murine toxin neutralizing mAb antibody epitope (Lim et al., 2005)

DSLSEEEKELLNRIQVDSS (SEQ ID NO: 18)

IQLF- a region of LFn thought to bind a protective human toxin neutralizing monoclonal antibody (unpublished)

SDVLEMYKAIGGKIYIVDGDITKHISLEAL (SEQ ID NO: 19)

PA2D3- a region of PA though to bind a protective human toxin neutralizing monoclonal antibody (unpublished)

IKLNAKMNILIRDKRFHYDRN (SEQ ID NO: 20)

PAD4loop/DR1- the region encompassing the small loop of Domain 4 which is essential for binding of PA to its receptor. This region also contains part of the epitope recognized by the toxin neutralizing murine mAb 14B7 (Rosovitz et al., 2003). This region also contains an immunodominant DR1 T cell epitope (unpublished data)

KKYNDKLPLYISNPNYKVNVYA (SEQ ID NO: 21)

F1- a region of F1, the second plague vaccine candidate, thought to bind a protective animal monoclonal antibody (unpublished)

AADLTASTTATATLVEPARITLTYKEGAPITIM (SEQ ID NO: 22)

LFn- The protective N terminal domain of LF (see above)

AGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVK
KEAAEKLLEKVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDAL
LHEHYVYAKEGYEPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSKINQPYQKFL

DVLNTIKNASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQ HRDVLQLYAPEAFNYMDKFNEQEINL (SEQ ID NO: 23)

[00153] To determine the immunogenicity and protective efficacy of this fusion protein, the protein was codon optimized for *Salmonella* and synthesized by Genescript as a Hist tagged protein. The complete amino acid sequence (SEQ ID NO: 24) is shown in Figure 31 and the *Salmonella* codon optimized gene sequence (SEQ ID NO: 25) is shown in Figure 32. The protein was subsequently expressed from *E. coli* and the purified protein was analyzed by Western blot (Figure 33) and used to immunize animals (Examples 14 and 15 below).

EXAMPLE 14
IMMUNOGENICITY STUDY OF LCRV-LFR4-IQLF-PA2D3-PAD4LOOP/DR1-F1-LFN FUSION PROTEIN

[00154] Groups of 10 BALB/c mice were immunized i.m. on days 0 and 28 with 10ug of components (1) LFD1, (2) PA, (3) LcrV-LFR4-IQLF-PA2D3-PAD4loop/DR1-F1- LFn and (4) Control (PBS) in combination with alum. Animals were tail bled on days –1, 13, 27, 42 and 56 and PA, LF, LcrV and F1 specific antibody responses were determined by ELISA and are set forth in Figure 33.

[00155] The results show that the mice that received the LcrV.PA.F1.LFD1 fusion mounted a comparable LF specific immune response to that seen in the mice which received only LFD1 suggesting that the addition of the LcrV.PA.F1 as a 5' fusion has no adverse effect on the LF specific antibody response. The same was the case for the LcrV specific response. It is noted that failure to induce a PA and F1 specific response is not surprising, as the presentation of these epitopes was not optimized.

EXAMPLE 15
IMMUNOGENICITY STUDY OF LCRV-LFR4-IQLF-PA2D3-PAD4LOOP/DR1-F1-LFN FUSION PROTEIN (2)

[00156] Groups of 12 A/J mice were immunized i.m. on days 0 and 14 with 10ug of protein in combination with alhydrogel. Protein utilized was as follows:

1.rPA

2.rLrcV-LFn

3.rF1-LFn

4.rLcrV-LFR4-IQLF-PA2D3-PAD4loop/DR1-F1- LFn

[00157] Animals are tail bled to determine the magnitude of vaccine specific antibody responses and subjected to live agent aerosol challenge 3 weeks after the last vaccine dose. Such a challenge consists of a lethal aerosol challenge with *Y. pestis* strain GB at 1x 10^3 - 1 x 10^4 cfu/mouse.

EXAMPLE 16
IMMUNOGENICITY IN MICE

[00158] Mice vaccinated in accordance with vaccines discussed herein can be tested for immunogenicity by exposure to a lethal aerosol challenge with *B. anthracis* strain STI spores at approx 1 x 10^4 spore/mouse.

EXAMPLE 17
IMMUNOGENICITY AND EFFICACY OF *SALMONELLA* EXPRESSING BOTH PA AND LFn

[00159] Since LF Domain 1 is protective against *B. anthracis,* co-expression of both PA and LF1 in *Salmonella* are evaluated. Initially, the LF protein is evaluated for expression in *Salmonella* alone. Thus, a synthetic LF Domain 1 gene (LFoN), codon-optimized for expression in *E. coli*, and the native LF Domain 1 gene (LFnN) have been cloned into pSEC10 and expression of LFnN and LFoN is demonstrated by Western blotting.

[00160] The immunogenicity of *Salmonella* construct expression LF Domain 1 is determined in the A/J mouse model following oral dosing with approximately 1 x 10^9 cfu ml^{-1}. Immunogenicity of each construct is measured by quantifying the specific IgG end-point titer for each group using an ELISA format. Subsequently, immunized mice are challenged with approximately 1 x 10^5 cfu (approximately 200 MLDs) of aerosolized *B. anthracis* STI spores and monitored for survival.

[00161] To determine the efficacy of a co-administration of PA and LFn two approaches are adopted. In the first mice are immunized with a combination of two Salmonella constructs expressing PA and LFn separately. Finally a construct is made expressing both PA and LFn.

EXAMPLE 18
IMMUNOGENICITY AND EFFICACY OF *SALMONELLA* EXPRESSING BOTH LcrV-PA-LFn

[00162] The Lcrv-PA-LFn fusion protein is cloned into the pSEC10 vector and expressed as ClyA fusion from *Salmonella*. The ability of this construct to confer protection against anthrax and plague is determined following oral dosing.

[00163] The immunogenicity of *Salmonella* constructs is determined in the A/J mouse model following oral dosing with approximately 1 x 10^9 cfu ml^{-1}. Immunogenicity of each construct is measured by quantifying the specific IgG end-point titer for each group using an ELISA format. Subsequently, immunized mice are challenged with either aerosolized anthrax (~1 x 10^5 cfu) or plague (1 x 10^4 cfu) and monitored for survival.

[00164] Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention. Accordingly, the invention is limited only by the following claims.

A SALMONELLA BASED ORAL VACCINE FOR ANTHRAX

What is claimed is:

1. A vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF).

2. The vaccine of claim 1, wherein codons in the nucleotide sequences are preferred by the *Salmonella*.

3. A method for enhancing antibody response for anthrax protective antigen (PA) in a subject, the method comprising administering a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF).

4. The method of claim 3, wherein codons in the nucleotide sequences are preferred by the *Salmonella*.

5. The method of claim 3, wherein the subject is human.

6. A vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding at least a fragment of anthrax protective antigen (PA) and at least one nucleotide sequence encoding at least a fragment of a non lethal mutated form of anthrax lethal factor (LF).

7. The vaccine according to claim 6, wherein the fragment of anthrax PA is fused to the fragment of anthrax LF.

8. The vaccine according to claim 6, wherein the vaccine induces a CTL response in a subject.

9. The vaccine of claim 6, wherein the mutated form of LF is wild type LF comprising replacement of amino acid 687 glutamic acid encoded by the codon GAA with cysteine encoded by the codon TGC.

10. The vaccine of claim 6, wherein codons in the nucleotide sequences are preferred by the *Salmonella*.

11. A vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding at least a fragment of anthrax protective antigen (PA) and at least one nucleotide sequence encoding at least a fragment of a non lethal mutated form of anthrax lethal factor (LF), wherein the fragments of the protective antigen and lethal factor include at least one active epitope.

12. The vaccine according to claim 11, wherein the active epitope is a furin cleavage site of the protective antigen.

13. The vaccine according to claim 11, wherein the active epitope is host cell binding domain of the protective antigen.

14. The vaccine according to claim 11, wherein the active epitope is the N-terminal region of the lethal factor.

15. The vaccine according to claim 11, wherein the active epitope include T and B cell epitopes.

16. The vaccine according to claim 11, further comprising a nucleotide sequence encoding for C3D on the C-terminal end of the nucleotide sequence encoding PA and the nucleotide sequence encoding LF.

17. The vaccine of claim 11, wherein codons in the nucleotide sequences are preferred by the *Salmonella*.

18. A method for inducing a cellular immune response in a subject comprising administering a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF).

19. The method of claim 18, wherein the cellular immune response comprises increased secreting T lymphocytes.

20. The method of claim 18, wherein the subject is human.

21. A vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) or a fragment thereof and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof.

22. The vaccine of claim 21, wherein the PA or fragment thereof is fused to the LF or fragment thereof.

23. The vaccine of claim 21, wherein the mutated form of LF is wild type LF comprising replacement of amino acid 687 glutamic acid encoded by the codon GAA with cysteine encoded by the codon TGC.

24. The vaccine of claim 21, wherein the fragment of LF is Domain 1.

25. The vaccine of claim 21, wherein the PA is not a fragment and the LF is a fragment comprising Domain 1.

26. The vaccine according to claim 21, wherein the vaccine induces a CTL response in a subject.

27. The vaccine of claim 21, wherein the vaccine induces an anthrax toxin neutralizing activity.

28. The vaccine of claim 21, wherein codons in the nucleotide sequences are preferred by the *Salmonella*.

29. A vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) or a fragment thereof and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof, wherein the wherein the PA or fragment thereof and LF or fragment thereof include at least one active epitope.

30. The vaccine of claim 29, wherein the mutated form of LF is wild type LF comprising replacement of amino acid 687 glutamic acid encoded by the codon GAA with cysteine encoded by the codon TGC.

31. The vaccine of claim 29, wherein the fragment of LF is Domain 1.

32. The vaccine of claim 29, wherein the PA is not a fragment and the LF is a fragment comprising Domain 1.

33. The vaccine of claim 29, wherein the epitope is a T cell epitope.

34. The vaccine of claim 29, wherein the epitope is a B cell epitope.

35. The vaccine of claim 29, wherein codons in the nucleotide sequences are preferred by the *Salmonella*.

36. A method of stimulating antibody response in a subject, comprising administration of a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) or a fragment thereof and at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof, wherein the LF contains at least one active epitope, wherein at least one antibody to the epitope is stimulated.

37. The method of claim 36, wherein the mutated form of LF is wild type LF comprising replacement of amino acid 687 glutamic acid encoded by the codon GAA with cysteine encoded by the codon TGC.

38. The method of claim 36, wherein the fragment of LF is Domain 1.

39. The method of claim 36, wherein the PA is not a fragment and the LF is a fragment comprising Domain 1.

40. The method of claim 36, wherein the epitope is a T cell epitope.

41. The method of claim 36, wherein the epitope is a B cell epitope.

42. The method of claim 36, wherein the antibody is a monoclonal antibody.

43. The method of claim 42, wherein the monoclonal antibody is a human monoclonal antibody.

44. The method of claim 36, wherein the antibody confers immunity to the subject such that the subject is protected against a strain of anthrax subsequently introduced to the subject.

45. The method of claim 44, wherein the strain is *B. anthracis*.

46. The method of claim 44, wherein the strain is *B. cereus* G9241.

47. The method of claim 36, wherein codons in the nucleotide sequences are preferred by the *Salmonella*.

48. The method of claim 36, wherein the subject is human.

49. A vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding anthrax protective antigen (PA) Domain 4 or a fragment thereof.

50. The vaccine of claim 49 wherein the PA Domain 4 or fragment thereof includes at least one active epitope.

51. A vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) Domain 1 or a fragment thereof.

52. The vaccine of claim 51 wherein the LF Domain 1 or fragment thereof includes at least one active epitope.

53. A vaccine for the prevention of anthrax, comprising a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof and at least one nucleotide sequence encoding an antigen of an additional pathogen, or fragment thereof.

54. The vaccine of claim 53 wherein the at least one nucleotide sequence encoding LF or fragment thereof is fused to the at least one nucleotide sequence encoding an antigen of an additional pathogen, or fragment thereof.

55. The vaccine of claim 53 wherein the LF or fragment thereof includes at least one active epitope.

56. The vaccine of claim 53, wherein the LF is a fragment of full length LF and the fragment is Domain 1.

57. The vaccine of claim 53 wherein the additional pathogen is plague.

58. The vaccine of claim 57 wherein the antigen of plague or fragment thereof includes at least one active epitope.

59. The vaccine of claim 53 wherein the antigen is selected from F1 and LcrV antigens of plague.

60. A method of stimulating antibody response in a subject, comprising administration of a live, attenuated *Salmonella* comprising at least one nucleotide sequence encoding a non lethal mutated form of anthrax lethal factor (LF) or a fragment thereof, fused to a nucleotide sequence encoding an antigen of an additional pathogen, or fragment thereof wherein the LF contains at least one active epitope, and wherein at least one antibody to the epitope is stimulated and at least one antibody to the antigen of an additional pathogen is generated.

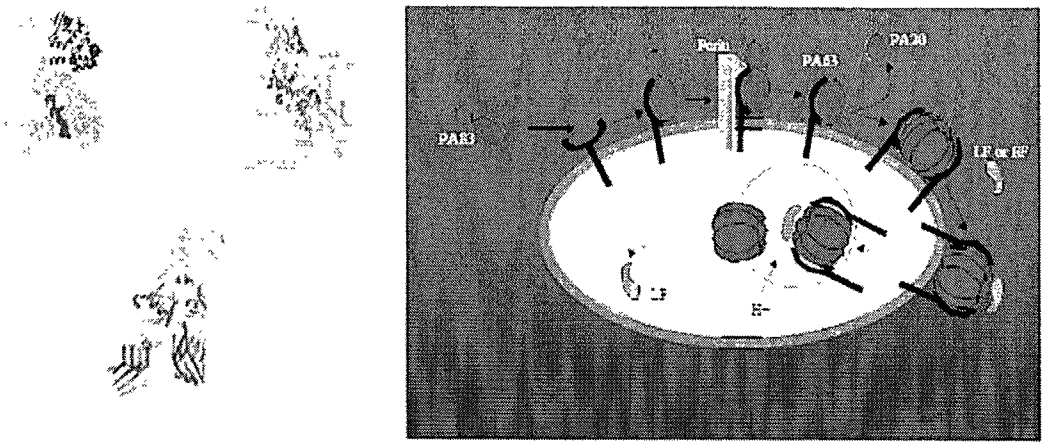
61. The method of claim 60, wherein the at least one antibody to the epitope confers immunity to the subject such that the subject is protected against anthrax, genetically modified *B. anthracis*, and anthrax-like strains subsequently introduced to the subject.

62. The method of claim 60, wherein the at least one antibody to the antigen of an additional pathogen confers immunity to the subject such that the subject is protected against a strain of the pathogen subsequently introduced to the subject.

63. The method of claim 60, wherein the additional pathogen is plague.

64. The method of claim 60, wherein the subject is human.

Figure 1
The Anthrax toxin cell uptake model



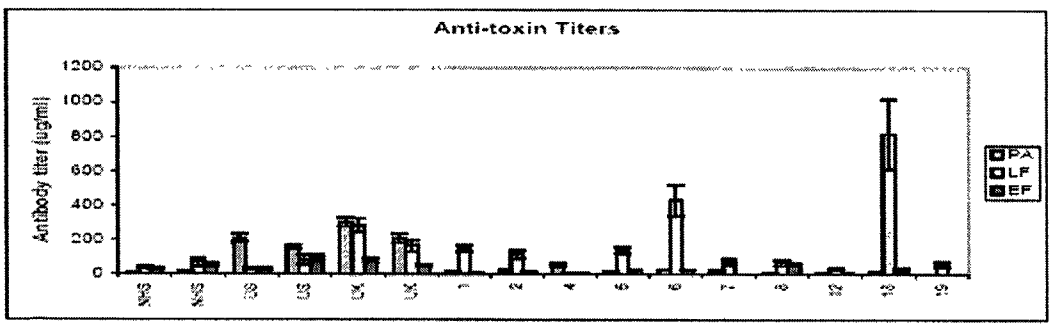


Figure 2. Human serum samples where obtained from vaccinated (US and UK licensed anthrax vaccines) and infected individuals who had been treated for cutaneous anthrax. Samples were analyzed for the presence of antibodies to PA, LF and EF by ELIZA.

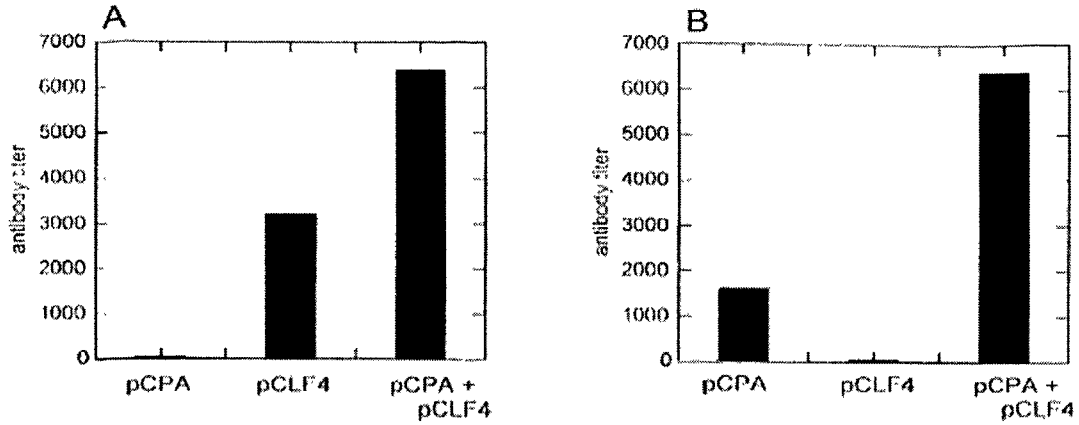


Figure 3. Titers of antibodies to LF domain 1 protein (A) or PA (B) in the sera of BALB/c mice immunized with DNA plasmids encoding PA (pCPA) LF Domain 1 (pCLF4) or a combination of pCPA and pCLF4 (Price *et al.*, 2001).

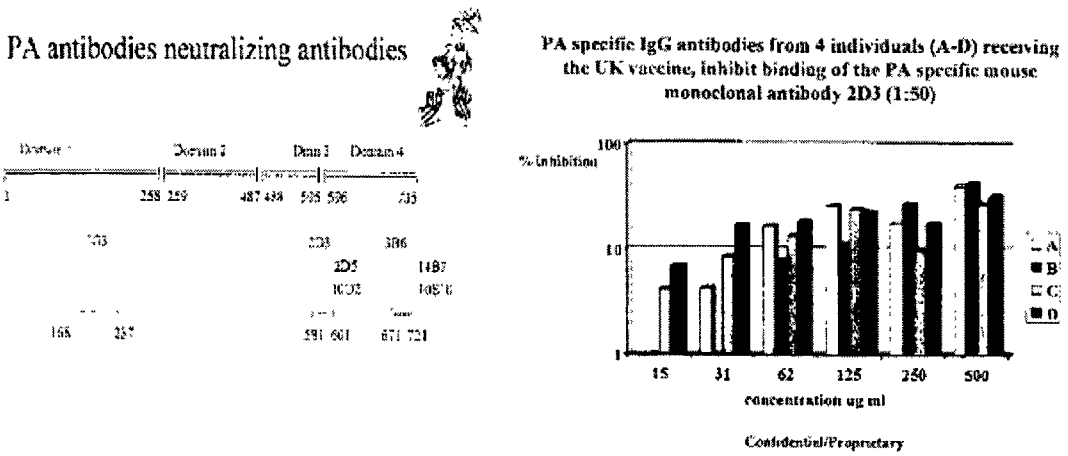


Figure 4, The known toxin neutralizing antibody domains of PA

Figure 5

The LF Domains expressed from *E. coli*

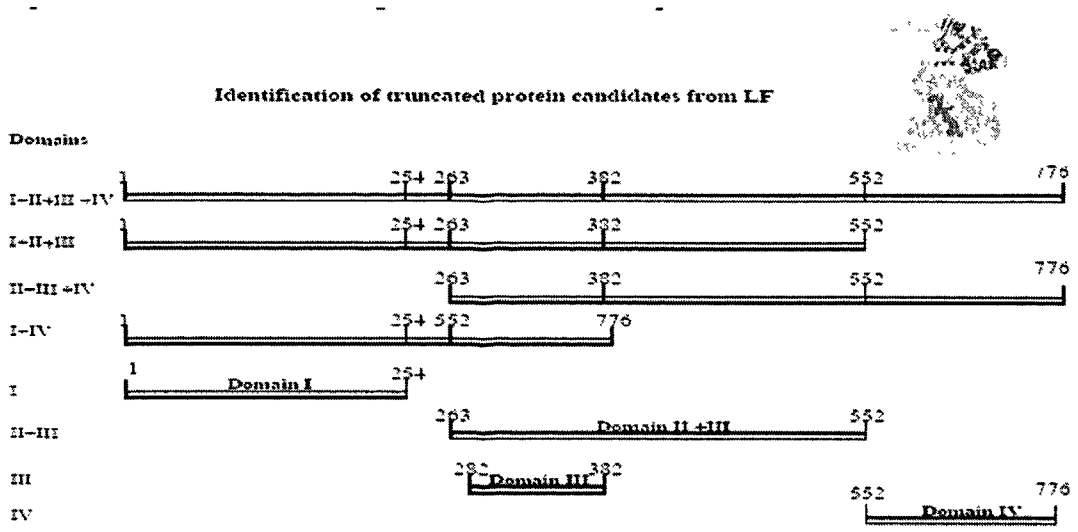


Figure 6

A LFn epitope delivery construct

Figure 7

A model of antigen uptake using the proposed *Salmonella* model

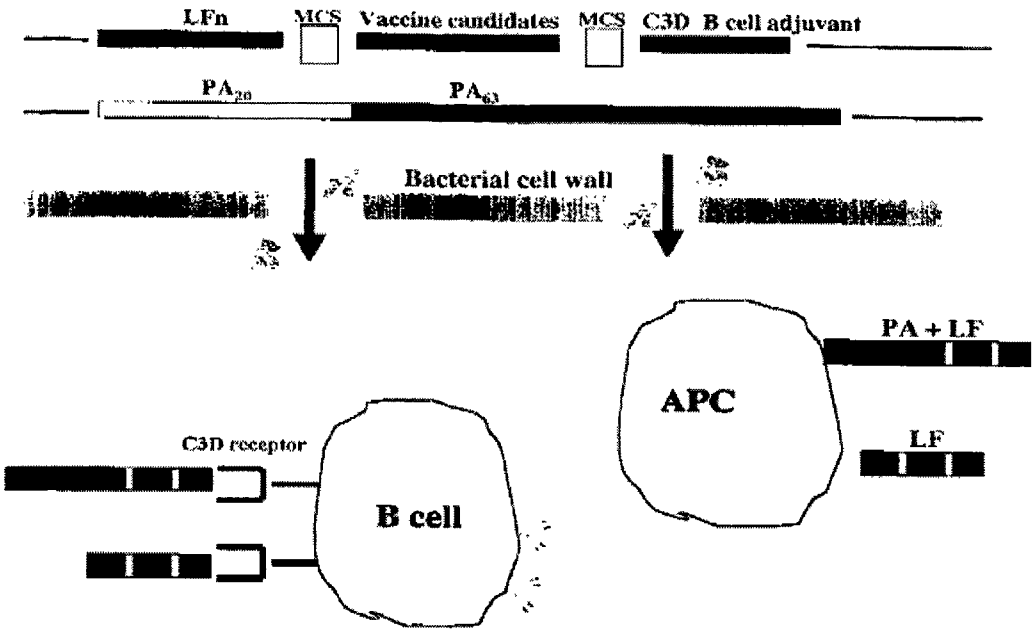


Figure 8

LF domain immunogenicity studies

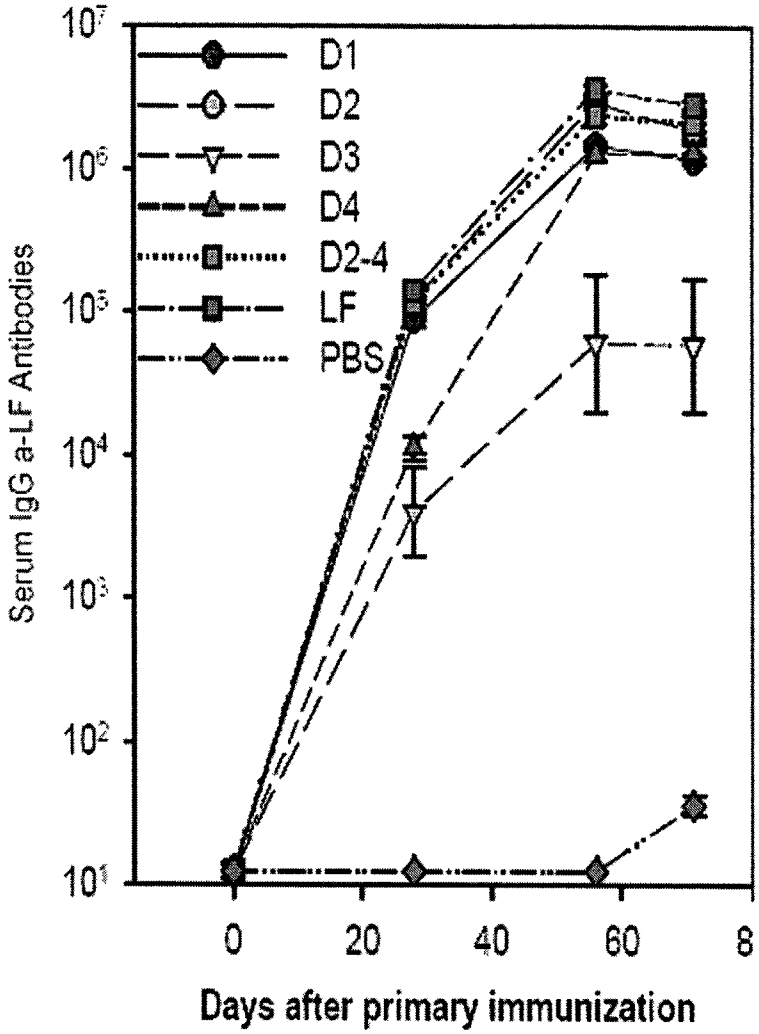
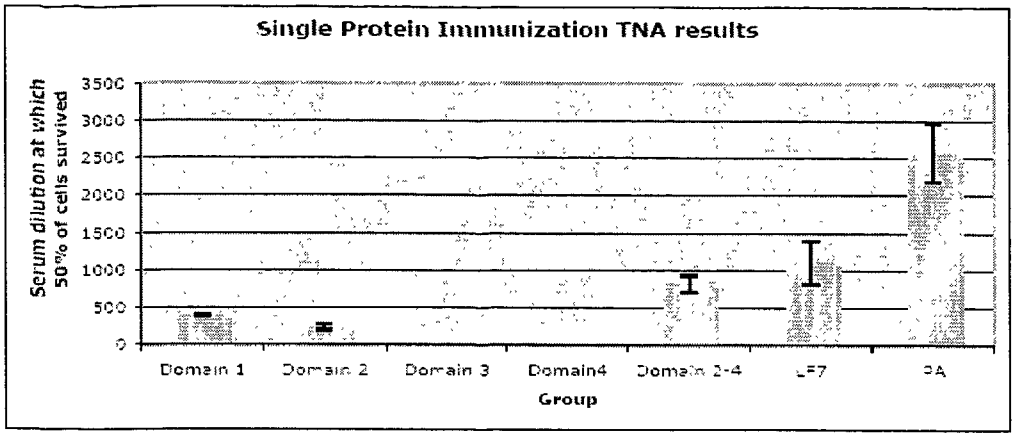


Figure 9

Group (10 Balb/c mice) Toxin Neutralization Titers from mice immunized with LF*, LF domains or PA



LF Domains 3 and 4 showed no detectable Toxin Neutralizing activity with a starting dilution of 1 in 100.

*biologically inactive

Figure 10

The immunogenicity of LF domains in the absence (A) and presence (B) of PA

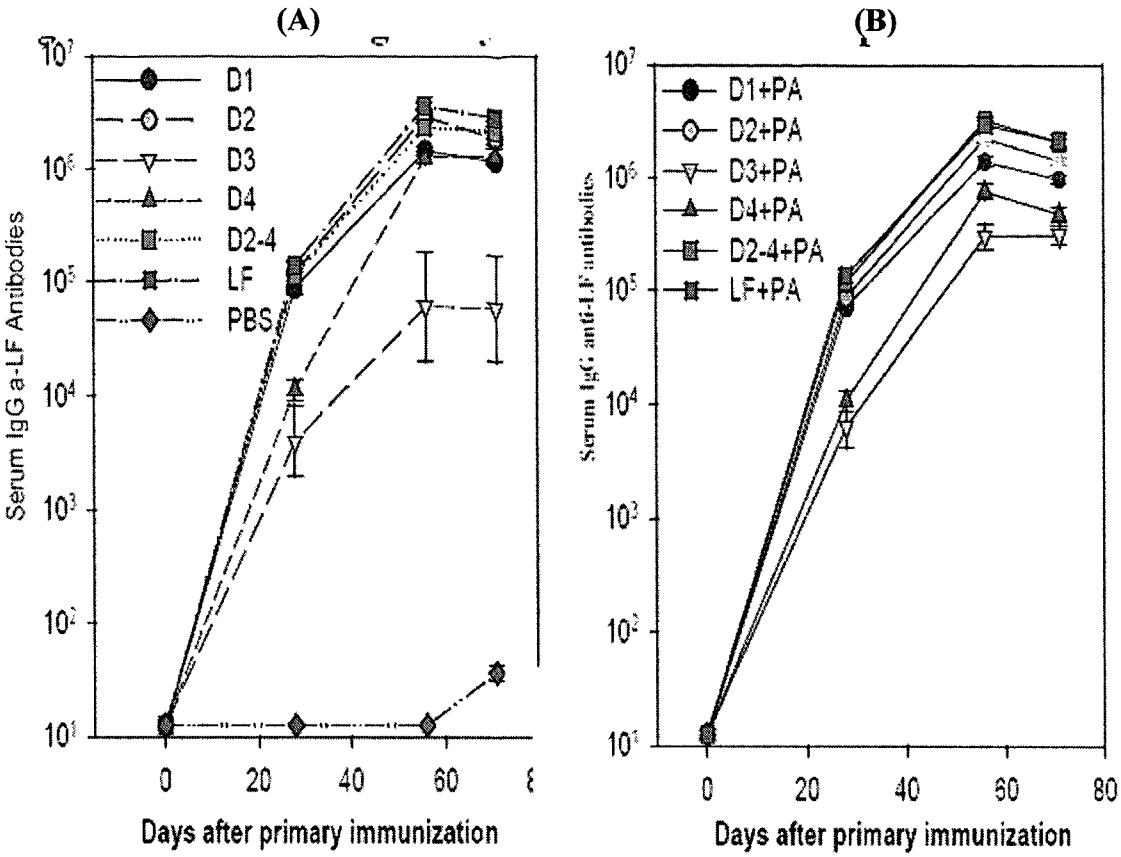


Figure 11

Group (10 Balb/c mice) Toxin Neutralization Titers from mice immunized with LF domains and PA

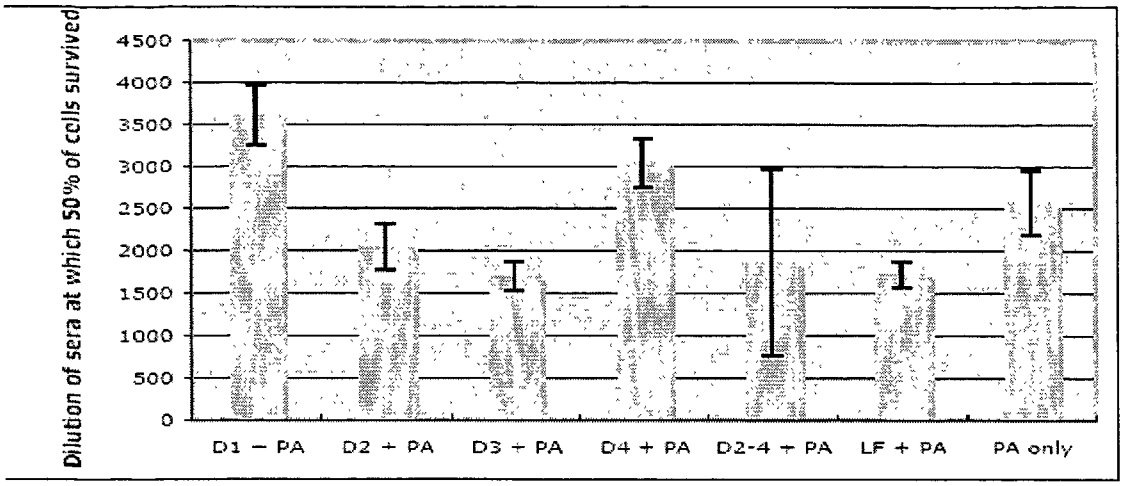


Figure 12

A summary of the TNA titers for LF D1 and full length biologically inactive LF in the presence of PA

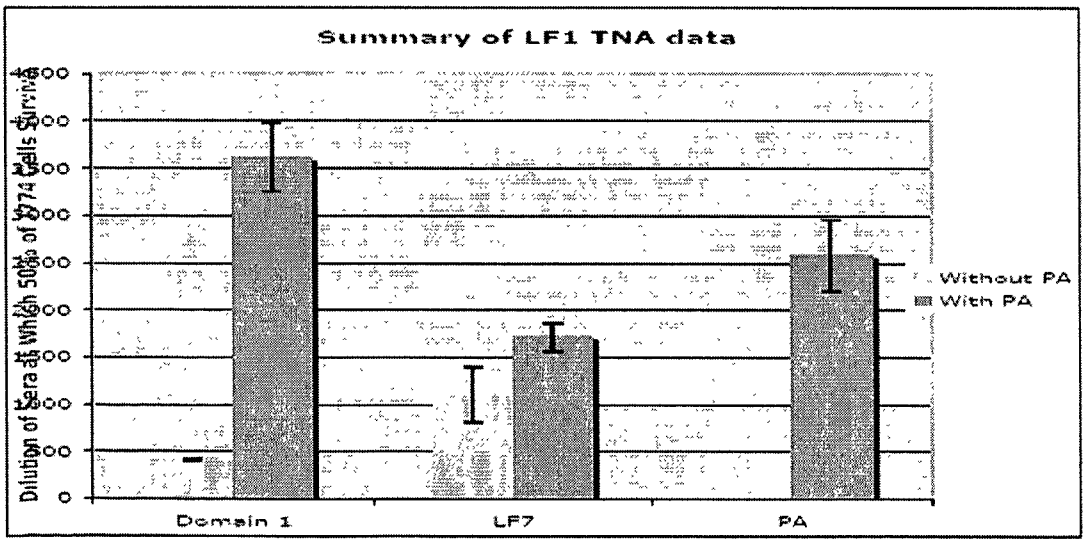


Figure 13

Survival of A/J mice challenged with *B. anthracis* STI spores by the *i.p.* route after immunization with rLF proteins

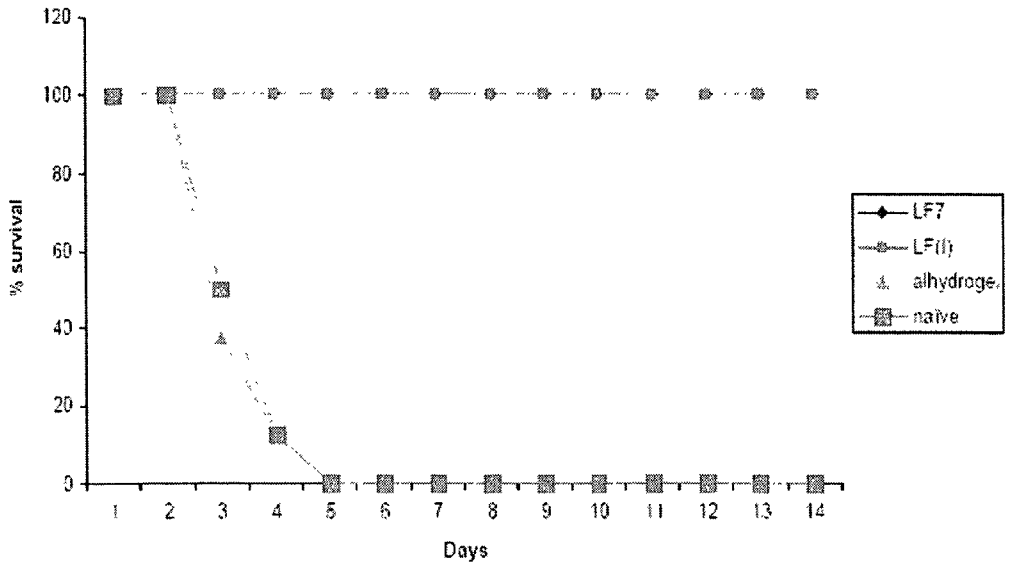


Figure 14

Anti-spore activity

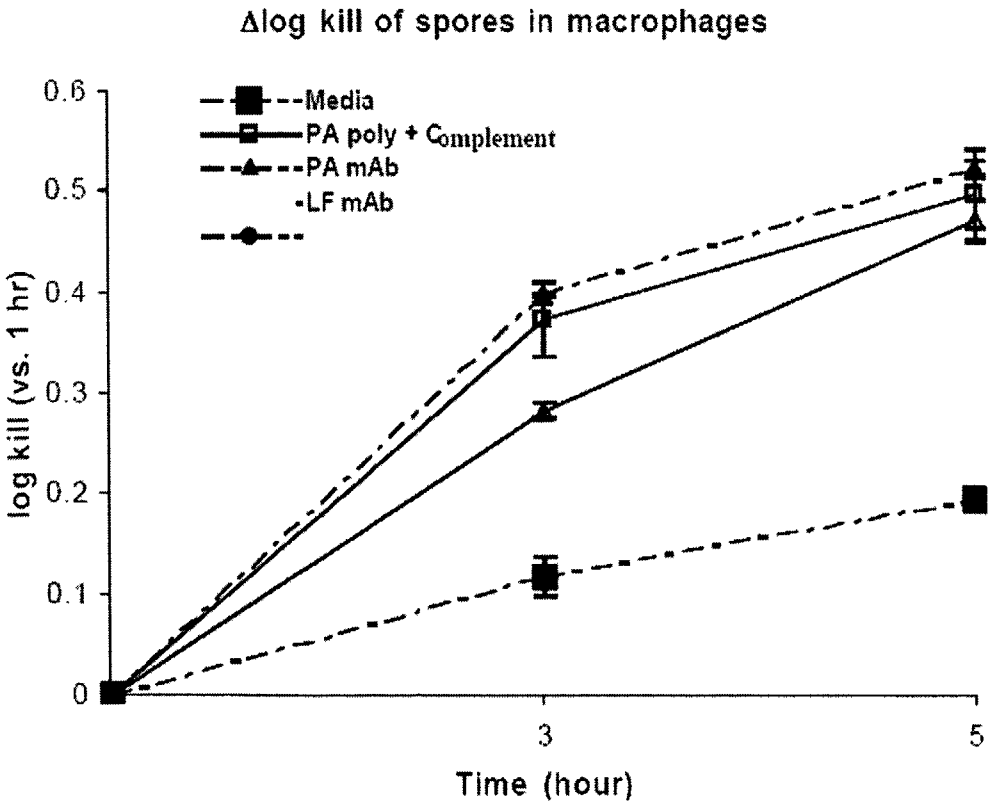
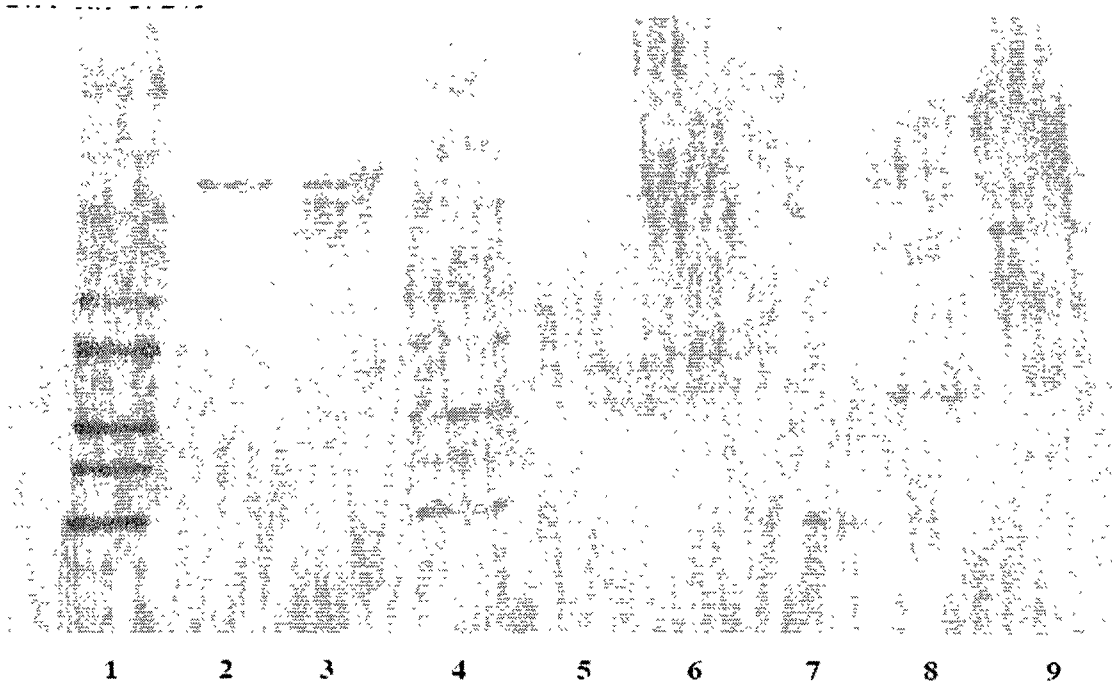


Figure 15

Western blot of PA and LF domains with serum from a rabbit exposed to *B. cereus* G9241



1=MW ladder; 2=PA; 3=LF; 4=MW ladder; 5=LFD1; 6=LFD2; 7=LFD3;8=LFD4; 9=LFD2-4

Figure 16

```
Organism: custom specified
Gene Name:
Sequence Type: dna
Optimization Region: 1 - 2331
Minimum Codon Frequency: 10
Cutoff for Secondary Structure: 80 (Local)
Cycles of Secondary Structure Optimization: 1
GC Range: 40 - 60
Consecutive Codon: 1
Repetitive Codon:
5' Additional Sequence: CCGAGGATCC
3' Additional Sequence: AAGCTTTCGG
Genetic Code: 1

RE Sites:BamHI(GGATCC), HindIII(AAGCTT)
RE Check Sites: SmaI(CCCGGG), EcoRV(GATATC)
RE Keep Sites:

Alignment (Optimized Region). The changed codons are indicated as red letters

Optimized 1     GCGGGCGGTCATGGTGATGTTGGTATGCAGGTTAAAGAGAAAGAGAAAAATAAAGATGAG
Original  1     GCGGGCGGTCATGGTGATGTAGGTATGCACGTAAAAGAGAAAGAGAAAAATAAAGATGAG

Optimized 61    AATAAACGTAAAGATGAAGAGCGTAATAAAACCCAGGAAGAGCATCTGAAAGAAATCATG
Original  61    AATAAGAGAAAAGATGAAGAACGAAATAAAACACAGGAAGAGCATTTAAAGGAAATCATG

Optimized 121   AAACATATTGTTAAAATTGAAGTTAAAGGTGAGGAAGCTGTTAAAAAAGAGGCAGCCGAA
Original  121   AAACACATTGTAAAAATAGAAGTAAAAGGGGAGGAAGCTGTTAAAAAAGAGGCAGCAGAA

Optimized 181   AAAATGCTGGAGAAATTCCTGAGCGATGTTCTGGAGATGTATAAAGCAATTGGTGGTAAA
Original  181   AAGCTACTTGAGAAAGTACCATCTGATGTTTTAGAGATGTATAAAGCAATTGGAGGAAAG

Optimized 241   ATTTATATTGTGGATGGTGATATTACCAAACATATTAGCCTGGAAGCACTGAGCGAAGAT
Original  241   ATATATATTGTGGATGGTGATATTACAAAACATATATCTTAGAAGCATTATCTGAAGAT

Optimized 301   AAGAAGAAAAATTAAAGACATCTATGGTAAAGATGCCCTGCTGCATGAACATTATATTTAT
Original  301   AAGAAAAAAATAAAAGACATTTATGGGAAAGATGCTTTATTACATGAACATTATGTATAT

Optimized 361   GCAAAAGAAGGCTATGAACCGGTTCTGGTTATCCAGAGCAGCGAAGATTATGTTGAAAAT
Original  361   GCAAAAGAAGGATATGAACCGGTACTTGTAATCCAATCTTCGGAAGATTATGTAGAAAAT

Optimized 421   ATTGAAAAAGCACTGAACGTTTATTATGAAATTGGTAAAATTCTGAGCCGTGATATTCTG
Original  421   ACTGAAAAGGCACTGAACGTTTATTATGAAATAGGTAAGATATTATCAAGGGATATTTTA

Optimized 481   AGCAAAAATTAATCAGCCGTATCAGAAATTTCTGGATGTTCTGAATACCATTAAAAATGCA
Original  481   AGTAAAAATTAATCAACCATATCAGAAATTTTTAGATGTATTAAATACCATTAAAAATGCA

Optimized 541   AGCGATAGCGATGGCTAGGATCTTCTGTTTACCAATCAGCTGAAAGAACATCCGATTGAC
Original  541   TCTGATTCAGATGGACAAGATCTTTTATTTACTAATCAGCTTAAGGAACATCCCACAGAT

Optimized 601   TTTATGGTTGAATTTCTGGAACATAATAGCAATGAGGTTCAGGAAGTTTTTGCGAAAGCC
Original  601   TTTTCTGTAGAATTCTTGGAACAAAATAGCAATGAGGTACAAGAAGTATTCGCGAAAGCT

Optimized 661   TTTGCATATTATATCGAGCCGCAGCATCGTGATGTTCTGCAGCTGTATGCACCGGAAGCC
Original  661   TTTGCATATTATATCGAGCCACAGCATCGTGATGTTTTACAGCTTTATGTACCGGAAGCT

Optimized 721   TTTAATCATATGGATAAGTTTAACGAACAGGAAATTAATCTGAAACCTGGAAGAGCTGAAA
Original  721   TTTAATTACATGGATAAATTTAACGAACAAGAAATAAATCTATCCTTGGAAGAACTTAAA
```

Figure 16 (cont.)

Figure 16 (cont.)

Figure 17

>Optimized Sequence Length: 2351, GC%:40.66

CCGAGGATCCGCGGGCGGTCATGGTGATGTTGGTATGCATGTTAAAGAGAAAGAGAAAAA
TAAAGATGAGAATAAACGTAAAGATGAAGAGCGTAATAAAACCCAGGAAGAGCATCTGAA
AGAAATCATGAAACATATTGTTAAAATTGAAGTTAAAGGCGAGGAAGCCGTTAAAAAAGA
GGCAGCCGAAAAACTGCTGGAGAAAGTTCCGAGCGATGTTCTGGAGATGTATAAAGCAAT
TGGCGGTAAAATCTATATTGTGGATGGTGATATTACCAAACATATTAGCCTGGAAGCACT
GAGCGAAGATAAGAAGAAAATTAAAGACATCTATGGCAAAGATGCCCTGCTGGCATGAACA
TTATGTTTATGCAAAAGAAGGCTATGAACCGGTTCTGGTTATCCAGAGCAGCGAAGATTA
TGTTGAAAATACCGAAAAAGCACTGAACGTTTATTATGAAATTGGTAAAATTCTGAGCCG
TGATATTCTGAGCAAAATTAATCAGCCGTATCAGAAATTTCTGGATGTTCTGAATACCAT
TAAAAATGCAAGCGATAGCGATGGCCAGGATCTGCTGTTTACCAATCAGCTGAAAGAACA
TCCGACCGACTTTAGCGTTGAATTTCTGGAACAGAATAGCAATGAGGTTCAGGAAGTTTT
TGCGAAAGCCTTTGCATATTATATCGAGCCGCAGCATCGTGATGTTCTGCAGCTGTATGC
ACCGGAAGCCTTTAATTATATGGATAAGTTTAACGAACAGGAAATTAATCTGAGCCTGGA
AGAGCTGAAAGATCAGCGTATGCTGAGCCGTTATGAAAAATGGGAAAAAATTAAACAGCA
TTATCAGCATTGGAGCGATAGCCTGAGCGAAGAGGGCCGTGGCCTGCTGAAAAAACTGCA
GATTCCGATTGAGCCGAAAAAAGATGACATTATCCATAGCCTGAGCCAGGAAGAGAAAGA
GCTGCTGAAACGTATTCAGATTGATAGCAGCGATTTTCTGAGCACCGAGGAAAAAGAGTT
TCTGAAAAAACTGCAGATTGATATTCGTGATAGCCTGAGCGAAGAGGAAAAAGAGCTGCT
GAATCGTATTCAGGTGGATAGCAGCAATCCGCTGAGCGAAAAAGAAAAAGAGTTTCTGAA
AAAACTGAAACTGGATATTCAGCCGTATGATATTAATCAGCGTCTGCAGGATACCGGCGG
TCTGATTGATAGCCCGAGCATTAATCTGGATGTTCGTAAACAGTATAAACGTGATATTCA
GAATATTGATGCCCTGCTGCATCAGAGCATTGGCAGCACCCTGTATAATAAAATCTATCT
GTATGAAAATATGAATATCAATAACCTGACCGCAACCCTGGGTGCGGATCTGGTTGATAG
CACCGATAATACCAAAATTAATCGTGGTATTTTTAATGAGTTTAAGAAAAATTTTAAATA
TAGCATTAGCAGCAACTATATGATTGTTGATATTAATGAACGTCCGGCTACTGGATAATGA
GCGTCTGAAATGGCGTATCCAGCTGAGCCCGGATACCGCTGCAGGCTATCTGGAAAATGG
CAAACTGATTCTGCAGCGTAACATCGGTCTGGAAATTAAAGATGTTCAGATTATCAAAGA
GAGCGAAAAAGAATATATTCGTATTGATGCGAAAGTTGTGCCGAAAAGCAAAATTGATAC
CAAAATTCAGGAAGCACAGCTGAATATTAATCAGGAATGGAATAAAGCACTGGGCCTGCC
GAAATATACCAAACTGATTACCTTTAACGTGCATAATCGTTATGCAAGCAATATTGTTGA
AAGCGCCTATCTGATTCTGAATGAATGGAAAAATAACATTCAGAGCGATCTGATTAAAAA
AGTTACCAATTATCTGGTTGATGGTAATGGCCGTTTTGTTTTTACCGATATTACCCTGCC
GAATATTGCCGAACAGTATACCCATCAGGATGAGATCTATGAGCAGGTTCATAGCAAAGG
CCTGTATGTTCCGGAAAGCCGTAGCATTCTGCTGCATGGCCCGAGCAAAGGTGTTGAACT

GCGTAATGATAGCGAGGGTTTTATTCATTGTTTTGGCCATGCCGTGGATGACTATGCCGG
CTATCTGCTGGATAAAAACCAGAGCGATCTGGTTACCAATAGCAAAAAGTTTATTGATAT
TTTTAAAGAAGAGGGCAGCAATCTGACCAGCTATGGCCGTACCAATGAAGCGGAATTCTT
TGCAGAAGCCTTTCGTCTGATGCATAGCACCGACCATGCCGAACGTCTGAAAGTTCAGAA
AAATGCCCCGAAAACCTTTCAGTTTATTAACGATCAGATTAAGTTTATTATCAACAGCTA
AAAGCTTTCGG

Restriction Enzyme Sites:

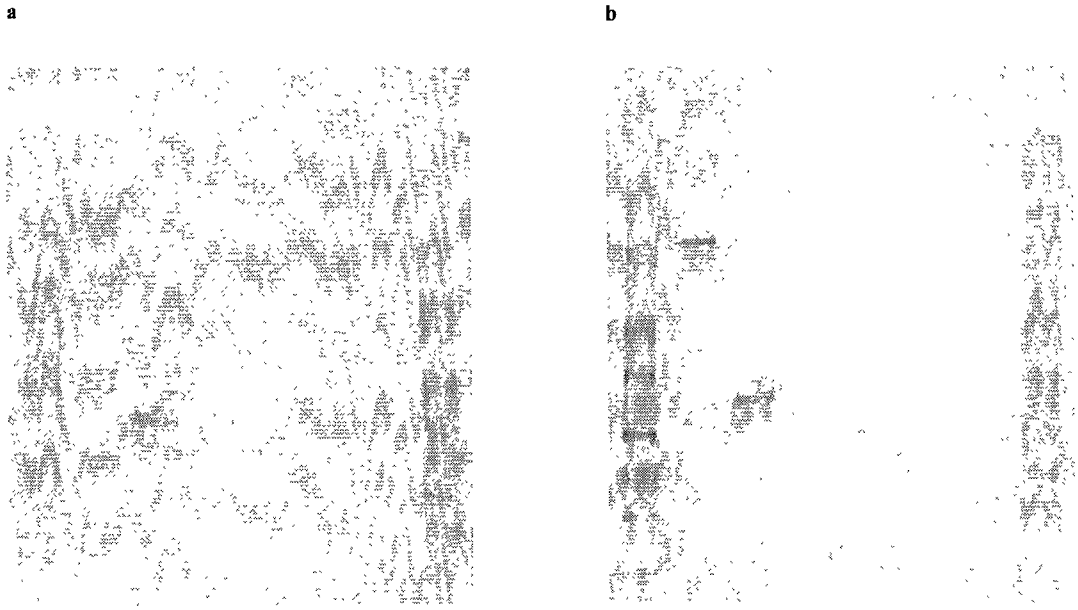
BamHI(GGATCC):1 (5)
HindIII(AAGCTT):1 (2342)
SmaI(CCCGGG):0
EcoRV(GATATC):0

Figure 18

Protein Alignment - Optimized (TOP) vs. Original (BOTTOM):

```
1      AGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAE
1      AGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAE

61     KLLEKVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDALLHEHYVY
61     KLLEKVPSDVLEMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDALLHEHYVY

121    AKEGYEPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNA
121    AKEGYEPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNA

181    SDSDGQDLLFINQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEA
181    SDSDGQDLLFINQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEA

241    FNYMDKFNEQEINLSLEELKDQRMLSRYEKWEKIKQHYQHWSDSLSEEGRGLLKKLQIPI
241    FNYMDKFNEQEINLSLEELKDQRMLSRYEKWEKIKQHYQHWSDSLSEEGRGLLKKLQIPI

301    EPKKDDIIHSLSQEEKELLKRIQIDSSDFLSTEEKEFLKKLQIDIRDSLSEEEKELLNRI
301    EPKKDDIIHSLSQEEKELLKRIQIDSSDFLSTEEKEFLKKLQIDIRDSLSEEEKELLNRI

361    QVDSSNPLSEKEKEFLKKLKLDIQPYDINQRLQDIGGLIDSPSINLDVRKQYKPDIQNII
361    QVDSSNPLSEKEKEFLKKLKLDIQPYDINQRLQDIGGLIDSPSINLDVRKQYKPDIQNID

421    ALLHQSIGSTLYNKIYLYENMNINNLIATLGADLVDSTINTKINRGIFNEFKKNFKYSIS
421    ALLHQSIGSTLYNKIYLYENMNINNLIATLGADLVDSTINTKINRGIFNEFKKNFKYSIS

481    SNYMIVDINERPALDNERLKWRIQLSPDTRAGYLENGKLILQRNIGLEIKDVQIIKQSEK
481    SNYMIVDINERPALDNERLKWRIQLSPDTRAGYLENGKLILQRNIGLEIKDVQIIKQSEK

541    EYIRIDAKVVPKSKIDTKIQEAQLNINQEWNKALGLPKYTKLITFNVHNRYASNIVESAY
541    EYIRIDAKVVPKSKIDTKIQEAQLNINQEWNKALGLPKYTKLITFNVHNRYASNIVESAY

601    LILNEWKNNIQSDLIKKVTNYLVDGNGRFVFTDITLPNIAEQYTHQDEIYEQVHSKGLYV
601    LILNEWKNNIQSDLIKKVTNYLVDGNGRFVFTDITLPNIAEQYTHQDEIYEQVHSKGLYV

661    PESRSILLHGPSKGVELRNDSEGFIHCFGHAVDDYAGYLLDKNQSDLVTNSKKFIDIFKE
661    PESRSILLHGPSKGVELRNDSEGFIHCFGHAVDDYAGYLLDKNQSDLVTNSKKFIDIFKE

721    EGSNLISYGRTNEAEFFAEAFRLMHSTDHAERLKVQKNAPKTFQFINDQIKFIINS*
721    EGSNLISYGRTNEAEFFAEAFRLMHSTDHAERLKVQKNAPKTFQFINDQIKFIINS*
```

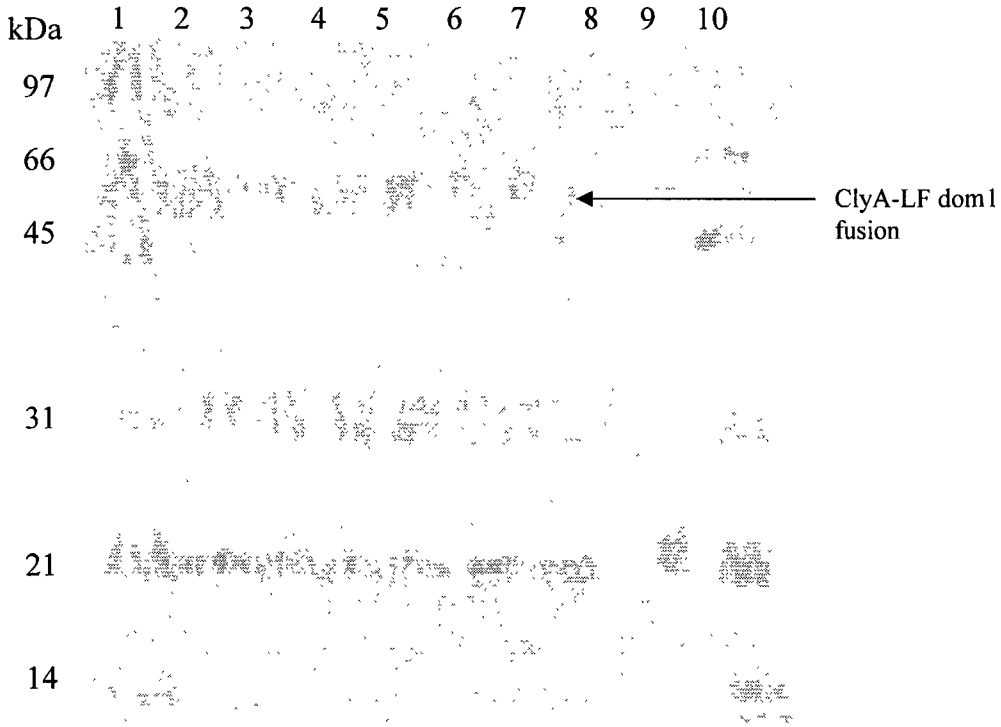
Figure 19



Domain recognition of IQNLF. Panels A and B are Western blots probed with IQNLF and loaded with a marker (left and right lanes) and equal amounts of full length rLF (lane 1), LF domain I (lane 2), LF domain II (lane 3), LF domain III (lane 4), LF domain IV (lane 5), and LF domains II-IV (lane 6). Panels A is a native blot while B is an SDS-denaturing blot.

Figure 20

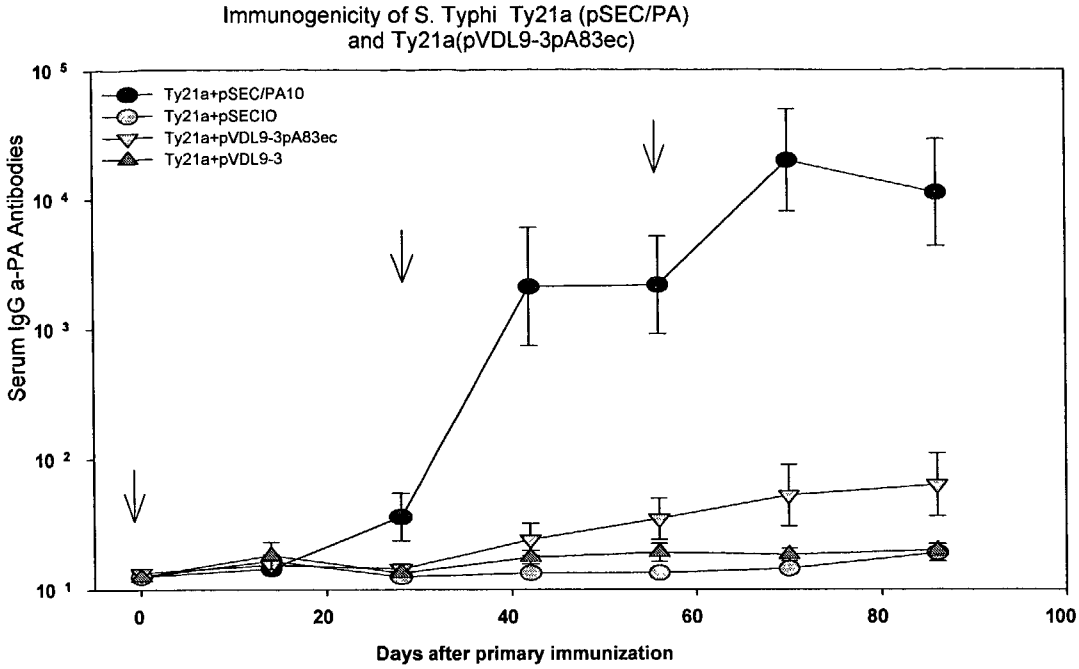
Expression of LF domain 1 in *S. enterica* serovar Typhimurium SL3261



Lanes: 1) Bio-Rad low range standards; 2) SL3261/pSEC10-LFnN#26; 3) SL3261/pSEC10-LFnN#29; 4) SL3261/pSEC10-LFnN#31; 5) SL3261/pSEC10-LFoN#26; 6) SL3261/pSEC10-LFoN#27; 7) SL3261/pSEC10-LFoN#33); 8) Recombinant LF dom1; 9) *E. coli* TOP10/pSEC10; 10) Bio-Rad low range standards. Probed with mouse monoclonal antibody to LF domain 1.

Figure 21

(A)



Balbc mice immunized i.n. on days 0,28 and 56 with 10 ^{9} cfu

(B)

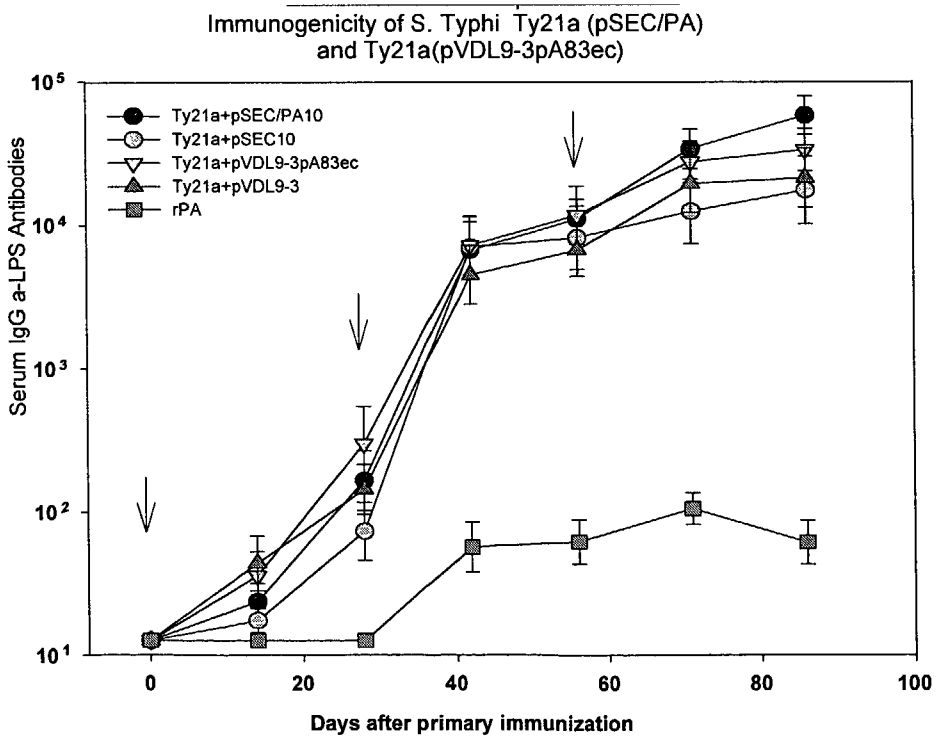


Figure 22

Heterologous prime boost. Mice orally primed with Salmonella expressing PA were boosted with a single I.m. dose of recombinant PA protein

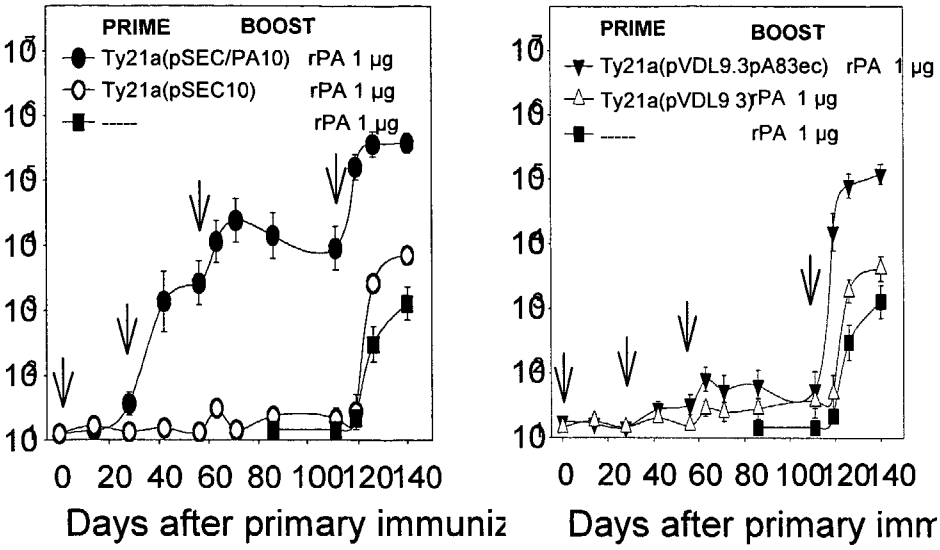
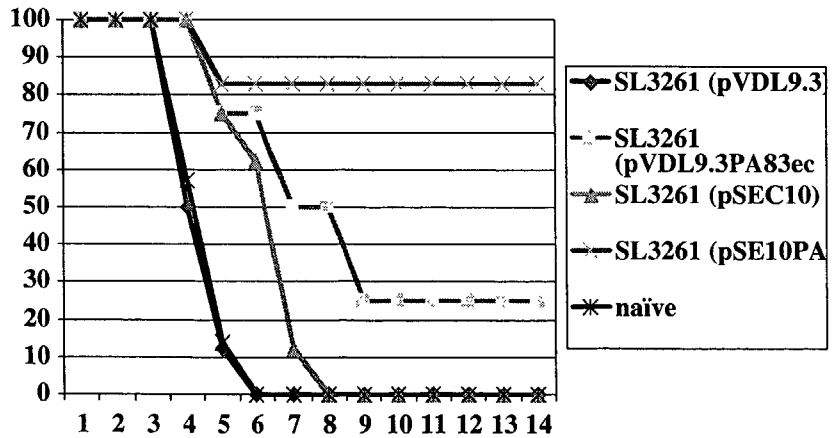


Figure 23

Survival rate of mice challenged with *B. anthracis* after oral inoculation with *S. typhimurium* expressing PA plasmids



Oral immunization (intra gastric gavage)

A/J mice (gps 8) received 1-5x10^9 cfu on days 0,14 and 28 and were challenged with a lethal spore aerosol (100 MLD) on day 80.

Figure 24

Codon Optimization Result

Organism: Salmonella enterica ;
Gene Name: LF1.PA4
Sequence Type: dna
Optimization Region: 7 - 1278
GC Range: 30 - 70
Addition 5' Sequence:
Addition 3' Sequence:
Cut Offs:
Secondary Structure Stack Cutoff: 30
Repeat Cutoff: 19
Relative Frequence Cutoff: 50
5' Splice Score Cutoff: 80
Genetic Code: 1
RE Sites and CIS Pattern:
BamHI(GGATCC),HindIII(AAGCTT),
RE Check Sites:
SmaI(CCCGGG),EcoRV(GATATC)
RE Keep Sites:

DNA Alignment (Optimized Region). The changed codons are indicated as red letters:

Optimized 7 GCCGGTGGTCATGGCGATGTTGGTATGCATGTTAAAGAAAAAGAAAAAAAAATAAAGATGAA
Original 7 GCGGGCGGTCATGGCGATGTTGGTATGCATGTTAAAGAGAAAGAGAAAAATAAAGATGAG

Optimized 67 AACAAACGTAAAGATGAAGAACGTAACAAAACCCAGGAAGAACATCTGAAAGAAATTATG
Original 67 AATAAACGTAAAGATGAAGAGCGTAATAAAACCCAGGAAGAGCATCTGAAATAAATCATG

Optimized 127 AAACATATTGTTAAAATTGAAGTTAAAGGTGAAGAAGCGGTTAAAAAAGAAGCGGCGGAA
Original 127 AAACATATTGTTAAAATTGAAGTTAAAGGCGAGGAAGCCGTTAAAAAAGAGGCAGCCGAA

Optimized 187 AAACTGCTGGAAAAAGTTCCGTCTGATGTTCTGGAAATGTATAAAGCTATTGGTGGTAAA
Original 187 AAACTGCTGGAGAAAGTTCCGAGCGATGTTCTGGAGATGTATAAAGCAATTGGCGGTAAA

Optimized 247 ATTTATATTGTTGATGGTGATATTACTAAACATATCTCTCTGGAAGCGCTGTCTGAAGAT
Original 247 ATCTATACTGTGGATGGTGATATTACCAAACATATTAGCCTGGAAGCACTGAGCGAAGAT

Optimized 307 AAAAAAAAAATTAAAGATATCTATGGTAAAGACGCGCTGCTGCATGAACATTATGTTCAT
Original 307 AAGAAGAAAATTAAAGACATCTATGGCAAAGATGCCCTGCTGCATGAACATTATGTTTAT

Optimized 367 GGTAAAGAAGGTTATGAACTGGTTCTGGTTATTCAGTCTTCTGAAGATTATGTTGAAAAA
Original 367 GCAAAAGAAGGCTATGAACCGGTTCTGGTTATCCAGAGCAGCGAAGATTATGTTGAAAAT

Optimized 427 ATTGAAAAAGCTCTGGAACGTTTATTATGAAATTGGTAAAATTCTGTCTCGTGATATTCTG
Original 427 ACCGAAAAAGCACTGAACGTTTATTATGAAATTGGTAAAATTCTGAGCCGTGATATTCTG

Optimized 487 TCTAAAATTAACCAGCCGTATCAGAAATTCTGGATGTTCTGAACACTATTAAAAAGGCG
Original 487 AGCAAAATTAATCAGCCGTATCAGAAATTCTGGATGTTCTGAATACCATTAAAAATGCA

Optimized 547 TCTGATTCTGATGGTCAGGATCTGCTGTTTACCAACCAGCTGAAAGAACATCCGACCGAT
Original 547 AGCGATAGCGATGGCCAGGATCTGCTGTTTACCAATCAGCTGAAAGAACATCCGACCGAC

Optimized 607 TTTTCTGTTGAATTTCTGGAACAGAACTCTAACGAAGTTCAGGAAGTTTTTGCTAAAGCG
Original 607 TTTAGCGTTGAATTTCTGGAACAGAATAGCAATGAGGTTCAGGAAGTTTTTGCGAAAGCC

Optimized 667 TTTGCGTATTATATTGAACCGGCAGCATCGTGATGTTCTGCAGCTGTATGCTCCGGAAGCG
Original 667 TTTGCATATTATATTGAGCCGGCAGCATCGTGATGTTCTGCAGCTGTATGCACCGGAAGCC

Figure 24 (cont.)

Figure 25

Protein Alignment (Optimized Region):

```
Optimized    1 AGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAE
Original     1 AGGHGDVGMHVKEKEKNKDENKRKDEERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAE

Optimized   61 KLLEKVPSDVLEMYKAIGGKIYIVDGDIIKHISLEALSEDKKKIKDIYGKDALLHEHYVY
Original    61 KLLEKVPSDVLEMYKAIGGKIYIVDGDIIKHISLEALSEDKKKIKDIYGKDALLHEHYVY

Optimized  121 AKEGYEPVLVIQSSEDYVENIEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNA
Original   121 AKEGYEPVLVIQSSEDYVENIEKALNVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNA

Optimized  181 SDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEA
Original   181 SISDGQDLLFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEA

Optimized  241 FNYMDKFNEQEINLSELGTTNIYTVLDKIKLNAKMNILIRDKRFHYDRNNIAVGADESVV
Original   241 FNYMDKFNEQEINLSELGTTNIYTVLDKIKLNAKMNILIRDKRFHYDRNNIAVGADESVV

Optimized  301 KEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLKEVINDRYDMLNISSLRQDG
Original   301 KEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIEDTEGLKEVINDRYDMLNISSLRQDG

Optimized  361 KIFIDFKKYNDKLPLYISNPNYKVNVYAVTKENTIINPSENGDTSTNGIKKILIFSKKGY
Original   361 KIFIDFKKYNDKLPLYISNPNYKVNVYAVTKENTIINPSENGDTSTNGIKKILIFSKKGY

Optimized  421 EIG*
Original   421 EIG*
```

Figure 26

Optimized Sequence: Length: 1284, GC%:35.28, Minimum Free Energy:-298.25

GGATCCGCTGGTGGTCATGGTGATGTTGGTATGCATGTTAAAGAAAAAGAAAAAAACAAA
GATGAAAACAAACGTAAAGATGAAGAACGTAACAAAACCCAGGAAGAACATCTGAAAGAA
ATTATGAAACATATTGTTAAAATTGAAGTTAAAGGTGAAGAAGCGGTTAAAAAAGAAGCG
GCGGAAAAACTGCTGGAAAAAGTTCCGTCTGATGTTCTGGAAATGTATAAAGCGATTGGT
GGTAAAATTTATATTGTTGATGGTGATATTACTAAACATATCTCTCTGGAAGCGCTGTCT
GAAGATAAAAAAAAAATCAAAGATATCTATGGTAAAGATGCGCTGCTGCATGAACATTAT
GTTTATGCGAAAGAAGGTTATGAACCGGTTCTGGTTATTCAGTCTTCTGAAGATTATGTT
GAAAACACTGAAAAAGCTCTGAACGTTTATTATGAAATTGGTAAAATTCTGTCTCGTGAT
ATTCTGTCTAAAATTAACCAGCGGTATCAGAAATTTCTGGATGTTCTGAACACTATTAAA
AACGCGTCTGATTCTGATGGTCAGGATCTGCTGTTTACCAACCAGCTGAAAGAACATCCG
ACCGATTTTTCTGTTGAATTTCTGGAACAGAACTCTAACGAAGTTCAGGAAGTTTTTGCT
AAAGCGTTTGCGTATTATATTGAACCGCAGCATCGTGATGTTCTGCAGCTGTATGCTCCG
GAAGCGTTTAACTATATGGATAAATTTAACGAACAGGAAATTAACCTGTCTGAACTGGGT
ACTACTAACATTTATACCGTTCTGGATAAAATTAAACTGAACGCTAAAATGAACATTCTG
ATTCGTGATAAACGTTTTCATTATGATCGTAACAACATCGCTGTTGGTGCTGATGAATCT
GTTGTTAAAGAAGCGCATCGTGAAGTTATTAACTCTTCTACCGAAGGTCTGCTGCTGAAC
ATTGATAAAGATATTCGTAAAATTCTGTCTGGTTATATTGTTGAAATTGAAGATACTGAA
GGTCTGAAAGAAGTTATCAACGATCGTTATGATATGCTGAACATTTCTTCTCTGCGTCAG
GATGGTAAAACCTTTATTGATTTTAAAAAATATAACGATAAACTGCCGCTGTATATTTCT
AACCCGAACTATAAAGTTAACGTTTATGCGGTTACCAAAGAAAACACTATTATTAATTGG
TCTGAAAACGGTGATACCTCTATTAACGGTATCAAAAAAATCCTGATTTTTTCTAAAAAA
GGTTATGAAATTGGTTAAAAGCTT

Restriction Enzymes:

Filtering Enzymes and CIS Pattern:

BamHI(GGATCC): 1 (1 - GGATCC)

HindIII(AAGCTT): 1 (1279 - AAGCTT)

Checking Enzymes:

SmaI(CCCGGG): 0

EcoRV(GATATC): 1 (322 - GATATC)

Keeping Enzymes:

Figure 27

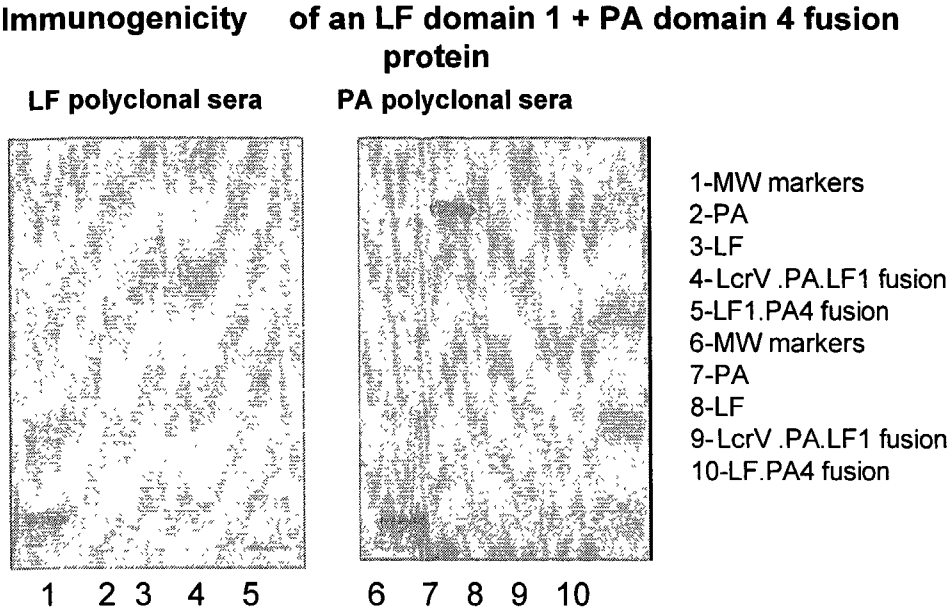


Figure 28

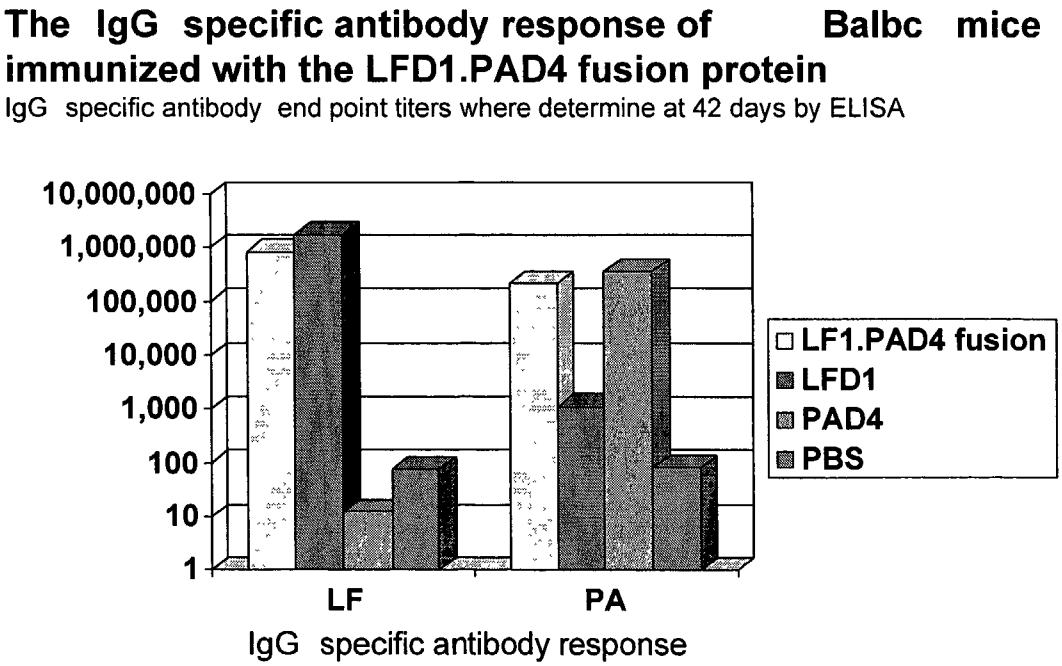


Figure 29

. LF_n/V and V/LF_n fusion proteins are immunogenic and elicit anti-LF and anti V serum IgG responses in BALB/c mice

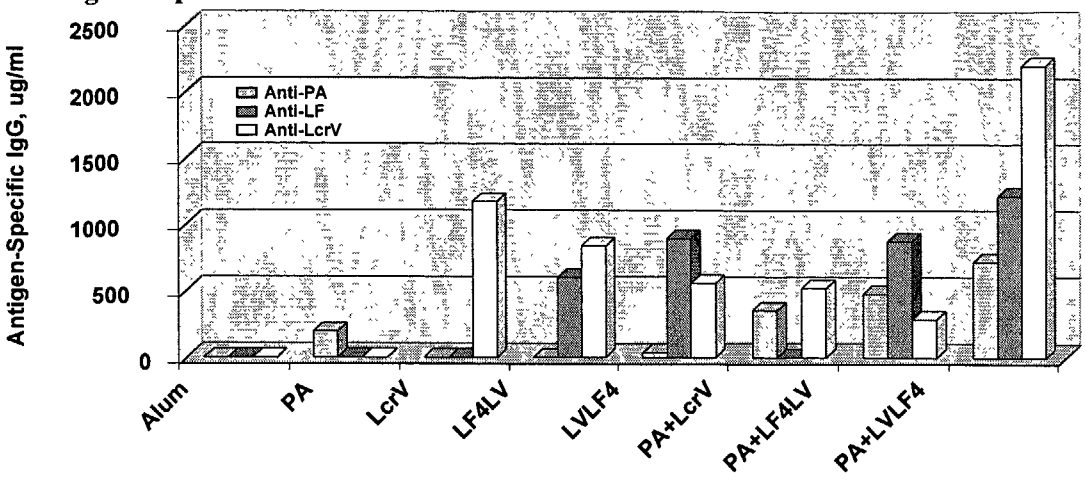


Figure 30
Multi-agent delivery

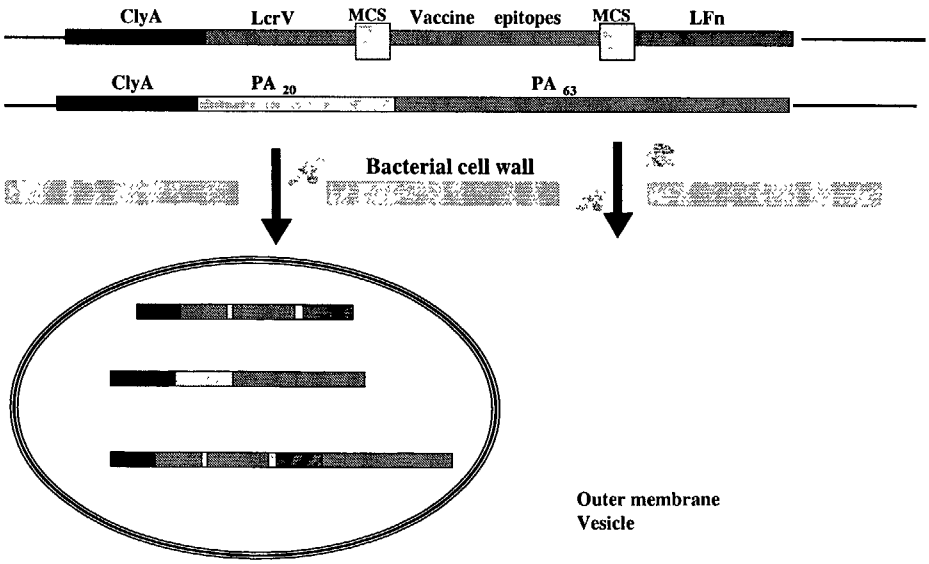


Figure 31
The LcrV-PA-F1-LFD1 fusion

Native codon usage

ATGAGAGGATCGCATCACCATCACCATCACGGATCCATGATTAGAGCCTACGAA
CAAAACCCACAACATTTTATTGAGGATCTAGAAAAAGTTAGGGTGGAACAACTT
ACTGGTCATGGTTCTTCAGTTTTAGAAGAATTGGTTCAGTTAGTCAAAGATAAA
AATATAGATATTTCCATTAAATATGATCCCAGAAAAGATTCGGAGGTTTTTGCCA
ATAGAGTAATTACTGATGATATCGAATTGCTCAAGAAAATCCTAGCTTATTTTCT
ACCCGAGGATGCCATTCTTAAAGGCGGTCATTATGACAACCAACTGCAAAATGG
CATCAAGCGAGTAAAAGAGTTCCTTGAATCATCGCCGAATACACAATGGGAATT
GCGGGCGTTCATGGCAGTAATGCATTTCTCTTTAACCGCCGATCGTATCGATGA
TGATATTTTGAAAGTGATTGTTGATTCAATGAATCATCATGGTGATGCCCGTAG
CAAGTTGCGTGAAGAATTAGCTGAGCTTACCGCCGAATTAAAGATTTATTCAGT
TATTCAAGCCGAAATTAATAAGCATCTGTCTAGTAGTGGCACCATAAATATCCA
TGATAAATCCATTAATCTCATGGATAAAAATTTATATGGTTATACAGATGAAGAG
ATTTTTAAAGCCAGCGCAGAGTACAAAATTCTCGAGAAAATGCCTCAAACCACC
ATTCAGGTGGATGGGAGCGAGAAAAAAATAGTCTCGATAAAGGACTTTCTTGGA
AGTGAGAATAAAAGAACCGGGGCGTTGGGTAATCTGAAAAACTCATACTCTTAT
AATAAAGATAATAATGAATTATCTCACTTTGCCACCACCTGCTCGGATAAGTCC
AGGCCGCTCAACGACTTGGTTAGCCAAAAAACAACTCAGCTGTCTGATATTACA
TCACGTTTTAATTCAGCTATTGAAGCACTGAACCGTTTCATTCAGAAATATGATT
CAGTGATGCAACGTCTGCTAGATGACACGTCTGGTAAAGCATGCGAGCTCGGTA
CCAGCGATGTTCTGGAGATGTATAAAGCAATTGGCGGTAAAATCTATATTGTGG
ATGGT GATATTACCAAACATATTAGCCTGGAAGCACTGGATAGCCTGAGCGAA
GAGGAAAAAGAGCTGCTGAATCGTATTCAGGTGGATAGCAGCATCAAATTAAAT
GCAAAAATGAATATTTTAATAAGAGATAAACGTTTTCATTATGATAGAAATAAAA
AATATAATGATAAATTACCGTTATATATAAGTAATCCCAATTATAAGGTAAATGT
ATATGCTGCGGCAGATTTAACTGCAAGCACCACTGCAACGGCAACTCTTGTTGA
ACCAGCCCGCATCACTCTTACATATAAGGAAGGCGCTCCAATTACAATTATGGC
GGGCGGTCATGGTGATGTAGGTATGCACGTAAAAGAGAAAGAGAAAAATAAAG
ATGAGAATAAGAGA AAAGATGAA GAA CGA AAT AAA ACA CAG GAA GAG CAT
TTA AAG GAA ATCATG AAA CAC ATT GTA AAA ATA GAA GTA AAA GGG GAG
GAA GCT GTTAAA AAA GAG GCA GCA GAA AAG CTA CTT GAG AAA GTA CCA
TCT GATGTT TTA GAG ATG TAT AAA GCA ATT GGA GGA AAG ATA TAT ATT
GTGGAT GGT GAT ATT ACA AAA CAT ATA TCT TTA GAA GCA TTA TCT
GAAGAT AAG AAA AAA ATA AAA GAC ATT TAT GGG AAA GAT GCT TTA TTA
CAT GAA CAT TAT GTA TAT GCA AAA GAA GGA TAT GAA CCC GTA CTT GTA
ATC CAA TCT TCG GAA GAT TAT GTA GAA AAT ACT GAA AAG GCA CTG AAC
GTT TAT TAT GAA ATA GGT AAG ATA TTA TCA AGG GAT ATT TTA AGT AAA
ATT AAT CAA CCA TAT CAG AAA TTT TTA GAT GTA TTA AAT ACC ATT AAA
AAT GCA TCT GAT TCA GAT GGA CAA GAT CTT TTA TTT ACT AAT CAG CTT
AAG GAA CAT CCC ACA GAC TTT TCT GTA GAA TTC TTG GAA CAA AAT AGC
AAT GAG GTA CAA GAA GTA TTT GCG AAA GCT TTT GCA TAT TAT ATC GAG
CCA CAG CAT CGT GAT GTT TTA CAG CTT TAT GCA CCG GAA GCT TTT AAT
TAC ATG GAT AAA TTT AAC GAA CAA GAA ATA AAT CTA TAA

(SEQ ID NO: 24)

Figure 32

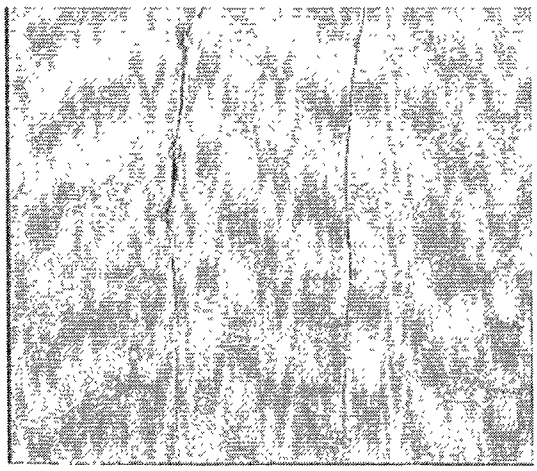
Fusion protein amino acid sequence which includes an N terminal 6 His tag for protein purification.

Met R G S H H H H H H G S Met I R A Y E Q N P Q H F I E D L E K V R V E Q L T G H G S S V L E E L V Q L V K D K N I D I S I K Y D P R K D S E V F A N R V I T D D I E L L K K I L A Y F L P E D A I L K G G H Y D N Q L Q N G I K R V K E F L E S S P N T Q W E L R A F Met A V Met H F S L T A D R I D D D I L K V I V D S Met N H H G D A R S K L R E E L A E L T A E L K I Y S V I Q A E I N K H L S S S G T I N I H D K S I N L Met D K N L Y G Y T D E E I F K A S A E Y K I L E K Met P Q T T I Q V D G S E K K I V S I K D F L G S E N K R T G A L G N L K N S Y S Y N K D N N E L S H F A T T C S D K S R P L N D L V S Q K T T Q L S D I T S R F N S A I E A L N R F I Q K Y D S V Met Q R L L D D T S G K A C E L G T S D V L E Met Y K A I G G K I Y I V D G D I T K H I S L E A L D S L S E E E K E L L N R I Q V D S S I K L N A K Met N I L I R D K R F H Y D R N K K Y N D K L P L Y I S N P N Y K V N V Y A A A D L T A S T T A T A T L V E P A R I T L T Y K E G A P I T I Met A G G H G D V G Met H V K E K E K N K D E N K R K D E E R N K T Q E E H L K E I Met K H I V K I E V K G E E A V K K E A A E K L L E K V P S D V L E Met Y K A I G G K I Y I V D G D I T K H I S L E A L S E D K K K I K D I Y G K D A L L H E H Y V Y A K E G Y E P V L V I Q S S E D Y V E N T E K A L N V Y Y E I G K I L S R D I L S K I N Q P Y Q K F L D V L N T I K N A S D S D G Q D L L F T N Q L K E H P T D F S V E F L E Q N S N E V Q E V F A K A F A Y Y I E P Q H R D V L Q L Y A P E A F N Y Met D K F N E Q E I N L Stop (SEQ ID NO: 25)

Figure 33

Immunogenicity of LcrV -PA-F1-LFD1 fusion protein

Fusion protein was probed with antigen specific immune serum



1 2 3 4 5 6

1 PA antisera
2. MW marker
3. LF antisera
4. MW marker
5. LcrV antisera
6. MW marker

Figure 34

The IgG specific antibody response of Balbc mice immunized with fusion proteins

IgG specific antibody end point titers where determine at 42 days by ELISA