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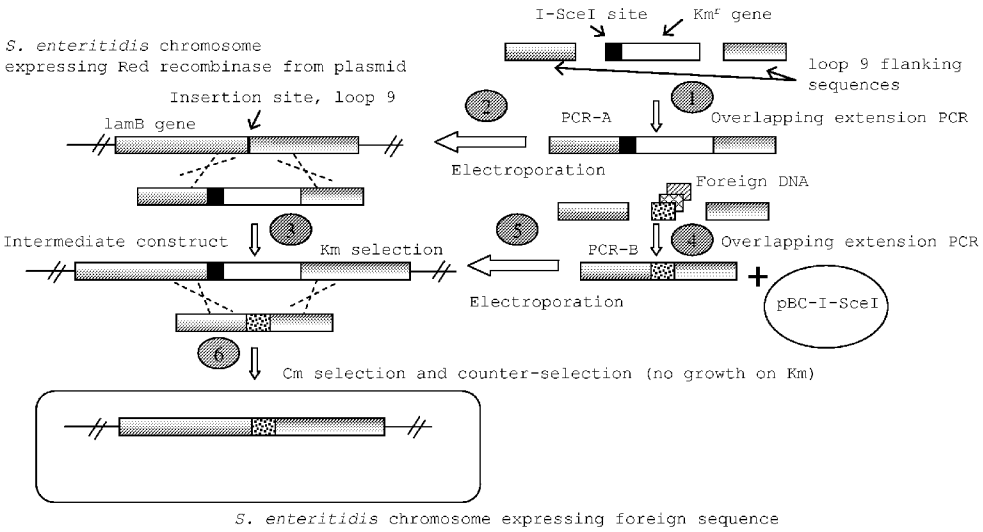

FIG. 1

(57) Abstract: Vaccines comprising *fli*C and CD 154 polypeptides and *Salmonella enteritidis* vaccine vectors comprising *fli*C polypeptides are provided. Also provided are methods of enhancing an immune response against flagellated bacteria and methods of reducing morbidity associated with infection with flagellated bacteria.

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COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO FLAGELLATED BACTERIUM

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application Serial No. 60/983,803, filed on October 30, 2007, which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with partial United States government support awarded by USDA/NRI Proposal # 2007-01953. The United States may have certain rights in this invention.

INTRODUCTION

Salmonella continues to be one of the most commonly reported bacterial causes of human food-borne infections worldwide, and epidemiological evidence indicates that poultry and poultry products are a significant source of human infection. In the United States, an estimated 1.4 million cases of human *Salmonellosis* are reported annually. Of these cases, *S. enterica* serovars Enteritidis (SE) and Typhimurium (ST) are the most commonly isolated, although a number of other serovars have also been shown to cause enteritis in humans.

Salmonella infrequently causes apparent clinical disease in poultry flocks. However, infection in young chicks with some *Salmonella* isolates results in 2% mortality within the first 48 hours post-hatch, and up to 20% morbidity within the first five days. Therefore, increasing the resistance of the poultry population against *Salmonella* will not only reduce the impact of low level disease on performance, but will also reduce the significant health risk for the human population as well.

SUMMARY

A vaccine comprising a *fliC* polynucleotide sequence encoding a first polypeptide and a second polynucleotide sequence encoding a CD154 polypeptide is disclosed. The CD154 polypeptide is capable of binding CD40 and has fewer than 50 amino acids, and includes amino acids 140-149 of SEQ ID NO: 8 or a homolog thereof.

In another aspect, a vaccine comprising a variant of *Salmonella enteritidis* 13A is disclosed. The *Salmonella* comprises a first polynucleotide sequence encoding a *fliC* polypeptide.

In yet another aspect, methods of enhancing the immune response against a flagellated bacterium in a subject by administering a bacterium are provided. The bacterium includes a first polynucleotide sequence encoding a *fliC* polypeptide and a second polynucleotide sequence encoding a CD154 polypeptide. The CD154 polypeptide is capable of binding CD40 and has fewer than 50 amino acids, and includes amino acids 140-149 of SEQ ID NO: 8 or a homolog thereof. The bacterium is administered in an amount effective to enhance the immune response of the subject to the flagellated bacterium.

In still another aspect, methods of enhancing the immune response against a flagellated bacterium in a subject by administering a variant of *Salmonella enteritidis* 13A are provided. The *Salmonella* includes a first polynucleotide sequence encoding a *fliC* polypeptide. The *Salmonella* is administered in an amount effective to enhance the immune response of the subject to the flagellated bacterium.

In a still further aspect, methods of reducing morbidity associated with infection with a flagellated bacterium in a subject by administering a bacterium are provided. The bacterium includes a first polynucleotide sequence encoding a *fliC* polypeptide and a second polynucleotide sequence encoding a CD154 polypeptide. The CD154 polypeptide is capable of binding CD40, and has fewer than 50 amino acids, and includes amino acids 140-149 of SEQ ID NO: 8 or a homolog thereof. The bacterium is administered in an amount effective to reduce the morbidity of the subject after infection with the flagellated bacterium.

In another aspect, methods of reducing the morbidity of infection with a flagellated bacterium in a subject by administering a variant of *Salmonella enteritidis* 13A are provided. The *Salmonella* includes a first polynucleotide sequence encoding a *fliC* polypeptide. The *Salmonella* is administered in an amount effective to reduce the morbidity of the subject after infection with the flagellated bacterium.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the scheme for making site-directed mutations in *Salmonella enteritidis*.

Figure 2 depicts the design scheme of the overlapping extension PCR method used to generate the *fliC* and *fliC* -CD154 insertions into loop 9 of the *lamB* polynucleotide.

Figure 3 is a bar graph showing the percent *Salmonella enteritidis* recovery as compared to the initial inoculum in the liver/spleen and cecal tonsils at 3 days post-inoculation with the indicated bacteria.

Figure 4 is a bar graph showing the percent *Salmonella enteritidis* recovery as compared to the initial inoculum in the liver/spleen and cecal tonsils at 21 days post-inoculation with the indicated bacteria.

DETAILED DESCRIPTION

Vaccination against *Salmonella* is difficult because over 2,000 serovars have been described and immunity to one serovar generally does not confer immunity to a distinct serovar. Development of a vaccine to protect humans, poultry and other domesticated animals from *Salmonellosis* is needed. A vaccine capable of protecting against multiple serovars would be optimal. A vaccine comprising a highly conserved region of *fliC*, a flagellar filament protein found on flagellated Salmonella, is provided.

Recombinant DNA technologies enable relatively easy manipulation of many bacterial and viral species. Some bacteria and viruses are mildly pathogenic or non-pathogenic, but are capable of generating a robust immune response. These bacteria and viruses make attractive vaccine vectors for eliciting an immune response to antigens. Bacterial or viral vaccine vectors may mimic a natural infection and produce robust and long lasting immunity. Vaccine vectors are often relatively inexpensive to produce and administer. In addition, such vectors can often carry more than one antigen and may provide protection against multiple infectious agents.

In one aspect, a vaccine comprising a first polynucleotide sequence encoding a *fliC* polypeptide and a second polynucleotide sequence encoding a CD154 polypeptide which is capable of binding CD40 is provided. In another aspect, the use of vaccine vectors, such as bacterial vectors, for vaccination and generation of immune responses against *Salmonella* and other flagellated pathogenic bacteria is disclosed. *Salmonella* strains make suitable vaccine vectors because bacterial genes may be mutated or attenuated to create bacteria with low to no pathogenesis to the infected or immunized subject, while maintaining immunogenicity.

The majority of *Salmonella* isolates contain two genes that encode flagellar (H) antigens, *fliC* and *flj*B, which are alternately expressed by a phase-variation mechanism. The phase 1 antigens are encoded by *fliC* whereas *flj*B encodes the phase 2 antigens. A conserved region within *fliC* that has almost 100% homology between multiple *Salmonella* serovars and

between *fliC* and *flj*B has been identified. This conserved region of *fliC* is depicted in SEQ ID NO: 1 and was used to generate several vaccine vectors as described in the Examples. Other possible polypeptides for use in vaccine vectors are disclosed in SEQ ID NO: 2 (a similar region of *fliC* from *E. coli*), SEQ ID NO: 10, SEQ ID NO: 11 or SEQ ID NO: 12. Immunogenic fragments of the *fliC* polypeptides may also be used to generate vaccines. In addition, there is extensive homology between this conserved region of *fliC* and flagellar sequences of other bacteria, such as *Shigella* and *E. coli*, such that a vaccine vector expressing *fliC* may enhance the immune response to flagellated bacteria generally. Therefore, expression of these protective epitopes on the surface of a *Salmonella* vaccine vector may induce protective immunity against multiple serovars of the organism and may allow immunization against all flagellated bacterium.

Involvement of dendritic cells (DCs) is essential for the initiation of a powerful immune response as they possess the unique ability to activate naïve T cells, causing T cell expansion and differentiation into effector cells. It is the role of the DC, which is an antigen presenting cell (APC) found in virtually all tissues of the body, to capture antigens, transport them to associated lymphoid tissue, and then present them to naïve T cells. Upon activation by DCs, T cells expand, differentiate into effector cells, leave the secondary immune organs, and enter peripheral tissues. Activated cytotoxic T cells (CTLs) are able to destroy virus-infected cells, tumor cells or even APCs infected with intracellular parasites (e.g., *Salmonella*) and have been shown to be critical in the protection against viral infection.

CD40 is a member of the TNF-receptor family of molecules and is expressed on a variety of cell types, including professional antigen-presenting cells (APCs), such as DCs and B cells. Interaction of CD40 with its ligand CD154 is extremely important and stimulatory for both humoral and cellular immunity. Stimulation of DCs via CD40, expressed on the surface of DCs, can be simulated by anti-CD40 antibodies. In the body, however, this occurs by interaction with the natural ligand for CD40 (i.e. CD154) expressed on the surface of activated T-cells. Interestingly, the CD40-binding regions of CD154 have been identified. The CD40-binding region of CD154 may be expressed on the surface of a vaccine vector, such as a *Salmonella* vaccine vector, and may result in an enhanced immune response against a co-presented peptide sequence.

Salmonella can survive the gastrointestinal tract of the host and give rise to a mucosal immune response. Oral vaccines using a *Salmonella* vector produce a robust mucosal immune response and are relatively easy to administer to both animals and humans.

However, many of the current attenuated *Salmonella* vaccine strains are not as effective in generating a strong protective immune response as compared to their more virulent counterparts. Virulent strains provide a robust immune response but may also cause significant morbidity to the vaccinated subject. A *Salmonella* strain that could be used for effective mucosal, e.g., oral, vaccination would provide a vector that could be used to readily vaccinate a subject against one or more pathogenic agents, such as flagellated bacteria. Alternatively, a method of limiting an infection caused by a *Salmonella* vaccine vector would also be useful. Provided herein are methods of limiting an infection caused by a *Salmonella* vaccine vector by administering the *Salmonella* vaccine vector to a subject and administering a second vaccine vector comprising a first polynucleotide sequence encoding a *fliC* polypeptide. Administration of the second vaccine vector enhances the immune response to *Salmonella* and limits the infection caused by the *Salmonella* vaccine vector. The second vaccine vector may be administered before, at the same time as or after the *Salmonella* vaccine vector.

A *Salmonella enteritidis* strain useful as a vaccine vector, and various recombinant vaccine vectors made using this strain, are described. Specifically, a *Salmonella enteritidis* 13A (SE13A) capable of expressing an exogenous *fliC* polypeptide is provided. In addition, a vaccine vector and methods of enhancing an immune response in a subject by administering the vaccine vector comprising a first polynucleotide sequence encoding a *fliC* polypeptide and a second polynucleotide sequence encoding a polypeptide of CD154 or a homolog thereof that is capable of binding to CD40 are disclosed. The vaccine vectors may be used to enhance an immune response against *Salmonella* or another flagellated bacterium, such as *Escherichia coli* or *Shigella*, or may be used to reduce the morbidity associated with a flagellated bacterial infection.

A wild-type isolate of *Salmonella*, *Salmonella enteritidis* 13A (SE13A) (deposited with the American Type Culture Collection (ATCC) on September 13, 2006, deposit number PTA-7871), was selected based upon its unusual ability to cause mucosal colonization and submucosal translocation in chickens, permitting robust presentation of associated antigens or epitopes in commercial chickens. Importantly, this wild-type *Salmonella* isolate causes no clinically detectable disease or loss of performance in commercial chickens, indicating little disease-causing potential of the wild-type *Salmonella* in vertebrate animals.

The SE13A isolate may be further attenuated by inactivating at least one gene necessary for sustained replication of the bacteria outside of laboratory or manufacturing

conditions. Attenuated or variant *Salmonella* strains that can be used as vaccine vectors are described below. SE13A was used to generate attenuated *Salmonella* strains to develop vaccines and generate enhanced immune responses. SE13A is invasive, non-pathogenic for poultry and causes no measurable morbidity. These features result in an enhanced immune response as compared to non-invasive bacterial vectors. Attenuation of SE13A by mutation of genes that limit the ability of the bacterium to spread may increase the safety of the vaccine. For example, SE13A strains with mutations in *aroA* and/or *htrA* retain the ability to generate an immune response, but have limited replication in the host. Thus, the attenuation increases the safety of the vaccine vector without compromising the immunogenicity.

Mutations may be made in a variety of other *Salmonella* genes including, but not limited to, *cya*, *crp*, *asd*, *cdt*, *phoP*, *phoQ*, *ompR*, outer membrane proteins, *dam*, *htrA* or other stress related genes, *aro*, *pur* and *gua*. As shown in the Examples, mutations in *aroA* and *htrA* were found to attenuate SE13A. The *aro* genes are enzymes involved in the shikimate biosynthesis pathway or the aromatase pathway and *aro* mutants are auxotrophic for the aromatic amino acids tryptophan, tyrosine and phenylalanine. *htrA* is a stress response gene that encodes a periplasmic protease that degrades aberrant proteins. Mutants in *htrA* are also attenuated and display increased sensitivity to hydrogen peroxide.

The mutations in *aroA* and *htrA* described in the Examples are deletion mutations, but the mutations can be made in a variety of ways. Suitably, the mutations are non-reverting mutations that cannot be repaired in a single step. Suitable mutations include deletions, inversions, insertions and substitutions. A vaccine vector may include more than one mutation, for example a vaccine vector may contain mutations in both *aroA* and *htrA*. Methods of making such mutations are well known in the art.

SE13A or the attenuated SE13A variants may be used as vaccine vectors. Polynucleotides encoding *fliC* polypeptide antigens and other antigens from any number of pathogenic organisms may be inserted into the bacteria and expressed by the bacteria to generate antigenic polypeptides. The polynucleotides may be inserted into the chromosome of the bacteria or encoded on plasmids or other extrachromosomal DNA. Suitably, polynucleotides encoding fliC antigens are inserted into a bacterial polynucleotide that is expressed. Suitably, the bacterial polynucleotide encodes a transmembrane protein, and the polynucleotide encoding the fliC antigen is inserted into the bacterial polynucleotide sequence to allow expression of the fliC antigen on the surface of the bacteria. For example, the polynucleotide encoding *fliC* may be inserted in frame into the bacterial polynucleotide in

a region encoding an external loop region of a transmembrane protein such that the bacterial polynucleotide sequence remains in frame. See Example 1.

Alternatively, the first polynucleotide encoding *fliC* antigen may be inserted into a polynucleotide encoding a secreted polypeptide. Those of skill in the art will appreciate that the polynucleotide encoding the *fliC* antigen could be inserted in a wide variety of bacterial polynucleotides to provide expression and presentation of the *fliC* antigen to the immune cells of a subject treated with the bacterial vaccine vector. In the Examples, a polynucleotide encoding a *fliC* antigen was inserted into loop 9 of the *lamB* gene of SE13A. The polynucleotide encoding the *fliC* antigen may be included in a single copy or more than one copy. In the Examples, a bacterial vaccine vector containing a single copy of the *fliC* antigen inserted into loop 9 of *lamB* is described. Alternatively, copies of an epitope may be inserted into the bacterial vaccine vector at more than one location. The copies of the polynucleotide may be linked together or separated by a linker. Suitable linkers are known to those of skill in the art and include, but are not limited to a repeated amino acid, such as 1-10 serine residues.

As described in more detail below, a vaccine vector may include a CD154 polypeptide that is capable of binding CD40 in the subject and stimulating the subject to respond to the vaccine vector and its associated antigen. As described above, these polynucleotides may be inserted into the chromosome of the vaccine vector or maintained extrachromosomally. One of skill in the art will appreciate that these polynucleotides can be inserted in a variety of endogenous polynucleotides and expressed in different parts of the vaccine vector such as the cell wall or may be secreted. The polynucleotide encoding a CD154 polypeptide capable of enhancing the immune response to a foreign antigen may also encode the foreign antigen. The polynucleotide encoding a CD154 polypeptide may be linked to the polynucleotide encoding the *fliC* antigen, such that in the vaccine vector the CD154 polypeptide and the *fliC* antigen are present on the same polynucleotide. In the Examples, a polynucleotide encoding a polypeptide of CD154 that is capable of binding to CD40 also encodes the *fliC* antigen. See SEQ ID NOS: 1, 2, 9, 10 and 11 in the attached sequence listing. In the Examples, the polynucleotide encoding the *fliC* antigen and the polynucleotide encoding the CD154 polypeptide are both inserted in loop 9 of the *lamB* gene. Those of skill in the art will appreciate that bacterial polynucleotides encoding other transmembrane proteins and other loops of the *lamB* gene may also be used.

The SE13A bacteria include an exogenous polynucleotide encoding a *fliC* polypeptide that is a portion of the full-length *fliC* polypeptide natively associated with *Salmonella*. Suitably a polynucleotide encoding a portion of the *fliC* polypeptide or the entire *fliC* polypeptide may be inserted into the vaccine vector. In the Examples, a seven amino acid polypeptide (SEQ ID NO:1) was incorporated into SE13A. Suitably, the portion of the *fliC* polypeptide inserted into the vaccine vector is an immunogenic fragment. An immunogenic fragment is a peptide or polypeptide capable of eliciting a cellular or humoral immune response. Suitably, an immunogenic fragment of *fliC* may be 6 or more amino acids, 10 or more amino acids, 15 or more amino acids or 20 or more amino acids of the full-length protein sequence.

Other suitable epitopes for inclusion in a *fliC* vaccine vector include, but are not limited to, polynucleotides encoding other bacterial polypeptides. One of skill in the art will appreciate that a variety of sequences may be used in combination with any other antigen and may also be used in conjunction with polypeptides encoding immune stimulatory peptides such as a polypeptide of CD154.

As discussed above, a polynucleotide encoding a CD154 polypeptide that is capable of enhancing the immune response to the antigen may be included in the vaccine vector. Suitably, the CD154 polypeptide is fewer than 50 amino acids long, more suitably fewer than 40, fewer than 30 or fewer than 20 amino acids in length. The polypeptide may be between 10 and 15 amino acids, between 10 and 20 amino acids or between 10 and 25 amino acids in length. The CD154 sequence and CD40 binding region are not highly conserved among the various species. The CD154 amino acid sequences of chicken and human are provided in SEQ ID NO: 9 and SEQ ID NO: 8, respectively.

The CD40 binding regions of CD154 have been determined for a number of species, including human, chicken, duck, mouse and cattle and are shown in SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, respectively. Although there is variability in the sequences in the CD40 binding region between species, the human CD154 polypeptide was able to enhance the immune response in chickens. Therefore, one may practice the invention using species specific CD154 polypeptides or a heterologous CD154 polypeptide.

In the Examples, several SE13A recombinant bacteria were generated. In each of the SE13A strains containing polynucleotides encoding both the *fliC* and CD154 peptides, the fliC polypepetide and the CD154 polypeptide were encoded on the same polynucleotide and

were in frame with each other and with the *Salmonella lamB* polynucleotide in which they were inserted. In alternative embodiments, the CD154 polypeptide and the fliC polypeptide may be encoded by distinct polynucleotides. SE13A *aroA htrA fliC* contains a deletion in *aroA* and *htrA* and encodes both the *fliC* epitope (SEQ ID NO:1) and optionally the CD154 polypeptide (SEQ ID NO: 3) inserted into loop 9 of *lamB*.

Compositions comprising an attenuated *Salmonella* strain and a pharmaceutically acceptable carrier are also provided. A pharmaceutically acceptable carrier is any carrier suitable for *in vivo* administration. Examples of pharmaceutically acceptable carriers suitable for use in the composition include, but are not limited to, water, buffered solutions, glucose solutions or bacterial culture fluids. Additional components of the compositions may suitably include, for example, excipients such as stabilizers, preservatives, diluents, emulsifiers and lubricants. Examples of pharmaceutically acceptable carriers or diluents include stabilizers such as carbohydrates (e.g., sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein-containing agents such as bovine serum or skimmed milk and buffers (e.g., phosphate buffer). Especially when such stabilizers are added to the compositions, the composition is suitable for freeze-drying or spray-drying.

Methods of enhancing immune responses in a subject by administering a vaccine vector containing a *fliC* polypeptide and a CD154 polypeptide capable of binding to CD40 and activating CD40 are also provided. The vaccine vector comprising the polynucleotide encoding a CD154 polypeptide capable of binding to CD40 is administered to a subject in an amount effective to enhance the immune response of the subject to the vaccine. Suitably, the vaccine vector contains a polynucleotide encoding a polypeptide including amino acids 140-149 of the human CD154 polypeptide (SEQ ID NO: 8) or a homolog thereof. As noted above and demonstrated in the Examples, the CD40 binding region of CD154 is not conserved among species, yet heterologous CD154 was capable of enhancing the immune response (i.e. human sequence used in a chicken). Therefore, a homologue of amino acid 140-149 derived from one species may be used to stimulate an immune response in a distinct species.

Several suitable polypeptides are identified herein. Suitably, the polynucleotide encodes a CD154 polypeptide from the same species as the subject. Suitably, a polynucleotide encoding the polypeptide of SEQ ID NO: 3 is used in human subjects, a polynucleotide encoding the polypeptide of SEQ ID NO: 4 is used in chickens, a polynucleotide encoding the polypeptide of SEQ ID NO: 5 is used in ducks, a polynucleotide encoding the polypeptide of SEQ ID NO: 6 is used in mice, and a polynucleotide encoding

the polypeptide of SEQ ID NO: 7 is used in cows. In the Examples, the human CD154 polypeptide (SEQ ID NO: 3) was used in a chicken vaccine vector and was demonstrated to enhance the immune response to a foreign antigen. Thus other heterologous combinations of CD154 polypeptides and subjects may be useful in the methods of the invention. The CD154 polypeptide may be used to enhance the immune response in the subject to any foreign antigen or antigenic polypeptide present in the vaccine vector in addition to the *fliC* polypeptide. One of skill in the art will appreciate that the CD154 polypeptide could be used to enhance the immune response to more than one antigenic polypeptide present in a vaccine vector.

The polypeptide from CD154 stimulates an immune response at least in part by binding to its receptor, CD40. The Examples used a polypeptide homologous to the CD154 polypeptide which is expressed on immune cells of the subject and which is capable of binding to the CD40 receptor on macrophages and other antigen presenting cells. Binding of this ligand-receptor complex stimulates macrophage (and macrophage lineage cells such as dendritic cells) to enhance phagocytosis and antigen presentation while increasing cytokine secretions known to activate other local immune cells (such as B-lymphocytes). As such, molecules associated with the CD154 peptide are preferentially targeted for immune response and expanded antibody production.

Potential vaccine vectors for use in the methods include, but are not limited to, *Salmonella* (*Salmonella enteritidis*), *Shigella, Escherichia (E. coli), Yersinia, Bordetella, Lactococcus, Lactobacillus, Bacillus, Streptococcus, Vibrio (Vibrio cholerae), Listeria*, adenovirus, poxvirus, herpesvirus, alphavirus, and adeno-associated virus.

In addition, methods of enhancing an immune response against flagellated bacteria and methods of reducing morbidity associated with subsequent infection with a flagellated bacterium are disclosed. Briefly, the methods comprise administering to a subject a vaccine vector comprising a polynucleotide sequence encoding a *fliC* polypeptide in an effective amount. The *fliC* polypeptides include SEQ ID NO:1-2 and 10-12. The insertion of the *fliC* polypeptides into the vaccine vector may be accomplished in a variety of ways known to those of skill in the art, including but not limited to, the scarless site-directed mutation system described in BMC Biotechnol. 2007 Sept, 17: 7(1): 59, Scarless and Site-directed Mutagenesis in Salmonella enteritidis chromosome, which is incorporated herein by reference in its entirety. The vaccine vector may also be engineered to express the *fliC* polypeptides in conjunction with polynucleotides capable of enhancing the immune response

as discussed above, such as in SEQ ID NO:8 and SEQ ID NO:9. In particular, a polypeptide of CD154 capable of binding CD40 may be expressed by the vaccine vector to enhance the immune response of the subject to the fliC polypeptide. Optionally, the vaccine vector is a bacterium, such as *Salmonella enteritidis*.

The useful dosage of the vaccine vector to be administered will vary depending on the age, weight and species of the subject, the mode and route of administration and the type of pathogen against which an immune response is sought. The composition may be administered in any dose sufficient to evoke an immune response. For bacterial vaccines, it is envisioned that doses ranging from 10^3 to 10^{10} bacteria, from 10^4 to 10^9 bacteria, or from 10^5 to 10^7 bacteria are suitable. The composition may be administered only once or may be administered two or more times to increase the immune response. For example, the composition may be administered two or more times separated by one week, two weeks, or by three or more weeks. The bacteria are suitably viable prior to administration, but in some embodiments the bacteria may be killed prior to administration. In some embodiments, the bacteria may be able to replicate in the subject, while in other embodiments the bacteria may not be capable of replicating in the subject.

For administration to animals or humans, the compositions may be administered by a variety of means including, but not limited to, intranasally, mucosally, by spraying, intradermally, parenterally, subcutaneously, orally, by aerosol, or intramuscularly. Eye-drop administration or addition to drinking water or food are additionally suitable. For chickens, the compositions may be administered in ovo.

Some embodiments of the invention provide methods of enhancing immune responses in a subject. Suitable subjects may include, but are not limited to, vertebrates, suitably mammals, suitably a human, and birds, suitably poultry such as chickens. Other animal models of infection may also be used. Enhancing an immune response includes, but is not limited to, inducing a therapeutic or prophylactic effect that is mediated by the immune system of the subject. Specifically, enhancing an immune response may include enhanced production of antibodies, enhanced class switching of antibody heavy chains, maturation of antigen presenting cells, stimulation of helper T cells, stimulation of cytolytic T cells or induction of T and B cell memory.

It is envisioned that several epitopes or antigens from the same or different pathogens may be administered in combination in a single vaccine vector to generate an enhanced immune response against multiple antigens. Recombinant vaccine vectors may encode

antigens from multiple pathogenic microorganisms, viruses or tumor associated antigens. Administration of vaccine vectors capable of expressing multiple antigens has the advantage of inducing immunity against two or more diseases at the same time. For example, live attenuated bacteria, such as *Salmonella enteritidis* 13A, provide a suitable vaccine vector for eliciting an immune response against multiple antigens.

Bacterial vaccines may be constructed using exogenous polynucleotides encoding antigens which may be inserted into the bacterial genome at any non-essential site or alternatively may be carried on a plasmid using methods well known in the art. One suitable site for insertion of polynucleotides is within external portions of transmembrane proteins or coupled to sequences that target the exogenous polynucleotide for secretory pathways. One example of a suitable transmembrane protein for insertion of polynucleotides is the *lamB* gene. In the Examples, fliC and CD154 polynucleotides were inserted into loop 9 of the *lamB* sequence.

Exogenous polynucleotides include, but are not limited to, polynucleotides encoding antigens selected from pathogenic microorganisms or viruses and include polynucleotides of the vaccine vector which are expressed in such a way that an effective immune response is generated. Such polynucleotides may be derived from pathogenic viruses such as influenza (e.g., M2e, hemagglutinin, or neuraminidase), herpesviruses (e.g., the genes encoding the structural proteins of herpesviruses), retroviruses (e.g., the gp160 envelope protein), adenoviruses, paramyxoviruses, coronaviruses and the like. Exogenous polynucleotides can also be obtained from pathogenic bacteria, e.g., genes encoding bacterial proteins such as toxins, and outer membrane proteins. Further, exogenous polynucleotides from parasites are attractive candidates for use of a vector vaccine.

Polynucleotides encoding polypeptides involved in triggering the immune system may also be included in a vaccine vector, such as a live attenuated *Salmonella* vaccine. The polynucleotides may encode immune system molecules known for their stimulatory effects, such as an interleukin, Tumor Necrosis Factor, or an interferon, or another polynucleotide involved in immune-regulation. The vaccine vector may also include polynucleotides encoding peptides known to stimulate an immune response, such as the CD154 polypeptide described herein.

The following examples are meant only to be illustrative and are not meant as limitations on the scope of the invention or of the appended claims.

EXAMPLES

Example 1. Construction of fliC and fliC/CD154 inserts.

Strains and Culture Conditions

All plasmids were first maintained in TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA, USA) unless described otherwise. *Salmonella enteritidis* 13A was used for introduction of mutations. *Salmonella enteritidis* strain 13A was a field isolate available from USDA/APHIS/NVSL and deposited with the ATCC as deposit number PTA-7871. Bacteria carrying plasmid pKD46 were grown at 30°C. Other bacteria were grown at 37°C. Plasmid curing was conducted at 37°C.

Luria-Bertani (LB) media was used for routine growth of cells, and SOC media (Invitrogen, Carlsbad, CA, USA) was used for phenotypic expression after electroporation. When appropriate, the following antibiotics were added to the media: ampicillin (Amp) at 100μg/ml, kanamycin (Km) at 50μg/ml, and chloramphenicol (Cm) at 25μg/ml.

Plasmids

Plasmids pKD46, pKD13, and pBC-I-SceI were described previously (Datsenko and Wanner, PNAS 2000, 97:6640-6645 and Kang et al., J Bacteriol 2004, 186:4921-4930, both of which are incorporated herein by reference in their entireties). Plasmid pKD46 encodes Red recombinase enzymes which mediate homologous recombination of incoming linear DNA with chromosomal DNA. This plasmid also contains the Ampicillin resistance gene and is temperature-sensitive so that it requires 30°C for maintenance in the cell. Plasmid pKD13 served as a template for amplification of the Km resistance (Km^r) gene used in overlapping PCR. Plasmid pBC-I-SceI, which is maintained in the cell at 37°C, produces the I-SceI enzyme, which cleaves the following 18 base pair, rare recognition sequence: 5′-TAGGGATAACAGGGTAAT-3′ (SEQ ID NO:13). Plasmid pBC-I-SceI also contains the chloramphenicol resistance (Cm^r) gene.

PCR

All primers used for PCR are listed in Table 1. Typically, PCR was performed using approximately 0.1μg of purified genomic, plasmid or PCR-generated DNA (Qiagen, Valencia, CA, USA), 1x cloned *Pfu* polymerase buffer, 5U *Pfu* polymerase (Stratagene La Jolla, CA, USA), 1mM dNTPs (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and 1.2μM of each primer in a total volume of 50 μL. The DNA engine thermal cycler (Bio-Rad,

Hercules, CA, USA) was used with the following amplification conditions: 94°C for 2 minutes; 30 cycles of 94°C sec for 30 sec, 58°C for 60 sec, 72°C for 90 sec per 1 kb; and 72°C for 10 minutes for final extension. Each PCR product was gel purified (Qiagen, Valencia, CA, USA) and either eluted in 25μL EB buffer for preparation of templates used in overlapping extension PCR or in 50μL EB buffer, ethanol precipitated and suspended in 5μL of ddH_2O for electroporation into *S. enteritidis*.

Table 1. Primer sequences

Primer	Amplified region	Primer sequence
lam-up-f	loop 9 up	5'TGTACAAGTGGACGCCAATC 3' (SEQ ID NO: 14)
lam-up-f		5'*GTTATCGCCGTCTTTGATATAGCC* 3' (SEQ ID NO: 15)
lam-dn-f	looop 9 dn	5'*ATTTCCCGTTATGCCGCAGC* 3' (SEQ ID NO: 16)
lam-dn-f		5'GTTAAACAGAGGGCGACGAG 3' (SEQ ID NO: 17)
Km-f	I-SceI/Km^r gene	5'*GCTATATCAAAGACGGCGATAAC***TAACTATAAC GGTCCTAAGGTAGCGA**ATTTCCGGGGATCCGTC GA 3' (SEQ ID NO: 18)
Km-r		5'*GCTGCGGCATAACGGGAAAT* TGTAGGCTGGAGC TGCTTCG 3' (SEQ ID NO: 19)
Kan4f	inside Km^r gene: sequencing	5'CAAAAGCGCTCTGAAGTTCC 3' (SEQ ID NO: 20)
Kan4r		5'GCGTGAGGGGATCTTGAAGT 3' (SEQ ID NO: 21)
fliC up reverse	fliC/ loop 9 up	5'CGGTTCTGTACGGAGGAGGA*GTTATCGCCGTCT TTGATATAGCC* 3' (SEQ ID NO: 22
fliC down forward	fliC/ loop 9 down	5'TCCTCCTCCGTACAGAACCGTTTCAACTCCGCTA TTACCAACCTGGGCAACACCTCCTCCTCC*ATTTCC CGTTATGCCGCAGC* 3' (SEQ ID NO: 23)
fliC hCD154 up reverse	fliC-hCD154/ loop 9 up	5'GGAGGTGTTGCCCAGGTTGGTAATAGCGGAGTT GAAACGGTTCTGTACGGAGGAGGA*GTTATCGCCG TCTTTGATATAGCC* 3' (SEQ ID NO: 24)
fliC hCD154 up reverse	fliC-hCD154/ loop 9 down	5'CCAACCTGGGCAACACCTCCTCCTCCTGGGCAG AAAAAGGTTATTATACCATGTCTTCCTCCTCC*ATT TCCCGTTATGCCGCAGC* 3' (SEQ ID NO: 25)
fliC cCD154 up reverse	fliC-cCD154/ loop 9 up	5'GGAGGTGTTGCCCAGGTTGGTAATAGCGGAGTT GAAACGGTTCTGTACGGAGGAGGA*GTTATCGCCG TCTTTGATATAGCC* 3' (SEQ ID NO: 26)
fliC cCD154 up reverse	fliC-cCD154/ loop 9 down	5'CCAACCTGGGCAACACCTCCTCCTCC TGGATGACCACCTCCTATGCGCCGACCTCCTCCT CCTCC*ATTTCCCGTTATGCCGCAGC* 3' (SEQ ID NO: 27)
lam 3f	outer regions of loop 9: sequencing	5'GCCATCTCGCTTGGTGATAA 3' (SEQ ID NO: 28)
lam 3r		5'CGCTGGTATTTTGCGGTACA 3' (SEQ ID NO: 29)

In Table 1, italicized nucleotides are complementary to either side of the *lamB* gene loop 9 insertion site, which corresponds to nucleotide 1257 using *S. typhimurium* as an annotated reference genome. Bold font nucleotides represent the I-SceI recognition site in the Km-f primer.

Electroporation

Transformation of pKD46 into *S. enteritidis* was the first step carried out so that Red recombinase enzymes could be used for mediating recombination of subsequent mutations. Plasmid pKD46 was harvested from *E. coli* BW25113 (Datsenko and Wanner, PNAS 2000, 97:6640-6645) using a plasmid preparation kit (Qiagen Valencia, CA, USA). Then 0.5μL of pKD46 DNA was used for transformation into *S. enteritidis* 13A which had been prepared for electroporation. (Datsenko and Wanner, PNAS 2000, 97:6640-6645). Briefly, cells were inoculated into 10-15mL of 2X YT broth and grown at 37°C overnight. Then 100μL of overnight culture was re-inoculated into 10mL fresh 2X YT broth at 37°C for 3-4 hours. Cells to be transformed with pKD46 plasmid were heated at 50°C for 25 minutes to help inactivate host restriction. Cells were washed five times in ddH_2O water and resuspended in 60μL of 10% glycerol. Cells were then pulsed at 2400-2450kV for 1-6ms, incubated in SOC for 2-3 hours at 30°C and plated on LB media with appropriate antibiotics. *S. enteritidis* transformants with pKD46 were maintained at 30°C. When these transformants were prepared for additional electroporation reactions, all steps were the same except that 15% arabinose was added to induce Red recombinase enzymes one hour prior to washing, and cells did not undergo the 50°C heat step.

Loop 9 up- I-SceI/ Km^r- Loop 9 down Construct

Introduction of I-SceI enzyme recognition site along with the Km^r gene into loop 9 of the *lamB* gene was done by combining the Red recombinase system (Datsenko and Wanner, PNAS 2000, 97:6640-6645, which is incorporated herein by reference in its entirety) and overlapping PCR (Horton et al., BioTechniques 1990, 8:528-535, which is incorporated herein by reference in its entirety). The insertion site corresponds to nucleotide 1257 of the *lamB* gene using *Salmonella typhimurium* LT2 (S. *typhimurium*) as an annotated reference genome. First, the upstream and downstream regions immediately flanking the loop 9 insertion site (loop 9 up and loop 9 down, respectively) were amplified separately. Primers used were lam-up-f and lam-up-r for loop 9 up and lam-dn-f and lam-dn-r for loop 9 down. Then the Km^r gene from pKD13 plasmid was amplified using primers Km-f and Km-r. Here,

the I-SceI enzyme site was synthetically added to the 5' end of Km-f primer then preceded by a region complimentary to the loop-up-r primer. Likewise, a region complimentary to the loop-dn-f primer was added to the 5' end of Km-r primer. The complimentary regions allow all 3 PCR products to anneal when used as templates in one PCR reaction. Figure 2a represents this design scheme. PCR fragments consisting of loop 9 up- I-SceI/ Km^r- loop 9 down sequence (PCR-A) were electroporated into *S. enteritidis* cells, which harbored pKD46 and were induced by arabinose, and then plated on LB with Km plates. To verify the correct sequence orientation of the mutation, we performed colony PCR with primer pairs Kan4F/lam3f and Kan4R/lam3r, where Kan4F and Kan4R are Km^r gene-specific primers and lam3f and lam3r are primers located outside the *lamB* loop 9 region. These PCR fragments were gel purified (Qiagen, Valencia, CA, USA) and used for DNA sequencing.

Loop 9 up- fliC or CD154s or combination sequence- Loop 9 down Construct

The final overlapping PCR fragment, PCR-B, contained the added fliC antigen (or combination with CD154 sequences flanked by loop 9 up and down regions (Figure 2b). Combination sequences consisted of *fliC* polynucleotide comprising SEQ ID NO:1 and a CD154 polynucleotide sequence along with spacers such as Glycine (Gly) or Serine (Ser) residues.

To shorten the amount of steps for construction of the next fragment, the *fliC* or *fliC*-CD154 sequence was synthetically added to the 5' end of the lam-dn-f primer and preceded by the complimentary region to the loop-up-r primer. The previously used PCR product for loop 9 up could be used together with the newly constructed PCR product in which *fliC* or *fliC*-CD154 were incorporated at the 5' end of loop 9 down to perform the final PCR reaction. However, for other insert sequences (referred to as combination sequences), an extra PCR step was needed, due to the longer lengths of insert sequences, to amplify loop 9 up with added nucleotides specific to insertion sequences connected to loop-up-r primer. The coding sequence for Gly (GGT) and Serine (TCC) as well as all other amino acids were chosen based on compiled data of the most frequently used codons in *E. coli* and *Salmonella typhimurium* proteins. See Table 1 for further details of primer design.

Genomic Replacement of I-SceI/ Km^r with fliC or fliC-CD154

PCR-B products were electroporated into *S. enteritidis* cells along with plasmid pBC-I-SceI at a molar ratio of approximately 40:1 (Kang et al., J Bacteriol 2004, 186:4921-4930, which is incorporated herein by reference in its entirety). Clones for each PCR-B

recombination mutation were chosen according to the ability to grow on Cm plates but not on Km plates, due to the replacement of PCR-B for the Km^r encoding PCR-A sequence. Modified regions in the selected clones were PCR-amplified, and DNA sequences were determined using primers lam3f and lam3r located outside the loop 9 down and up amplified regions.

I-SceI site/ Km^r insertion mutation

The first mutation step involved designing a PCR fragment, PCR-A, which would serve as the carrier of the I-SceI site/ Km^r cassette to be inserted into the *lamB* site. PCR-A consisted of the I-SceI enzyme recognition site adjacent to the Km^r gene with approximately 200- 300bp of flanking DNA on each end homologous to the upstream and downstream regions of *lamB* loop 9 insertion site (loop 9 up and loop 9 down, respectively). The fragment was introduced into *S. enteritidis* cells expressing Red recombinase enzymes and Km^r colonies were selected. After screening a few colonies by colony PCR, positive clones were sequenced for the desired inserted I-SceI site/ Km^r sequence, and the identified mutant was selected and designated as SE164.

Genomic Replacement of I-SceI/ Km^r with fliC or fliC-CD154

The second mutation step required constructing a PCR fragment, referred to as PCR-B and shown in Figure 2B, consisting of the final insertion sequence, *fliC* or *fliC*-CD154, flanked by *lamB* homologous fragments. PCR-B amplicons have no selection marker and must be counter-selected after replacement for the previous I-SceI site/ Km^r mutation in SE164. Plasmid pBC-I-SceI encodes the Cm^r gene and the I-SceI enzyme, which will cut the genome at the I-SceI site of SE164. Therefore, pBC-I-SceI was electroporated into SE164 along with PCR-B. After recombination of PCR-B to replace PCR-A, positive clones were chosen based on the ability to grow on Cm but not on Km. After DNA sequencing of mutants to confirm successful recombination of PCR-B, the strains were designated *fliC*, *fliC*-cCD154 and *fliC*-hCD154. Ten random clones for each the *fliC* and *fliC*-CD154 insertion were used for PCR with lam 3f and lam 3r then digested using unique restriction enzymes sites for each insertion sequence and 100% of clones tested by digestion were positive for the desired mutation sequence. Sequencing results demonstrated that the insertion of *fliC* or *fliC*-CD154 was exactly into the loop 9 region without the addition of extraneous nucleotides.

Example 2. Attenuation of fliC or fliC-CD154 mutants/inserts.

Attenuation of SE13A was achieved by deletion mutation of the *aroA* gene and/or the *htrA* gene. Mutation of the *aroA* gene, a key gene in the chorismic acid pathway of bacteria, results in a severe metabolic deficiency which affects seven separate biochemical pathways. Mutation of the *htrA* gene reduces the cell's ability to withstand exposure to low and high temperatures, low pH, and oxidative and DNA damaging agents and reduces the bacteria's virulence.

To achieve deletion mutations in SE13A, the target gene sequence in the bacterial genome of *S. enteritidis* was replaced with the Km resistant gene sequence. This was completed using overlapping extension PCR and electroporation of the PCR products as described above. The Km resistance gene was targeted into the genomic region containing the genes of interest (*aroA* or *htrA*) by flanking the Km resistance gene with 200-300 base pairs of sequences homologous to the genes of interest. Once Km resistant mutants were obtained, the *aroA* or *htrA* deletion mutations were confirmed by DNA sequencing. The attenuated strains were also tested *in vivo* with regards to clearance time. Both of the attenuated strains had quicker clearance times than did the wildtype 13A strain, but both were able to colonize the liver, spleen, and cecal tonsils of chickens after oral infection. In addition, an attenuated strain lacking both *aroA* and *htrA* was also isolated.

Example 3. Colonization of chickens

Day-of-hatch chicks (40 per group) were inoculated with about 1x 10^8 cfu of various Salmonella isolates or saline control. The Salmonella isolates included the following: wtSE represents the original field isolate of *Salmonella enteritidis* 13A (SE13A); fliC represents the double attenuated (i.e., aroA and htrA) wild type SE13A expressing fliC in the lamB loop (cell surface); fliC-CD154C represents the double attenuated wild type SE13A similarly expressing fliC and a chicken CD154 oligopeptide.

On days 3 and 21 post-vaccination the cecal tonsils (CT) and the liver and spleen (L/S) were harvested and bacterial recovery assessed by a standard colony forming unit (cfu) assay in 10 animals per group. The results are shown in Figure 3 (Day 3) and Figure 4 (Day 21) and are reported as Percent wtSE recovery as measured by the number of cfu recovered compared to the inoculum. No (zero) Salmonellae were recovered from either the non-challenged controls (control) at day 3 or day 21 post-vaccination or the chicks inoculated with the fliC mutant by day 21 post-vaccination in the liver or spleen. These results suggest that the chicks are mounting an effective immune response against the *fliC* antigen that

allows them to clear the vaccine strain more rapidly than the wild-type SE13A. Different letters on the top of the bars in the graphs are indicative of significant differences from the wtSE ($p<0.05$). Therefore expression of *fliC* polypeptide on the surface of the bacterium induces a more robust immune response. Similar results were obtained when a human CD154 oligopeptide was used in place of the chicken CD154 peptide.

Serum was collected from the inoculated chicks and *fliC* specific antibody was measured by ELISA using standard procedures. *FliC* antibody production was not significantly increased in the animals vaccinated with *fliC* or *fliC*-CD154 containing SE.

CLAIMS

We claim:

1. A vaccine comprising a first polynucleotide sequence encoding a *fliC* polypeptide and a second polynucleotide sequence encoding a CD154 polypeptide capable of binding CD40, the CD154 polypeptide having fewer than 50 amino acids and comprising amino acids 140-149 of SEQ ID NO: 8 or a homolog thereof.

2. The vaccine of claim 1, wherein the *fliC* polypeptide comprises SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, an immunogenic fragment of SEQ ID NO: 1, an immunogenic fragment of SEQ ID NO: 2, an immunogenic fragment of SEQ ID NO: 10, an immunogenic fragment of SEQ ID NO: 11, or an immunogenic fragment of SEQ ID NO: 12.

3. The vaccine of claim 1 or 2, wherein the CD154 polypeptide comprises SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.

4. The vaccine of any of claims 1-3, wherein the vaccine is a bacterial vaccine.

5. The vaccine of claim 4, the bacterium comprising at least one of the *fliC* polypeptide and the CD154 polypeptide on its surface.

6. The vaccine of claim 4 or 5, wherein the bacterium is selected from the group consisting of Salmonella species, Escherichia species, Bacillus species and Lactobacillus species.

7. The vaccine of claim 6, wherein the bacterium is *Salmonella enteritidis.*

8. The vaccine of claim 7, wherein the bacterium is *Salmonella enteritidis* 13A selected from the group consisting of ATCC deposit number PTA-7871, ATCC deposit number PTA-7872, and ATCC deposit number PTA-7873.

9. The vaccine of any of the preceding claims, wherein at least one of the first polynucleotide and the second polynucleotide is inserted into a polynucleotide sequence encoding an external portion of a transmembrane protein.

10. The vaccine of claim 9, wherein the transmembrane protein is *lamB*.

11. The vaccine of any of the preceding claims, wherein the vaccine comprises more than one copy of the first polynucleotide sequence.

12. The vaccine of any of the preceding claims, wherein the vaccine comprises more than one copy of the second polynucleotide sequence.

13. The vaccine of any of the preceding claims, wherein the first polynucleotide sequence is linked in frame to the second polynucleotide sequence.

14. A vaccine comprising a variant of *Salmonella enteritidis* 13A, wherein the Salmonella comprises an exogenous first polynucleotide sequence encoding a *fliC* polypeptide.

15. The vaccine of claim 14, wherein the *fliC* polypeptide comprises SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, an immunogenic fragment of SEQ ID NO: 1, an immunogenic fragment of SEQ ID NO: 2, an immunogenic fragment of SEQ ID NO: 10, an immunogenic fragment of SEQ ID NO: 11, or an immunogenic fragment of SEQ ID NO: 12.

16. The vaccine of any of claims 14 or15, wherein the vaccine comprises more than one copy of the first polynucleotide sequence.

17. The vaccine of any of claims 14-16, wherein the first polynucleotide sequence is inserted into a polynucleotide sequence encoding an external portion of a transmembrane protein.

18. The vaccine of claim 17, wherein the transmembrane protein is lamB.

19. The vaccine of any of claims 14-18, further comprising a second polynucleotide sequence encoding a CD154 polypeptide capable of binding CD40 , the CD154 polypeptide having fewer than 50 amino acids and comprising amino acids 140-149 of SEQ ID NO: 8 or a homolog thereof.

20. The vaccine of claim 19, wherein the CD154 polypeptide comprises SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.

21. The vaccine of any of claims 19-20, wherein the first polynucleotide sequence is linked in frame to the second polynucleotide sequence.

22. The vaccine of any of claims 19-21, wherein the second polynucleotide sequence is inserted into a polynucleotide sequence encoding an external portion of a transmembrane protein.

23. The vaccine of claim 22, wherein the transmembrane protein is *lamB*.

24. The vaccine of any of claims 19-23, wherein the vaccine comprises more than one copy of the second polynucleotide sequence.

25. A method of enhancing the immune response against a flagellated bacterium in a subject comprising administering to the subject a vaccine vector comprising a first polynucleotide sequence encoding a *fliC* polypeptide and a second polynucleotide sequence encoding a CD154 polypeptide capable of binding CD40, the CD154 polypeptide having fewer than 50 amino acids and comprising amino acids 140-149 of SEQ ID NO: 8 or a homolog thereof, and the vaccine vector being administered in an amount effective to enhance the immune response of the subject to the flagellated bacterium.

26. The method of claim 25, wherein the *fliC* polypeptide comprises SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, an immunogenic fragment of SEQ ID NO: 1, an immunogenic fragment of SEQ ID NO: 2, an immunogenic fragment of SEQ ID NO: 10, an immunogenic fragment of SEQ ID NO: 11, or an immunogenic fragment of SEQ ID NO: 12.

27. The method of claim 25 or 26, wherein the CD154 polypeptide comprises SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.

28. The method of any of claims 25-27, wherein the vaccine vector is a bacterial vaccine vector.

29. The method of claim 28, the bacterium comprising at least one of the *fliC* polypeptide and the CD154 polypeptide on its surface.

30. The method of claims 28 or 29, wherein the bacterium is selected from the group consisting of Salmonella species, Escherichia species, Bacillus species and Lactobacillus species.

31. The method of claim 30, wherein the bacterium is *Salmonella enteritidis.*

32. The method of claim 31, wherein the bacterium is *Salmonella enteritidis* 13A selected from the group consisting of ATCC deposit number PTA-7871, ATCC deposit number PTA-7872, and ATCC deposit number PTA-7873.

33. The method of any of claims 25-32, wherein at least one of the first polynucleotide and the second polynucleotide is inserted into a polynucleotide sequence encoding an external portion of a transmembrane protein.

34. The method of claim 33, wherein the transmembrane protein is *lamB*.

35. The method of any of claims 25-34, wherein the vaccine comprises more than one copy of the first polynucleotide sequence.

36. The method of any of claims 25-35, wherein the vaccine comprises more than one copy of the second polynucleotide sequence.

37. The method of any of claims 25-36, wherein the first polynucleotide sequence is linked in frame to the second polynucleotide sequence.

38. The method of any of claims 25-37, wherein the bacterium is administered by a method selected from the group consisting of oral, intranasal, parenteral, and in ovo.

39. The method of any of claims 25-38, wherein the enhanced immune response comprises an enhanced antibody response.

40. The method of any of claims 25-39, wherein the enhanced immune response comprises an enhanced T cell response.

41. The method of any of claims 25-40, wherein the subject is a member of a poultry species.

42. The method of claim 41, wherein the poultry species is a chicken.

43. The method of any of claims 25-40, wherein the subject is a mammal.

44. The method of claim 43, wherein the subject is a human.

45. The method of any of claims 25-44, wherein from about 10^4 to about 10^9 bacteria are administered to the subject.

46. The method of any of claims 25-45, wherein from about 10^5 to about 10^7 bacteria are administered to the subject.

47. The method of any of claims 25-46, wherein the bacterium is killed prior to administration to the subject.

48. The method of any of claims 25-46, wherein the bacterium is not capable of replicating in the subject.

49. A method of enhancing the immune response against a flagellated bacterium in a subject comprising administering to the subject a variant of *Salmonella enteritidis* 13A comprising an exogenous first polynucleotide sequence encoding a *fliC* polypeptide in an amount effective to enhance the immune response of the subject to the flagellated bacterium.

50. The method of claim 49, wherein the *fliC* polypeptide comprises SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, an immunogenic fragment of SEQ ID NO: 1, an immunogenic fragment of SEQ ID NO: 2, an immunogenic fragment of SEQ ID NO: 10, an immunogenic fragment of SEQ ID NO: 11, or an immunogenic fragment of SEQ ID NO: 12.

51. The method of any of claims 49 or 50, wherein the vaccine comprises more than one copy of the first polynucleotide sequence.

52. The method of any of claims 49-51, wherein the first polynucleotide sequence is inserted into a polynucleotide sequence encoding an external portion of a transmembrane protein.

53. The method of claim 52, wherein the transmembrane protein is *lamB*.

54. The method of any of claims 49-53, further comprising a second polynucleotide sequence encoding a CD154 polypeptide capable of binding CD40 , the CD154 polypeptide having fewer than 50 amino acids and comprising amino acids 140-149 of SEQ ID NO: 8, or a homolog thereof.

55. The method of claim 54, wherein the CD154 polypeptide comprises SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7.

56. The method of any of claims 54-55, wherein the first polynucleotide sequence is linked in frame to the second polynucleotide sequence.

57. The method of any of claims 49-56, wherein the bacterium is administered by a method selected from the group consisting of oral, intranasal, parenteral, and in ovo.

58. The method of any of claims 49-57, wherein the enhanced immune response comprises an enhanced antibody response.

59. The method of any of claims 49-58, wherein the enhanced immune response comprises an enhanced T cell response.

60. The method of any of claims 49-59, wherein the subject is member of a poultry species.

61. The method of claim 60, wherein the poultry species is a chicken.

62. The method of any of claims 49-59, wherein the subject is a mammal.

63. The method of claim 62, wherein the subject is a human.

64. The method of any of claims 49-63, wherein from about 10^4 to about 10^9 bacteria are administered to the subject.

65. The method of any of claims 49-64, wherein the bacterium is killed prior to administration to the subject.

66. The method of any of claims 49-64, wherein the bacterium is not capable of replicating in the subject.

67. A method of reducing morbidity associated with infection with a flagellated bacterium in a subject comprising administering to the subject a vaccine vector comprising a first polynucleotide sequence encoding a *fliC* polypeptide and a second polynucleotide sequence encoding a CD154 polypeptide capable of binding CD40, the CD154 polypeptide having fewer than 50 amino acids and comprising amino acids 140-149 of SEQ ID NO: 8 or a homolog thereof, and the bacterium being administered in an amount effective to enhance the immune response of the subject to the flagellated bacterium.

68. A method of reducing morbidity associated with infection with a flagellated bacterium in a subject comprising administering to the subject a variant of *Salmonella enteritidis* 13A comprising an exogenous first polynucleotide sequence encoding a *fliC* polypeptide in an amount effective to enhance the immune response of the subject to the flagellated bacterium.

69. A method of limiting an infection caused by a Salmonella vaccine vector comprising administering the Salmonella vaccine vector to a subject and administering a second vaccine vector comprising a first polynucleotide sequence encoding a *fliC* polypeptide, wherein the second vaccine vector enhances the immune response to Salmonella and limits the infection caused by the Salmonella vaccine vector.

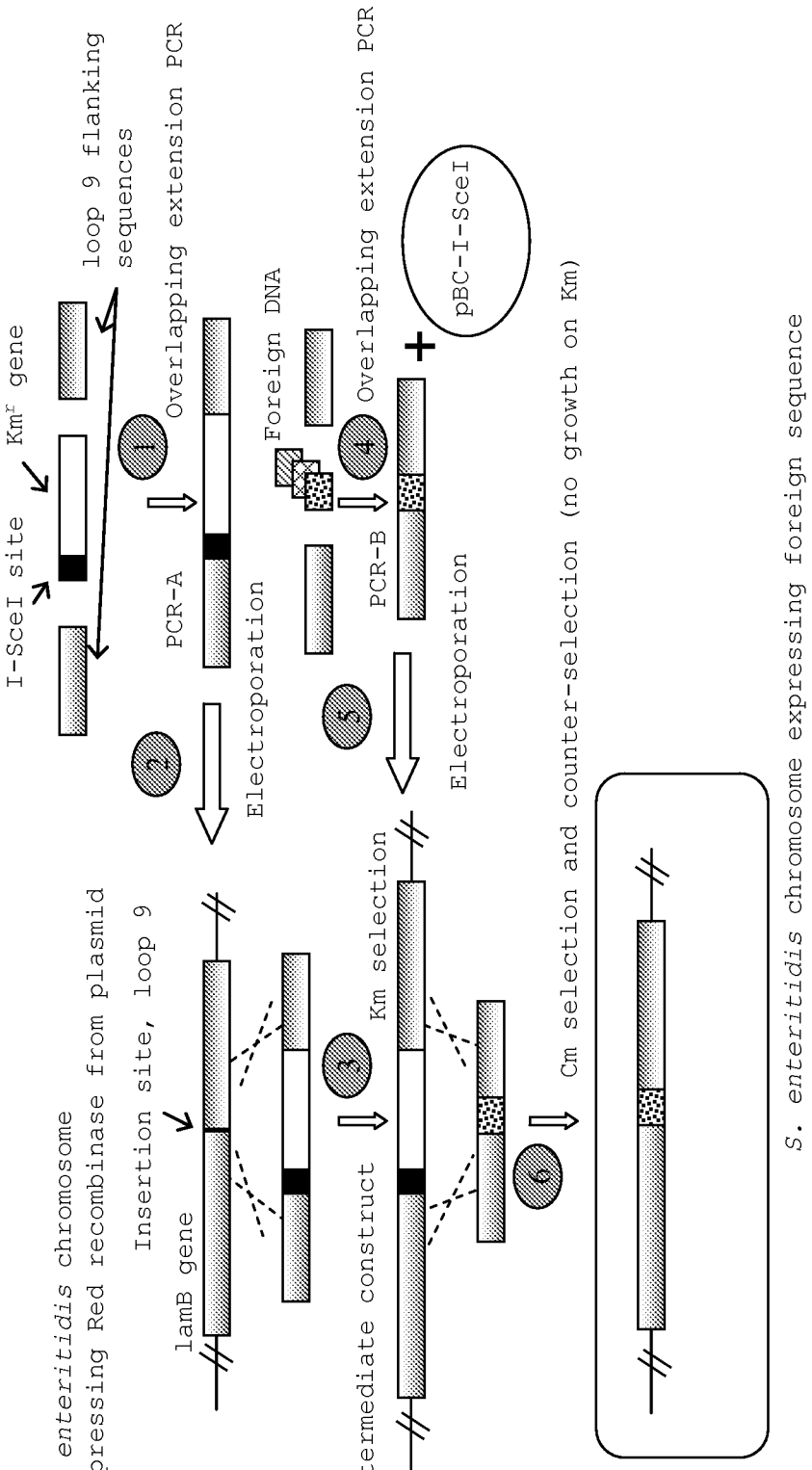
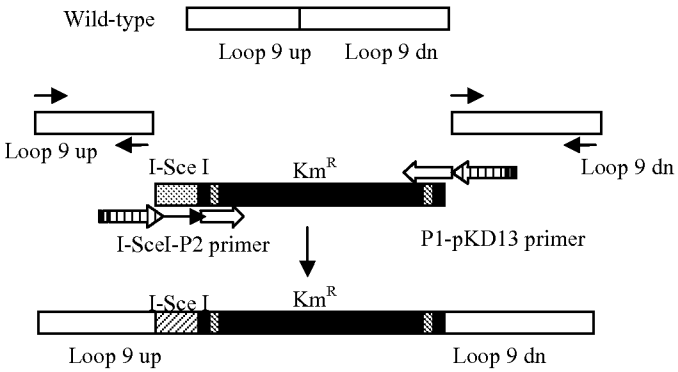
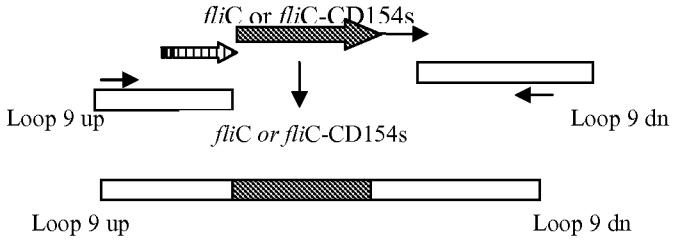

S. enteritidis chromosome
expressing Red recombinase from plasmid
Insertion site, loop 9
lamB gene
I-SceI site
Km^r gene
loop 9 flanking sequences
1
Overlapping extension PCR
PCR-A
2
Electroporation
Foreign DNA
PCR-B
4
Overlapping extension PCR
pBC-I-SceI
5
Electroporation
Intermediate construct
3
Km selection
6
Cm selection and counter-selection (no growth on Km)
S. enteritidis chromosome expressing foreign sequence

FIG. 1



PCR-A

FIG. 2A



PCR-B

FIG. 2B

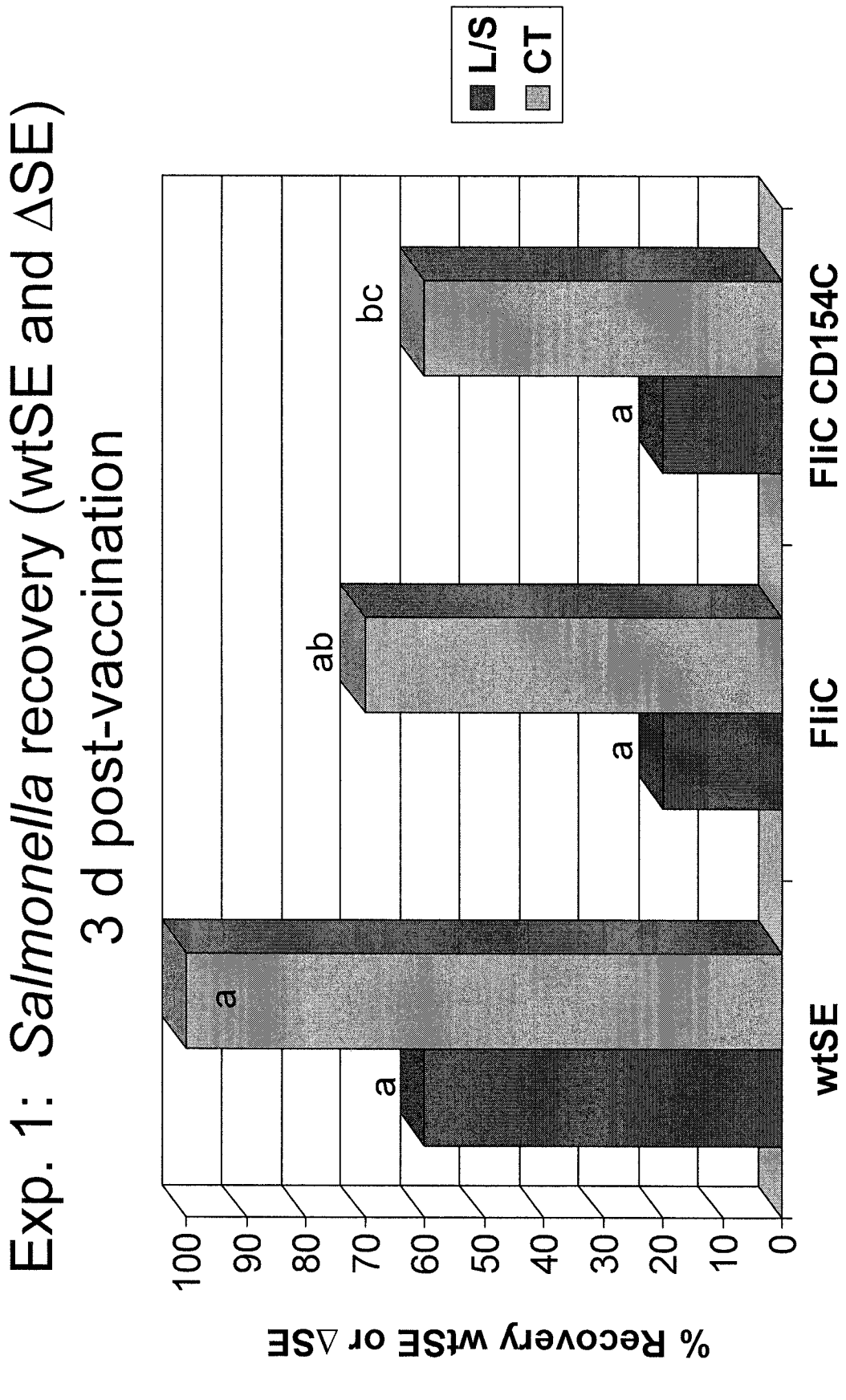

Exp. 1: Salmonella recovery (wtSE and ΔSE)
3 d post-vaccination
L/S
CT
% Recovery wtSE or ΔSE
100
90
80
70
60
50
40
30
20
10
0
a
a
ab
a
bc
a
wtSE
FliC
FliC CD154C
P-value ≤ 0.05

FIG. 3

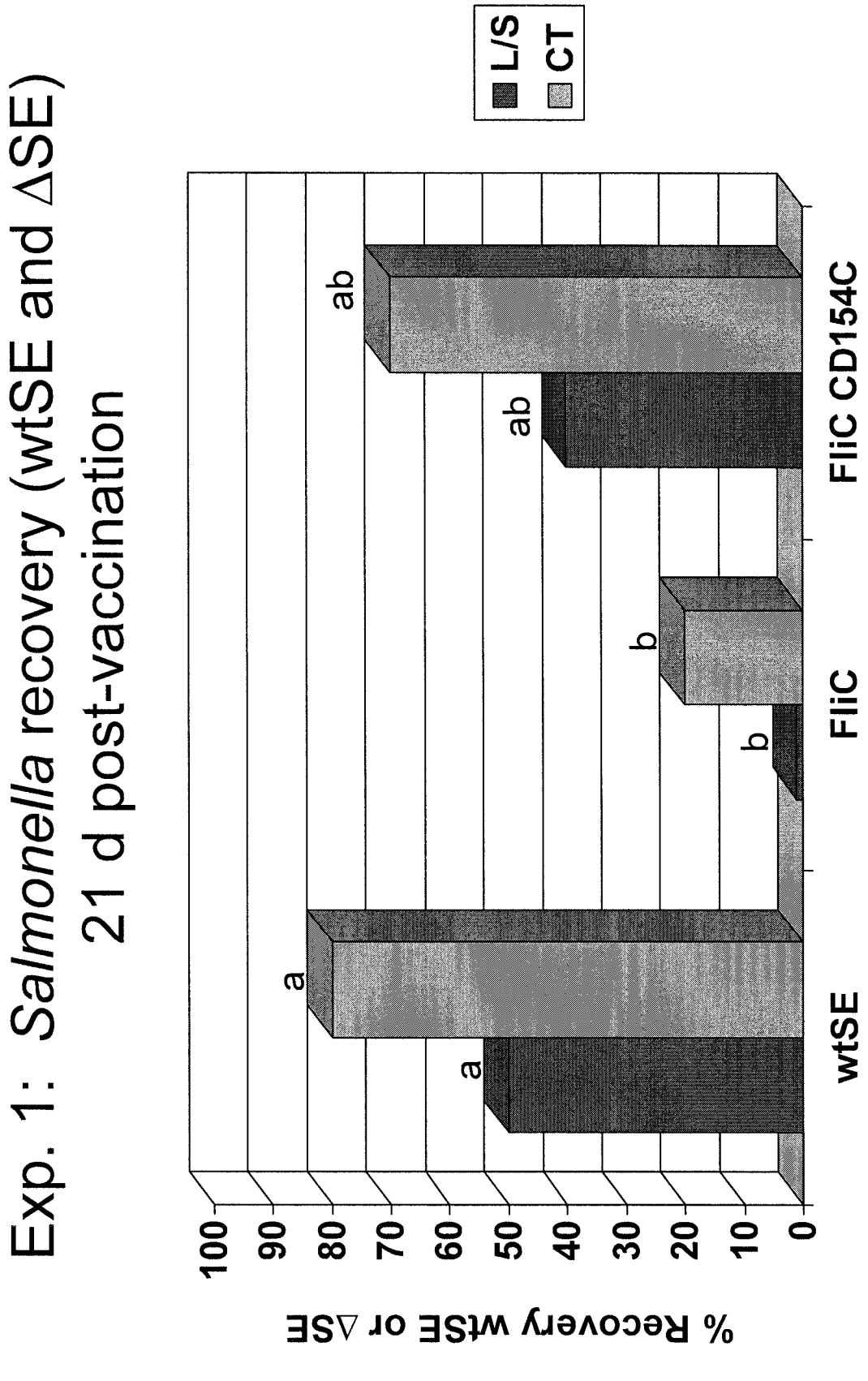

Exp. 1: Salmonella recovery (wtSE and ΔSE)
21 d post-vaccination
L/S
CT
% Recovery wtSE or ΔSE
100
90
80
70
60
50
40
30
20
10
0
a
a
b
b
ab
ab
wtSE
FliC
FliC CD154C
P-value ≤ 0.05

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/84813

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - B05D 5/12, H01L 21/00 (2009.01)
USPC - 427/8, 438/22, 257/79
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 427/8, 29/574, 438/22, 257/79
IPC(8): B05D 5/12, H01L 21/00 (2009.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 427/8, 29/574, 438/22, 257/79 (text search)
IPC(8): B05D 5/12, H01L 21/00 (2009.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST: US Patents full-text; US PGPubs full-text; EPO Abstracts; and JPO Abstracts; Google
Terms: self-healing universal non-uniformity treatment (SHUNT) system, nonuniformities/defects/irregularities, photovoltaic, self-healing electrolyte treatment (aniline materials,p-toluenesulphonic acid, perylene diimide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 7,098,058 B1 (KARPOV et al.) 29 August 2006 (29.08.2006), cols 5-11	2, 6, 13, 14, 16-18, 20-24 --- 1, 3-5, 7-12, 15, 19
Y	US 4,510,674 A (IZU et al.) 16 April 1985 (16.04.1985), col 2, ln 62 to col 3, ln 2; cols 7-12	1, 3-5, 7-12, 15
Y	US 5,800,632 A (ARAO et al.) 01 September 1998 (01.09.1998), col 3, lns 42-49; col 10, lns 2-3	19
Y	US 2007/0227586 A1 (ZAPALAC, JR.) 04 October 2007 (04.10.2007), para [0026]	7
Y	US 2006/0249202 A1 (YOO et al.) 09 November 2006 (09.11.2006), para [0033]	15
A	US 4,420,497 A (TICKLE) 13 December 1983 (13.12.1983), entire document	1-24

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 March 2009 (11.03.2009)	20 MAR 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/84813

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

Two claim groups were found:
Group I: Claims 1-24
Group II: Claims 25-42

-- see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Claims 1-24

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/84813

Continuation of Box No. III -- Observations where unity of invention is lacking

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

Group I: is directed to a A diagnostic and self-healing treatment system for a semiconductor device, the system comprising: i) a shunt busting/blocking treatment system, ii) a self-healing treatment system, and iii) an in-situ contact diagnostic system.

Group II: is directed to a diagnostic and self-healing treatment system for a semiconductor device having a substrate layer, a base electrode layer, a semiconductor layer, and at least one electrochemically active treatment material applied to at least a top surface of the semiconductor layer, the system comprising: at least a first conductive electrode lead configured to be removably connected to the base electrode layer; at least a second conductive electrode lead configured to be removably connected to the electrochemically active treatment material; at least a first external power source configured to be removably connected to the first conductive electrode lead and the second conductive electrode lead; and at least one device configured to conduct a non-contact diagnostic evaluation of the semiconductor device based on impedance measurements; and optionally at least a second external power source configured to be removably connected to the first conductive electrode lead and the second conductive electrode lead.

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

Group I does not include the inventive concept of at least a first conductive electrode lead configured to be removably connected to the base electrode layer; at least a second conductive electrode lead configured to be removably connected to the electrochemically active treatment material; at least a first external power source configured to be removably connected to the first conductive electrode lead and the second conductive electrode lead; and at least one device configured to conduct a non-contact diagnostic evaluation of the semiconductor device based on impedance measurements, as required by Group II.

Group II does not include the inventive concept of a shunt busting/blocking treatment system, as required by Group I.

Further, Groups I and II lack unity of invention, because even though the inventions of these groups require the technical feature of a self-healing semiconductor device, this technical feature is not a special technical feature as it does not make a contribution over the prior art in view of US 4,420,497 A (Tickle). Tickle discloses a self-healing semiconductor device as are known in the art (col 3, ln 63-col 4, ln 3).

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