Abstract:
The present invention provides polynucleotides comprising modified structural genes for flagellin. The recombinant genes are useful in the preparation of an expression vector for the expression of peptide epitopes as recombinant flagellin fusion proteins. The recombinant flagellin fusion proteins are useful in the production of vaccines.
FIELD OF THE INVENTION

The present invention relates to a polynucleotide comprising a structural gene for flagellin useful in the preparation of an expression vector for the expression of peptide epitopes as recombinant flagellin fusion proteins, and methods of use thereof for the production of vaccines.

BACKGROUND OF THE INVENTION

Flagellin based vaccines

Flagella are organelles involved in locomotion of bacterial cells. Bacterial flagella are helical filaments composed primarily of flagellin, the major structural component of the organelle accounting for more than 95% of the total mass. Flagellin has a mass of about 30-60 kD that arranges itself in a hollow cylinder to form the filament in bacterial flagellum. The structure of flagellin is responsible for the helical shape of the flagellar filament, which in turn is important for its proper function.

Structural analyses of bacterial flagella have revealed a common structural design among filaments isolated from different bacteria. The amino and carboxy-termini are structurally conserved and form the inner core of the flagellin protein. The termini are responsible for the flagellin's ability to polymerize into a filament. The central portion of the protein forms the outer surface of the flagellar filament. While the termini of the protein are similar between all bacterial flagellins, the central portion is wildly variable, hence the term hypervariable domain. Mutations in the flagellin structural gene produce changes in efficiency of filament formation, filament shape, sensitivity to flagellotropic phage, and/or the antigenic specificity of the flagella. Mammals often have acquired immune responses to flagellar antigens. A high degree of antigenic polymorphism is seen within a single genus, such as Salmonella, and is useful for identifying individual serotypes within a single species.

The structural and functional organization of the flagellum of the enteropathogenic bacteria Salmonella enterica serovar Typhimurium has been recently
reported (Ramos, 2004). The flagellar filament is composed of approximately 20,000 subunits of flagellin, which is encoded by the fliC gene.

Recombinant DNA technology involves insertion of DNA sequences into a DNA construct (vector) to produce a recombinant DNA molecule, which is capable of replicating in a host cell. The resulting product is a recombinant peptide or protein synthesized from the recombinant DNA. Certain vectors useful for the construction of recombinant DNA flagellin molecules are known.

US Patent No. 6,130,082 discloses recombinant flagellin fusion proteins comprising an epitope encoded by a functional flagellin structural gene and at least one epitope of a heterologous organism, which epitope is immunogenic upon introduction of the fusion protein into a vertebrate host. The epitope may be a small peptide of four amino acids, e.g. the Asn-Ala-Asn-Pro peptide representing the P. Falciparum immunodominant repeating epitope, or a large region of proteins encoding B-cell and T-cell epitopes. That invention is exemplified by a recombinant gene comprising a Salmonella H1-d flagellin structural gene into which a foreign epitope is inserted in place of DNA, which naturally occurs between the natural EcoRV restriction sites. EcoRV digestion produces blunt ends for cloning.

US Patent No. 4,886,748 teaches a DNA sequence comprising the hag gene which encodes flagellin of Escherichia coli or the hag gene lacking the DNA between base pairs 583 and 1143; and a DNA sequence which encodes a heterologous peptide of about 15 amino acids, wherein said DNA sequence encodes a fused protein which is excreted through the cell wall of an Escherichia coli host.

US Patent No. 4,702,911 discloses the use of purified subunits of bacterial pili from Neisseria gonorrhoeae, Moraxella hovis, and Escherichia coli useful in preparing vaccines which exhibit increased cross protection against different bacterial strains.

US Patent No. 4,801,536, discloses the expression of fusion proteins comprising an N-terminal portion of a flagellin protein and a sequence encoding a heterologous protein, so that the protein is exported from the host cell into the extracellular medium.

International patent publication WO 93/20846 teaches recombinant influenza vaccines against a plurality of different influenza virus strains comprising at least one recombinant protein comprising the amino acid sequence of flagellin and at least one amino acid sequence of an epitope of influenza virus HA or NP, or an aggregate of said
chimeric protein. International patent publication WO 00/32228 teaches human synthetic peptide based influenza vaccine for intranasal administration comprising at least four epitopes of influenza virus, wherein the epitopes are as follows: (i) one B-cell haemagglutinin (HA) epitope; (ii) one T-helper haemagglutinin (HA) or nucleoprotein (NP) epitope that can bind to many HLA molecules; and (iii) at least two cytotoxic lymphocyte (CTL) nucleoprotein (NP) or matrix protein (M) epitopes that are restricted to the most prevalent HLA molecules in different human populations.

There remains an unmet need for recombinant genes useful for the production of recombinant flagellin fusion proteins, containing peptides in multiple copies or in linear arrays.

**SUMMARY OF THE INVENTION**

The present invention provides a polynucleotide comprising modified fliC genes useful in the preparation of an expression vector comprising heterologous peptide epitopes and preparation of recombinant flagellin fusion proteins. The recombinant fusion proteins are useful in the preparation of vaccines. The modifiedZ/C genes provide a cloning site for a peptide or a plurality of peptides, wherein each peptide comprises from about 5 amino acids to about 50 amino acids, and a plurality of unique restriction enzyme sites, wherein at least one site has a cohesive overlapping end, allowing oriented cloning of the insert or inserts.

The modified genes may be used to construct a prokaryotic expression vector comprising a modified *Salmonella* flagellin gene comprising a plurality of unique restriction enzyme sites useful for the insertion of at least one peptide epitope; and a selection marker. According to a specific embodiment, the present invention is exemplified using a kanamycin resistance gene, yet any other antibiotic resistant gene is suitable, as are other types of marker genes. The modified gene is useful for preparation of a recombinant expression vector comprising at least one heterologous peptide epitope. The recombinant expression vector can direct the synthesis of a recombinant flagellin fusion protein, for use in the preparation of a vaccine.

According to the present invention the peptide epitope is inserted into the flagellin hypervariable domain, which is non-essential to the function of the encoded flagellin. The resulting recombinant fusion proteins comprise a flagellin protein and at least one peptide epitope. The recombinant fusion protein can be used to elicit an
immune response to the peptide epitope upon introduction of said fusion protein into a vertebrate host.

According to one aspect the present invention provides a modified Salmonella enterica serovar Typhi fliC gene comprising two unique DNA restriction sites within the hypervariable region, each unique restriction site flanking a natural EcoRV restriction site. According to one embodiment the modified fliC gene is set forth in SEQ ID NO:3. In some alternative embodiments the present invention provides a homolog to SEQ ID NO:3, the homolog having about 90%, about 95%, about 98% or about 99% homology to SEQ ID NO:3.

In one embodiment each of the two unique restriction enzyme sites, when cleaved, creates a 5’ nucleotide overhang. The present invention is exemplified with the Bsu36I and Eagl restriction enzyme sites. It is to be understood that other enzyme combinations are suitable. In one embodiment a first unique restriction site is a Bsu36I restriction site and a second unique restriction site is an Eagl restriction site. In other embodiments a peptide epitope is cloned into the Bsu36I and EcoRV restriction enzyme sites; or alternatively into the Eagl and EcoRV restriction enzyme sites.

According to another aspect the present invention provides a polynucleotide which is a prokaryotic expression vector comprising a modified Salmonella enterica serovar Typhi fliC gene set forth in SEQ ID NO:3 and a selection gene. In one embodiment the selection gene is a kanamycin resistance gene.

In certain embodiments, gene expression is driven by a promoter element. In some embodiments gene expression is driven by the beta-galactosidase promoter. In one embodiment the expression vector comprises a polynucleotide sequence set forth in SEQ ID NO:1 and is denoted herein pBVXOO. In another embodiment the expression vector comprises a polynucleotide sequence set forth in SEQ ID NO:2.

In some embodiments the peptide epitope is a natural or synthetic epitope. In some embodiments the peptide epitope is an artificial sequence. In some embodiments the peptide epitope is derived from tumor associated antigens, autoimmune disorder-associated antigens, or other antigens from human or animal origin. In some embodiments the peptide epitope derives from a heterologous organism; for example, a plant, a bacterium, a virus, a parasite, or a fungus. In some embodiments the heterologous organism is a virus. In certain embodiments the virus is an influenza virus.
The recombinant expression vector can be inserted into a host cell or organism that will support transcription and translation of the DNA into a recombinant flagellin fusion protein.

In another aspect the present invention provides a host cell comprising the recombinant expression vector according to the present invention. According to one embodiment the host cell is an E. coli bacterium carrying the plasmid pBVXOO whose polynucleotide sequence is set forth in SEQ ID NO:1, deposited on April 3, 2007 at the Collection Nationale de Cultures de Microorganismes (CNMC), Institut Pasteur, Paris, France, with the identification reference E. Coli XL1 Blue/pBVXOO and was assigned the registration number CNMC 1-3735.

The recombinant expression vector may be expressed in a flagella-deficient bacterial host. In one embodiment the bacterial host is a flagella-deficient E. coli host. In another embodiment the bacterial host is a flagella-deficient Salmonella or Shigella host.

According to some embodiments the recombinant flagellin polypeptide is a functional flagellin that is exported to the cell surface to form bacterial flagella. According to other embodiments the recombinant flagellin polypeptide is non-functional and forms aggregates comprising several such flagellin polypeptides in the bacterial cell.

In yet another aspect the invention provides recombinant flagellin proteins encoded by the polynucleotide, and the use of the proteins in vaccine formulations, for protection against infection by the heterologous organism or for protection against conditions or disorders caused by an antigen of the heterologous organism. The recombinant flagellin proteins according to the present invention are useful for presenting a desired epitope in an immunogenic form, to elicit an immune response.

In one embodiment the recombinant flagellin protein of the invention elicits an immune response selected from a cellular response, a humoral response, or a combination of a cellular and humoral response.

In yet another aspect the present invention provides a vaccine comprising at least one recombinant flagellin protein according to the present invention. The vaccine is useful for protection against a viral, bacterial, parasitic, or fungal organism, or for protection against conditions or disorders caused by an antigen of the heterologous organism.
In yet another aspect the present invention provides an immunogenic composition comprising at least one recombinant flagellin protein according to the present invention. The immunogenic composition is useful in the treatment or alleviation of diseases including autoimmune disease, cancer, allergy, prion disease and neurodegenerative disease such as Alzheimer's disease.

In some embodiments, the recombinant flagellin genes of the invention can be expressed by attenuated bacteria, in live oral vaccine formulations. In other specific embodiments, the recombinant flagellin fusion proteins can be formulated for use in vaccines in unit dosage form.

In another aspect the present invention provides a method for inducing an immune response and conferring protection against a heterologous organism in a subject, wherein the method comprises administering to the subject a vaccine comprising at least one recombinant flagellin fusion protein according to the principles of the present invention.

In certain embodiments the vaccine protects a subject against a viral disease. In some embodiments the vaccine protects a subject from influenza virus.

Routes of administration of the vaccine and the immunogenic composition include, but are not limited to intranasal, intramuscular, oral, intravenous, topical, mucosal intraperitoneal, subcutaneous, and transdermal delivery. Preferred routes of administration include oral, intranasal and intramuscular administration. In some embodiments the vaccine and the immunogenic composition are formulated for intranasal administration. In other embodiments the vaccine and the immunogenic composition are formulated for intramuscular administration.

These and other embodiments of the present invention will become apparent in conjunction with the description and claims that follow.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows SDS-PAGE of lysate fractions of flagella-deficient bacteria transformed with a vector carrying the modified fliC gene (pBND12.9), a negative control (pTRcHisA, the empty vector), and a positive control (pLS408).

Figure 2 shows Western analysis with an anti-flagellin antibody of the supernatant fraction of lysed flagella-deficient bacteria transformed with a vector
carrying the modified fliC gene (pBND12.9) and a negative control (pTRcHisA, the empty vector).

Figure 3 depicts a partial restriction map of the expression vector pBWXOO.

Figure 4 shows SDS-PAGE of lysate fractions of flagella-deficient bacteria transformed with a vector carrying the modified fliC gene (pBWXOO), a negative control (pBNDS.1) and a positive control (pBND12.9).

Figure 5 shows Western analysis with an anti-flagellin antibody of the supernatant fraction of lysed flagella-deficient bacteria transformed with a vector carrying the modified fliC gene (pBWXOO), a negative control (pBNDS.1) and a positive control (pBND12.9).

Figure 6 shows Western analysis with an anti-flagellin antibody of protein expression in membrane preparations of flagella-deficient bacteria transformed with a vector carrying the modified fliC gene (pBWXOO), a negative control (pBNDS.1) and a positive control (pBND12.9).

Figure 7 demonstrates the serum titer of antibodies to influenza peptide HA 91-108 in rabbits vaccinated with recombinant flagellin expressing this peptide epitope.

Figure 8 demonstrates the serum titer of anti-flagella antibodies in rabbits vaccinated with recombinant flagellin expressing influenza virus epitope HA 91-108.

Figure 9 demonstrates the serum titer of antibodies to influenza peptide HA 91-108 in mice vaccinated with recombinant flagellin expressing this peptide epitope. Pooled sera from each experimental group was tested for IgG titer specific to the HA 91-108 peptide after the third immunization.

Figure 10 demonstrates the serum titer of antibodies to H3N2 virus (A/Texas/1/77) in mice vaccinated with recombinant flagellin expressing influenza virus epitope HA 91-108. Pooled sera from each experimental group was tested for IgG titer specific to the H3N2 influenza virus after second and third immunization.

Figure 11 shows the results of a cell lysis assay by NK cells of mice immunized with a mixture of recombinant flagella expressing separately 6 influenza epitopes (HA91-108, HA354-372, HA307-319, NP335-350, NP380-393, and M1 2-12).

Figure 12 shows the results of a T-cell proliferation assay of T-cells isolated from mice immunized with recombinant flagellin expressing influenza epitope NP 335-350,
following stimulation with H3N2 virus (fig. 12A) or with the immunizing peptide (fig. 12B).

Figure 13 demonstrates IFN-gamma secretion from proliferating splenocytes of mice immunized with recombinant flagella expressing different peptide epitopes or a combination of 6 epitopes.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention provides a polynucleotide comprising a modified flagellin gene and use thereof to prepare an expression vector comprising at least one heterologous peptide epitope or a plurality of peptide epitopes and their subsequent expression as recombinant flagellin fusion proteins. The expression vector now disclosed has several advantages over the vectors known in the art, among the advantages are:

a) a cloning site for peptide or plurality of peptides wherein each peptide has a total length of from about 5 amino acids to about 50 amino acids;

b) two unique restriction enzyme sites, wherein at least one site has a cohesive overlapping end, for oriented cloning;

c) a kanamycin resistant gene replaces the ampicillin resistant gene, thereby obviating any health problems attendant with penicillin allergies;

d) a high copy number plasmid.

The recombinant genes expressed by the present expression vector encode a recombinant flagellin fusion protein comprising a polynucleotide encoding a flagellin structural gene and a nucleotide sequence encoding a peptide epitope of a heterologous organism, which recombinant flagellin fusion protein is immunogenic upon introduction of the fusion protein into a vertebrate host.

**Definitions**

For convenience and clarity certain terms employed in the specification, examples and claims are described herein.

"Nucleic acid sequence" or "polynucleotide" as used herein refers to an oligonucleotide or nucleotide and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand. Similarly, "amino acid sequence" as used herein refers to an
oligopeptide, peptide, polypeptide, or protein sequence, and fragments or portions thereof, and to naturally occurring, synthetic or recombinant molecules.

The term "oligonucleotide" is defined, as a molecule comprised of two or more deoxyribonucleotides and/or ribonucleotides, preferably more than six. Its exact size will depend upon many factors, which, in turn, depend upon the ultimate function and use of the oligonucleotide. The upper limit may be 15, 20, 30, 40, 50, 60, 70, 80, 90, or 150 nucleotides.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally or produced synthetically, which is capable of acting as a point of initiation of oligonucleotide synthesis of a primer extension product, complementary to a nucleic acid strand which serves as a template. Primer extension is initiated in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent.

The term "recombinant proteins or polypeptides" refers to proteins or polypeptides produced by recombinant DNA techniques. Within the scope of the present invention the recombinant proteins or polypeptides are produced from cells, microbial or mammalian, transformed by an exogenous recombinant DNA expression construct encoding the desired protein or polypeptide. Proteins or polypeptides expressed in most bacterial cultures will typically be free of glycan. Proteins or polypeptides expressed in yeast may have a glycosylation pattern different from that expressed in mammalian cells.

The term "peptide epitope" refers to a sequence of amino acid residues capable of eliciting an immune response in a host into which it is introduced. The peptide can be an artificial sequence or one derived from a natural protein. A peptide epitope refers to a sequence comprising at least one epitope. A "peptide epitope" is not intended to be limiting to a single amino acid sequence and may refer to a single peptide sequence or to a plurality of peptide sequences.

Peptide epitopes can be classified as B-cell, T-cell or both B cell and T cell, depending on the type of immune response they elicit. The definition of B cell or T cell epitope is not unequivocal; for example, an epitope can induce antibody production but at the same time that epitope can possess a sequence that enables binding to the human HLA molecule, rendering it accessible to CTLs, hence a dual B cell and T cell
classification for that particular epitope. A peptide epitope can comprise any amino acid sequence that elicits an immune response. Therefore, a peptide epitope can have an amino sequence derived from a peptide or protein of a known organism or alternatively, the peptide epitope may comprise an artificial sequence.

The term "immunogenicity" or "immunogenic" relates to the ability of a substance to stimulate or elicit an immune response. Immunogenicity is measured, for example, by determining the presence of antibodies specific for the substance. The presence of antibodies is detected by methods known in the art, for example using an ELISA assay.

The term "recombinant flagellin" refers to a flagellin polypeptide comprising a peptide epitope embedded within its sequence. The term "recombinant expression vector or plasmid" is a replicable DNA vector or plasmid construct used either to amplify or to express DNA encoding a protein or a polypeptide. An expression vector or plasmid contains DNA control sequences and a coding sequence. DNA control sequences include promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, and enhancers. Recombinant expression systems as defined herein will express the proteins or polypeptides of the invention upon induction of the regulatory elements.

The term "host cells comprising a vector" includes cells that have been transformed or transfected with exogenous DNA. Exogenous DNA may or may not be integrated (i.e., covalently linked) to chromosomal DNA making up the genome of the host cell. In prokaryotes and yeast, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid, or stably integrated into chromosomal DNA.

The term "inclusion bodies" refers to inactive aggregates of protein that may form when a recombinant polypeptide is expressed in a prokaryote. While the cDNA may properly code for a translatable mRNA, the protein that results may not fold correctly, or the hydrophobicity of the added peptide epitopes may cause the recombinant polypeptide to become insoluble. Inclusion bodies are easily purified by methods well known in the art.

The polypeptide sequence of the modified flagellin includes amino acid sequences substantially the same as the sequence set forth in SEQ ID NO:22. The term "substantially the same" refers to amino acid sequences that retain the activity of flagellin as described herein, e.g., the ability to induce an immune response in a
mammalian host. The flagellin polypeptides of the invention include conservative variations of the polypeptide sequence. The term "conservative variation" as used herein denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include the substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like.

The invention also provides polynucleotides encoding the flagellin polypeptides described herein. The polynucleotide sequence of the modified flagellin fliC gene is set forth in SEQ ID NO:3. A polynucleotide includes DNA, cDNA and RNA sequences which encode flagellin polypeptides. It is to be understood that all polynucleotides encoding flagellin polypeptides are also included herein, as long as they encode a polypeptide with flagellin activity. Such polynucleotides include naturally occurring, synthetic, and intentionally manipulated polynucleotides. For example, flagellin polynucleotide may be subjected to site-directed mutagenesis. The polynucleotides of the invention include sequences that are degenerate as a result of the genetic code. There are 20 natural amino acids, most of which are specified by more than one codon. Therefore, all degenerate nucleotide sequences are included in the invention as long as the amino acid sequence of flagellin polypeptide encoded by the nucleotide sequence is functionally unchanged.

Specifically disclosed herein is a polynucleotide sequence encoding a modified flagellin polypeptide. In one embodiment the modified fliC gene has a nucleotide sequence set forth in SEQ ID NO:3 and encoding a polypeptide of 490 amino acids, set forth in SEQ ID NO:22.

By "host cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, the expression vector of the present invention. Transformation of a host cell with recombinant DNA may be carried out by conventional techniques as are well known to those skilled in the art. Where the host is prokaryotic, such as E. coli, cells which are competent for DNA uptake can be prepared from cells harvested after exponential growth phase and subsequently treated by using procedures well known in the art. For example, host cells can be made competent for transformation by CaCl₂, MgCl₂ or RbCl treatment. Alternatively, cells can be made competent for electroporation by removing all traces of salt.
Any DNA sequence which encodes a natural or artificial epitope is suitable for use in the expression vector of the present invention. In some embodiments the peptide epitope derives from a protein or peptide sequence of a heterologous organism, which when expressed as a recombinant flagellin fusion protein in the expression vector of the present invention, produces protective immunity against such organism or against a condition or disorder caused by an antigen. In other embodiments, the peptide epitope derives from a protein or a peptide sequence of a syngeneic organism or an allogeneic organism.

In a preferred embodiment, the heterologous organism is a pathogenic microorganism. For example, such a heterologous epitope may be found in plants, bacteria, parasites, viruses, or fungi which are the causative agents of diseases or disorders. Such plants, bacteria, parasites, viruses, or fungi include, but are not limited to, those listed in Table 1. In addition, peptide epitopes of allergens and cancer cells can be used.

Table 1: Heterologous Organisms

<table>
<thead>
<tr>
<th>PARASITES</th>
<th>BACTERIA</th>
<th>VIRUSES</th>
<th>FUNGI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmodium spp.</td>
<td>Vibrio cholerae</td>
<td>Influenza A virus</td>
<td>Candida spp.</td>
</tr>
<tr>
<td>Eimeria spp.</td>
<td>Streptococcus pyogenes</td>
<td>Influenza B virus</td>
<td>Cryptococcus spp.</td>
</tr>
<tr>
<td>Schistosoma spp.</td>
<td>Neisseria meningitidis</td>
<td>Avian Influenza Virus</td>
<td>Blastomyces spp.</td>
</tr>
<tr>
<td>Trypanosoma spp.</td>
<td>Neisseria gonorrhoeae</td>
<td>Human Immunodeficiency virus, type I and II</td>
<td>Histoplasma spp.</td>
</tr>
<tr>
<td>Babesia spp.</td>
<td>Corynebacteria diphtheriae</td>
<td>Simian Immunodeficiency virus</td>
<td>Coccidioides spp.</td>
</tr>
<tr>
<td>Leishmania spp.</td>
<td>Clostridium tetani</td>
<td>Human T lymphocyte virus, type I, II and III</td>
<td>Paracoccidioides spp.</td>
</tr>
<tr>
<td>Cryptosporidia spp.</td>
<td>Branhamella catarrhalis</td>
<td>Respiratory syncytial virus</td>
<td>Aspergillus spp.</td>
</tr>
<tr>
<td>Toxoplasma spp.</td>
<td>Bordetella pertussis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pneumocystis spp.</td>
<td>Haemophilus spp. (e.g., influenzae)</td>
<td>Hepatitis B virus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chlamydia spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enterotoxigenic Escherichia coli</td>
<td>Non-A, Non-B Hepatitis Virus</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Herpes simplex virus, type I</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Herpes simplex virus, type II</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cytomegalovirus (CMV)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>West Nile Virus</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Parainfluenza virus</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Poliovirus</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rotavirus</td>
</tr>
</tbody>
</table>
In one embodiment the epitope is selected from a viral epitope. In one preferred embodiment the epitope is selected from an influenza A or B virus. Non-limiting examples of influenza epitopes are shown in table 2.

<table>
<thead>
<tr>
<th>#</th>
<th>Epitope type</th>
<th>Epitope location</th>
<th>Amino acids sequence</th>
<th>No. AA</th>
<th>Nucleotide sequence</th>
<th>No. bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B-Cell</td>
<td>HA 91-108</td>
<td>SKAFSNCYPY DVPDYASL (SEQ ID NO:23)</td>
<td>18</td>
<td>TCTAAAGCCTTCTCTAA CTGTTATCCTTTATGATGT TCCGGATTACGCTCTTA GAT (SEQ ID NO:30)</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td>B-Cell, CTL</td>
<td>M2 1-18</td>
<td>MSLLTTEVETPI RNEWGCR (SEQ ID NO:24)</td>
<td>18</td>
<td>ATGAGCCTGCTGACCG AAGTGGAAAACCCGATT CGTAACGAATGGGTTG TCGTGAT (SEQ ID NO:31)</td>
<td>54</td>
</tr>
<tr>
<td>3</td>
<td>T-helper</td>
<td>HA 307-319</td>
<td>PKYVKQNTILK LAT (SEQ ID NO:25)</td>
<td>13</td>
<td>CCGAAATATGTTAAG GTAACACTCTGAAATTA GCTACT (SEQ ID NO:32)</td>
<td>39</td>
</tr>
<tr>
<td>4</td>
<td>CTL</td>
<td>NP 335-350</td>
<td>SAAFEDLRVLS FIRGY (SEQ ID NO:26)</td>
<td>16</td>
<td>TCTGCTGCTTTCAAG ATCTCGCTGCTGCTCT TCATTGCCTGAT (SEQ ID NO:33)</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>CTL</td>
<td>NP 380-393</td>
<td>ELRSRYWAIRT RSG (SEQ ID NO:27)</td>
<td>14</td>
<td>GATATAGAAGACAGAT ATTGGCTATTACAAT AGAAGCCGT (SEQ ID NO:34)</td>
<td>42</td>
</tr>
<tr>
<td>6</td>
<td>CTL</td>
<td>M1 58-66</td>
<td>GILGFVFTL (SEQ ID NO:28)</td>
<td>9</td>
<td>GGTATCTGGTTCTGCT GTTCAACCTCGAT (SEQ ID NO:35)</td>
<td>27</td>
</tr>
<tr>
<td>7</td>
<td>Influenza B</td>
<td>HA 354-372</td>
<td>PAKLKKERGFF GAIAGFLE (SEQ ID NO:29)</td>
<td>19</td>
<td>CCGTCTAATGCTGA AAGAAGCTGGTTCCTGCGTCGTATGCTGTT CCTGGAAGAT (SEQ ID NO:5)</td>
<td>57</td>
</tr>
</tbody>
</table>
DNA sequences encoding peptides, which are known to contain antigenic determinants, can be cloned into the expression vector of the present invention. An antigen itself need not be highly immunogenic. The flagellin may function as a carrier molecule in conferring immunogenicity on epitopes that are poor immunogens per se. The DNA sequences can encode artificial peptides, i.e. peptides whose sequences are not derived from a natural protein. The recombinant flagellin fusion proteins in the vaccine formulations of the invention can also comprise a peptide epitope of a heterologous organism, in order to produce a recombinant flagellin fusion protein which, when introduced into a vertebrate host, induces an immune response that protects against a condition or disorder caused by an antigen containing the epitope. For example, in an embodiment of the invention, recombinant flagellin fusion proteins may comprise a peptide epitope of a toxin such as snake venom, bee venom, an allergy-inducing antigen or any other antigen to which an immune response is desired. In one embodiment, a tumor-specific antigen can be expressed as a recombinant flagellin fusion protein, for induction of a protective immune response to cancer.

The gene sequences encoding the heterologous epitope to be expressed as a recombinant flagellin according to the present invention can be isolated by techniques known in the art including, but not limited to, chemical synthesis, or by purification from genomic DNA of the microorganism, by cDNA synthesis from RNA of the microorganism, and by recombinant DNA methods.

Construction of Recombinant Flagellin Genes

In the construction of a recombinant flagellin gene of the present invention, the hypervariable domain of the flagellin gene has a heterologous sequence inserted within. The hypervariable domain of the $\text{fl}i\text{C}$ gene was modified to include unique restriction enzyme sites that when digested result in cohesive sequences that allow for oriented cloning of the insert.

In some embodiments of the present invention, a DNA sequence encoding at least one heterologous epitope is inserted into the hypervariable region of the flagellin monomer. According to one embodiment the recombinant flagellin monomers retain the ability to form intact flagella. The ability to assemble into flagella would, in the context of a live vaccine formulation, result in the presentation of a high concentration of the heterologous epitope, which exists within each flagellin monomer, to the immune system of the mammalian host. Without wishing to be bound to theory, presentation of the
epitope or epitopes as an organized polymeric structure affords immunogen with higher immunogenic potential when compared to presentation of a peptide epitope per se.

Various procedures for the purification of the intact flagella are known the art. In one embodiment, the recombinant flagellin molecules expressed by a parental, flagellin-deficient nonmotile strain of bacteria produce functional flagella.

According to another embodiment, the recombinant flagellin monomers form inclusion bodies instead of assembling into functional flagella. Without wishing to be bound by theory, inclusion bodies are more likely to form under certain circumstances, such as when a plurality of peptide epitopes and/or multiple copies of one or more epitopes are inserted in the recombinant flagellin. Using multiple copies of a plurality of epitopes in a single recombinant polypeptide may be advantageous, for example, when a high proportion of epitope to flagellin in a multivalent vaccine is desired. Another advantage of the expression of recombinant flagellin in inclusion bodies is the ease of purification of said inclusion bodies.

Various procedures for the purification of inclusion bodies are known in the art. In some embodiments the inclusion bodies are recovered from bacterial lysates by centrifugation and are washed with detergents and chelating agents to remove as much bacterial protein as possible from the aggregated recombinant protein. To obtain soluble protein, the washed inclusion bodies are dissolved in denaturing agents and the released protein is then refolded by gradual removal of the denaturing reagents by dilution or dialysis (Molecular cloning: a laboratory manual, 3rd edition, Sambrook, J. and Russell, D. W., 2001; CSHL Press).

In some embodiments the recombinant flagellin gene is constructed using recombinant DNA techniques. For example, the relevant sequences of the flagellin gene and of the heterologous gene can, by techniques known in the art, be cleaved at appropriate sites with restriction enzymes, isolated, and ligated. If cohesive termini are generated by restriction enzyme digestion, no further modification of DNA before ligation may be needed. If however, cohesive termini of the DNA are not available for generation by restriction endonuclease digestion, or different restriction sites other than those available are preferred, any one of numerous techniques known in the art may be used to accomplish ligation of the heterologous DNA at the desired sites.

In one embodiment, PCR amplification of nucleotide sequence of an epitope is performed utilizing primers that will insert the desired restriction enzyme sites. A linker
may also be used to generate suitable restriction sites in the heterologous gene sequence. In another embodiment the nucleic acid encoding the desired epitope is chemically synthesized. The particular strategy for constructing gene fusions will depend on the specific peptide epitope to be inserted.

The transformation of bacterial hosts with the recombinant DNA molecules of the present invention that incorporate the recombinant flagellin gene enables generation of multiple copies of the recombinant flagellin fusion protein sequence.

The expression vector of the present invention was constructed using in vitro recombinant DNA and synthetic techniques.

Expression in Bacterial Hosts

The recombinant expression vector comprising the recombinant flagellin sequence can be transferred into a bacterial host cell where it can replicate and be expressed. This can be accomplished by any of numerous methods known in the art including but not limited to transformation, phage transduction, electroporation, etc.

In a specific embodiment, any attenuated bacterial host which expresses the recombinant flagellin can be formulated as live vaccines. Such bacteria include but are not limited to attenuated invasive strains and attenuated Shigella, Salmonella or Escherichia species.

Nucleic Acids

The present invention provides nucleic acid molecules encoding the recombinant flagellin fusion protein of the invention. An isolated nucleic acid sequence encoding a peptide can be obtained from its natural source, for example as a portion of a gene. A nucleic acid molecule can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Nucleic acid sequences include natural nucleic acid sequences and homologs thereof, comprising, but not limited to, natural allelic variants and modified nucleic acid sequences in which nucleotides have been inserted, deleted, substituted, and/or inverted.

A polynucleotide or oligonucleotide sequence can be deduced from the genetic code of a protein, however, the degeneracy of the code must be taken into account, and nucleic acid sequences of the invention also include sequences, which are degenerate as a result of the genetic code, which sequences may be readily determined by those of ordinary skill in the art.
As used herein, highly stringent conditions are those, which are tolerant of up to about 5% to about 25% sequence divergence, preferably up to about 5% to about 15%. Without limitation, examples of highly stringent (-10^0C below the calculated Tm of the hybrid) conditions use a wash solution of 0.1 X SSC (standard saline citrate) and 0.5% SDS at the appropriate Ti (incubation temperature) below the calculated Tm of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those, which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE), 5 X Denhardt's reagent, 0.5% SDS, 100 μg/ml denatured, fragmented salmon sperm DNA at an appropriate Ti. (See generally Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d edition, Cold Spring Harbor Press (1989) for suitable high stringency conditions).

Stringency conditions are a function of the temperature used in the hybridization experiment and washes, the molarity of the monovalent cations in the hybridization solution and in the wash solution(s) and the percentage of formamide in the hybridization solution. In general, sensitivity by hybridization with a probe is affected by the amount and specific activity of the probe, the amount of the target nucleic acid, the detectability of the label, the rate of hybridization, and the duration of the hybridization. The hybridization rate is maximized at a Ti of about 20°C -25°C below Tm for DNA:DNA hybrids and about 10^0C -15°C below Tm for DNA:RNA hybrids. It is also maximized by an ionic strength of about 1.5M Na+. The rate is directly proportional to duplex length and inversely proportional to the degree of mismatching.

Specificity in hybridization, however, is a function of the difference in stability between the desired hybrid and "background" hybrids. Hybrid stability is a function of duplex length, base composition, ionic strength, mismatching, and destabilizing agents (if any). The Tm of a perfect hybrid may be estimated for DNA:DNA hybrids using the equation of Meinkoth et al (1984).

Vaccines and Immunogenic Compositions

The invention further provides a method of eliciting an immune response (including humoral, mucosal and/or cell-mediated immune responses) by administering to a vertebrate host, a recombinant flagellar fusion protein per se. In another embodiment
a bacterium transfected to express a recombinant flagellin fusion protein of this invention in a physiologically acceptable carrier can be administered to the host.

In one embodiment the vaccine is an immunogenic composition eliciting an immune response for prophylactic or therapeutic treatment of a disease in an individual (reviewed in Sela and Hilleman, 2004). In a non-limiting example, the peptide epitope can be selected from a polypeptide specifically expressed or overexpressed in a cancer cell, in order to generate recombinant flagellin for the preparation of a cancer vaccine or a prion vaccine. A cancer vaccine is a method of treating the disease involving administration of an immunogenic composition comprising one or more antigens or peptide antigens. This induces the patient's immune system to attack and eliminate the cancerous cells. Accordingly, the peptide antigen can be selected from a prion epitope, epitopes associated with neurodegenerative diseases, and epitopes related to autoimmune diseases (see, for example, Ali and Larche, 2005; De Groot, 2006; Koide et al, 2005, Madan, et al, 2006). Accordingly, the present invention provides a method for inducing an immune response and conferring treatment of a disease in a subject, the disease selected from an autoimmune disease, a prion disease, a neurodegenerative disease, wherein the method comprises administering to the subject an immunogenic composition according to the invention.

**Live Bacteria as Vaccines**

In one embodiment the vaccine according to the present invention comprises a host cell comprising a recombinant expression vector of the present invention. The recombinant expression vector directs expression of recombinant flagellin comprising an epitope of a heterologous organism so as to elicit an immune (humoral and/or cell-mediated) response to the heterologous epitope that will protect against infections by the organism or disorders caused by an antigen of the organism.

In one embodiment, the host strain is an attenuated host strain such as *Salmonella* species. Other suitable species can include but are not limited to *Shigella* and E. coli. In one preferred embodiment, the recombinant flagellin genes are expressed by the host bacteria as flagellin monomers that assemble into functional flagella, allowing the heterologous epitope on the recombinant molecules to be presented in a large number of copies to the host immune system.

The live vaccine formulation can be univalent or multivalent. Multivalent vaccines can be prepared from a single or few recombinant bacteria which express one or
more heterologous epitopes, which may be of different organisms. A single bacterium can express more than one epitope of the same or different antigens. The various epitopes may be expressed within the same recombinant flagellin protein, on separate recombinant flagellin molecules encoded by the same or different expression vectors, or in different bacteria.

**Subunit Vaccines**

The recombinant flagellin fusion protein may be used as an immunogen in subunit vaccine formulations, which may be multivalent. The multivalent vaccine formulation can comprise recombinant flagella, or a recombinant flagellin monomer containing more than one peptide epitope, which epitope may be of different organisms, or several flagellin molecules, each encoding a different peptide epitope, etc.

Furthermore, isolated flagella samples can be solubilized to flagellin subunits and then reassociated to flagella in order to: (a) aid in the purification of the recombinant flagellins by removing undesirable contaminants; and/or (b) produce an immunogen for multivalent vaccine formulation, by association of recombinant flagellin monomers encoding different peptide epitopes.

Since peptides are usually poor immunogens, the efficacy of peptide-based vaccine depends on the adequate presentation of the epitopes to the immune system. Without wishing to be bound to theory, the at least one peptide epitope is expressed in the expression vector of the present invention, which provides both carrier and adjuvant function. The functionality of the flagellin precludes the need for additional adjuvant, yet if desired adjuvant may be added. Suitable adjuvants are known to one with skill in the art and may be selected from aluminum hydroxide gel; mineral gel; surface active agents including lysolecithin and pluronic polyols; polyanions; peptides; oil emulsions; and the like.

**Vaccine Formulation**

The vaccine can be formulated for administration in one of many different modes. According to one embodiment of the invention, the vaccine is administered intranasally or intramuscularly. The intranasal composition can be formulated, for example, in liquid form as nose drops, spray, or suitable for inhalation, as powder, as cream, or as emulsion. The composition can contain additives, including stabilizers, buffers, or preservatives. For application, the vaccine composition can be supplied in a
vessel appropriate for distribution of the recombinant flagellin in the form of nose drops or an aerosol. In certain preferred embodiments the vaccine is formulated for mucosal delivery, in particular intranasal delivery.

In another embodiment of the invention, the vaccine is administered orally and the vaccine may be presented, for example, in the form of a tablet or encased in a gelatin capsule or a microcapsule.

In yet another embodiment, the vaccine is formulated for parenteral administration. In some embodiments the vaccine is formulated for mass inoculation, for example for use with a jet-injector or a single use cartridge. The formulation of these modalities is general knowledge to those with skill in the art.

Liposomes provide another delivery system for antigen delivery and presentation. Liposomes are bilayered vesicles composed of phospholipids and other sterols surrounding a typically aqueous center where antigens or other products can be encapsulated. The liposome structure is highly versatile with many types range in nanometer to micrometer sizes, from about 25 nm to about 500 µm. Liposomes have been found to be effective in delivering therapeutic agents to dermal and mucosal surfaces. Liposomes can be further modified for targeted delivery by for example, incorporating specific antibodies into the surface membrane, or altered to encapsulate bacteria, viruses or parasites. The average survival time of the intact liposome structure can be extended with the inclusion of certain polymers, for example polyethylene glycol, allowing for prolonged release in vivo.

Microparticles and nanoparticles employ small biodegradable spheres which act as depots for vaccine delivery. The major advantage that polymer microspheres possess over other depot-effecting adjuvants is that they are extremely safe and have been approved by the Food and Drug Administration in the USA for use in human medicine as suitable sutures and for use as a biodegradable drug delivery system. The rates of copolymer hydrolysis are very well characterized, which in turn allows for the manufacture of microparticles with sustained antigen release over prolonged periods of time.

Parenteral administration of microparticles elicits long-lasting immunity, especially if they incorporate prolonged release characteristics. The rate of release can be modulated by the mixture of polymers and their relative molecular weights, which will hydrolyze over varying periods of time. Without wishing to be bound to theory, the
formulation of different sized particles (1 μm to 200 μm) may also contribute to long-
lasting immunological responses since large particles must be broken down into smaller
particles before being available for macrophage uptake. In this manner a single- injection
vaccine could be developed by integrating various particle sizes, thereby prolonging
antigen presentation and greatly benefiting livestock producers.

EXAMPLES

Materials and Methods

Bacterial strains and vectors. The construction of the expression vector pLS408
is described in US Pat. No. 6,130,082, herein incorporated by reference. Expression
vector pTrcHis A was from Invitrogen. Expression vector pBK-CMV was from Stratagene.

A flagella-deficient, non motile E.coli strain obtained by insertional mutation in
the flagellin^/VC gene was used for expression of the modified flagellin.

Preparation of bacterial samples for SDS-PAGE and Western blot analysis.
Cell lysates were prepared as follows: After transformation with the vector, expression
was induced with IPTG and cells were harvested 3-6.5 hours following IPTG induction.
Cells were suspended twice in RIPA buffer, frozen in liquid nitrogen and thawed at 37°C
(3 repetitions). Cells were then sonicated and lysates were centrifuged. Supernatants
were separated from pellets and loaded separately on 10% PAGE.

Bacterial cell membranes were prepared as follows: cells were suspended in PBS
(pH 1.5), rotated for 30 min at room temperature and centrifuged; supernatant was
separated and adjusted to pH 7.0 by titration with 1M NaOH. Following the addition of
sample buffer the suspensions were boiled and then loaded on a 10% PAGE

Western blot analysis was performed with a rabbit anti-flagellin antibody.

Vaccine preparation. The immune response induced by a recombinant flagellin
gene expressing the influenza virus HA 91-108 B cell epitope (SEQ ID NO:23) was
tested in rabbits and mice. Five different preparations of the vaccine, hereafter denoted
Fia 91-108, were used in the study. The same plasmid inserted with the sequence coding
for HA 91-108 was used for the production of all the preparations but the purification
procedure of the recombinant flagellin was different. The different procedures resulted in various amounts of endotoxin in the protein preparation. In addition, different procedures were employed for the precipitation of the proteins. In some cases the recombinant flagella were purified from the bacterial pellet and in others a soluble fraction was prepared from the bacterial growth medium. The vaccine preparations were administered either intranasally or intramuscularly, suspended in PBS or in adjuvants, or subcutaneously when emulsified in Freund's adjuvant.

EXAMPLE 1: Construction of Expression Vector PBVXOO

In one aspect the present invention provides a polynucleotide which is an expression vector, denoted pBVXOO, comprising a flagellin gene and a kanamycin resistant gene. The polynucleotide sequence of pBVXOO is disclosed in SEQ ID NO:1.

First step: fliC gene modification and cloning into pTrc-His A (Invitrogen)

The flagellin fliC gene was isolated from Salmonella enterica serovar Typhi (Salmonella typhi) strain CT18, having Genbank accession number AL627272. The polynucleotide sequence of the natural fliC gene is set forth in SEQ ID NO:2. The region of 48bp flanked by the two EcoRV restriction sites of SEQ ID NO:2 was deleted, thereby providing a shortened fli/C gene having SEQ ID NO:4.

The EcoRV restriction site was replaced with a nucleic acid sequence comprising an Eagl, an EcoRV and a Bsu36I restriction site, 5'-EagI-EcoRV-Bsu36I-3', the nucleic acid sequence set forth in SEQ ID NO:8 herein below:

5'-GATCTATTATCGCCCGAGCAATACGATATCAACCTAAGGATGGTC-B'

using the following primers:

SEQ ID NO:6 denoted herein 289-01/134_Ala_F having an Ncol restriction site at nucleotide positions 5-10:

5'-AACACCATGGCACAAGTCATTAATACAAAC-S'

SEQ ID NO:7 denoted herein 289-02/134_B2a_R having an Xhol restriction site at nucleotide positions 4-9:

5'-GGACTCGAGTTAACGCAGTAAAGAGAGGACGTTTG-S'
Table 3: initial PCR reactions

<table>
<thead>
<tr>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Template</th>
<th>Expected product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>289-01/134_Ala_F</td>
<td>289-04/Mut-A2B_R</td>
<td>fliC gene DNA (pLS408)</td>
<td>646 (mod_fliC-5' - region)</td>
</tr>
<tr>
<td>SEQ ID NO:6</td>
<td>SEQ ID NO:9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>289-03/Mut-A2B_F</td>
<td>289-02/134-B2a_R</td>
<td>fliC gene DNA (pLS408)</td>
<td>891 (mod_fliC-3' - region)</td>
</tr>
<tr>
<td>SEQ ID NO:8</td>
<td>SEQ ID NO:7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR products were run on a 1% agarose gel and extracted and quantitated. Equimolar quantities of the two products were mixed together and amplified with PFU Taq polymerase with the 289-01/134_Ala_F (SEQ ID NO:6) and 289-02/134-B2a_R (SEQ ID NO:7) primers.

The PCR product, which comprised the modified fliC gene, was 1488 bp long and was confirmed by digestion with Ncol and Xhol restriction enzymes. The polynucleotide sequence of the modified fliC is set forth in SEQ ID NO:3. The amino acid sequence of the modified/7zC is set forth in SEQ ID NO:22.

The pTrcHis A vector (Invitrogen) was digested with Ncol and Xhol restriction enzymes to give 119bp and 4295 bp fragments. The linear plasmid was gel purified. The PCR insert and linear vector were ligated, transformed into XL1Blue competent bacterial cells and plated on ampicillin/LB plates. Fifteen colonies were selected and tested by PCR. The DNA from three positive colonies was sent for full sequence analysis. The clones were sequenced and a construct having the correct polynucleotide sequence was
selected for further modifications. This construct is denoted herein pBND12.9 and its polynucleotide sequence is set forth in SEQ ID NO: 10.

Expression of modified fliC in a flagella-deficient E. coli strain

Expression of the modified fliC gene in the pBND12.9 vector was tested in the flagella-deficient E. coli strain. Cells were transformed with DNA of pBND12.9. Control transformations included pTrcHis A alone (negative control) and pLS408 (positive control). Expression was induced with IPTG and cells were harvested 3.0 and 6.5 hours following IPTG induction. Supernatants and pellets were analyzed by 10% SDS-PAGE with Coomassie staining (Figure 1). Isolated flagella from Salmonella cells transformed with pLS408 were used as positive control for flagellin.

Supernatants were prepared from bacteria transformed with the empty vector pTrcHis A and from the tested clone, pBND12.9, following 6.5 hrs of IPTG induction. Supernatants of each sample were analyzed by SDS-PAGE followed by Western blot using rabbit anti-flagellin antibody. The positive control is isolated flagella from Salmonella cells transformed with pLS408 (Figure 2).

Second step: construction of the bacterial expression vector pBVXOO

Objective: Creation of a bacterial expression vector with the following features: a MCS (multiple cloning site) region downstream to the Lac promoter; elimination of the beta-galactosidase (beta-Gal) encoding DNA; addition of an Ncol restriction site to the MCS; replacement of the beta lactamase (bla) gene with a kanamycin resistance gene. The pUC18 plasmid is a pBR322 derivative that replicates at a copy number several-fold higher than the parent during growth of Escherichia coli at 37°C.

The insertion of the kanamycin resistance gene included the modification of an EagI site located at the 5’-end of the kanamycin resistance gene open reading frame (ORF). The modification was performed using a silent change to keep the proline residue.

Stage 1: Elimination of beta-Gal N-terminal region, located upstream to the MCS site, and creation of an Ncol site:

The MCS region is flanked by EcoRI (5’) and HindIII (3’) sites. The beta-galactosidase N-terminal region is immediately upstream to EcoRI. The nearest unique restriction sites flanking the MCS region are BseYI (5’) and Ndel (3’). The following primers were utilized in site-directed mutagenesis:
SEQ ID NO: 11, denoted herein 290-01/18-MCS_Fl having the nucleotide sequence:

\[ 5'-GTTCGTGCACACAG CCCAGC TTGGAG-S' \]

SEQ ID NO: 12, denoted herein 290-02/18-MCS_Rl having the nucleotide sequence:

\[ 5'-CTGAGAGTGACACATATGCCGTG-S' \]

SEQ ID NO: 13, denoted herein 290-03/18MCSmutF having the nucleotide sequence:

\[ 5'-CAGGAAACA GCCATGG CCATCATTAC GAATTC -3' \]

SEQ ID NO: 14, denoted herein 290-04/18MCSmutR having the nucleotide sequence:

\[ 5'-GAATTC GTAATGATG GCCATGG CTGTTTCCTG-S' \]

Two PCR reactions were performed using pUC18 as template:

1: 290-01/18-MCS_Fl + 290-04/18MCSmutR for the creation of the 5'-part of the modified region.

2: 290-02/18-MCS_Rl + 290-03/18MCSmutF for the creation of the 3'-part of the modified region.

The two PCR products have a 32 nucleotide overlap. They were purified and mixed at equimolar quantities and then the whole modified region was amplified using primers: 290-01/18-MCS_Fl + 290-02/18-MCS_Rl.

The amplification product and the pUC18 were restricted with BseYI and Ndel restriction enzymes. Following ligation and transformation, three colonies were sequenced and the desired correct clone was selected, herein denoted pBND6.3.

Stage 2: Replacement of ampicillin resistant gene (Amp) with a kanamycin gene:

The Amp gene was removed from pBND6.3 by cutting with Sspl and Bsal restriction enzymes. The 1945 bp fragment was purified. Next, the kanamycin resistance gene (kana) was amplified using pBK-CMV plasmid (Stratagene) as template with the following primers:

Forward primer: Kana_Sscp1_F (SEQ ID NO: 15), which contains the kana N-terminal starting from ATG and the necessary part of the Amp promoter derived from
pUC18 plus the Spl site. In addition, a silent nucleotide change was inserted into the kana coding region in order to eliminate an Eagl site by choosing a less used proline codon.

5'-TTCAA _TAATATT_ GAAAAAGGAAGATGATGATTGAACAAGATGGAT
TGCACGC AGTTCTC _CAGCCG_ CTTG-3'

Reverse primer: Kana_BsaI_R (SEQ ID NO: 16)

5'-AATGATACCGC _GAGACC_ TCAGAAGAACTCGTCAAGAAGGCCGATAG-B'

The amplified PCR product having 838 bp was digested with Sspl and Bsal, gel isolated and ligated to pBND6.3 digested with the same enzymes. Transformed cells were plated on LB-Kana plates and three positive clones were sequenced. One positive clone was used for the subsequent analysis, pBND8.1, having a nucleotide sequence set forth in SEQ ID NO: 17.

Stage 3: cloning and expression of the modified yZ/C gene into pBND 8.1

The expression vector pBND 8.1 was digested with BamHI and Ncol restriction enzymes. Ncol digestion was performed under conditions that allow only partial digestion in order to isolate a polynucleotide which had not been cleaved at the Ncol site existing within the kanamycin resistance coding sequence.

The modified fliC gene was isolated from construct pBND12.9 by restriction with Ncol and BgIII. The two fragments were ligated and the resulting construct contains a unique XhoI site that was inserted with the fliC gene fragment and is new in pUC18. On the other hand, both BamHI and BgIII sites were obliterated during ligation, as were the EcoRI, Kpnl, Sad and Smal restriction enzyme sites.

Resulting clones were partially sequenced (at the junction regions) and a correct clone having a nucleotide sequence set forth in SEQ ID NO:1 was selected and named pBVXOO. A partial restriction map of pBVXOO is shown in figure 3.

Expression of pBVXOO in a flagella-deficient E. coli strain

Expression of the modified fliC gene from the pBVXOO construct was tested in the flagella-deficient E. coli strain. Cells were transformed with the pBVXOO vector. Control transformations included: a negative control (transformation with pBND 8.1, which does not contain a fliC gene) and a positive control (transformation with pBND12.9). Expression was induced with IPTG and cells were harvested 4 hours
following IPTG induction. Supernatants and pellets were analyzed by 10% SDS-PAGE with Coomassie staining (Figure 4). Supernatants were analyzed by Western blotting with a rabbit anti-flagellin antibody (Figure 5). The positive control is isolated flagella from Salmonella cells transformed with pLS408.

Membranal samples were analyzed by Western blotting with a rabbit anti-flagellin antibody (Figure 6). The positive control is isolated flagella from Salmonella cells transformed with pLS408.

EXAMPLE 2: Construction of Expression vector pBND18.1

A second expression vector was prepared with the intention of transferring the 3.6Kb EcoRI insert of pLS408 into pBND18.1, and replacing the fliC gene region of pLS408 with the corresponding modified fliC gene set forth in SEQ ID NO:3.

Stage 1: The restriction sites, HindIII and Kpnl were removed from pBND18.1 by restriction with both enzymes and blunting the resulting cohesive ends using T4 DNA polymerase (the HindIII overhang was filled-in and Kpnl overhang was digested). Resulting blunt ends were re-ligated and plasmid DNA was transformed into XL1Blue cells. Two transformed clones were sequenced and named pBND13.1 and pBND13.2, whose multicloning sites (MCS) are set forth in SEQ ID NOS: 18 and 19, respectively.

Clone 13.1 is the result of the self ligation of the blunt ended plasmid (as described above) whereas clone 13.2 is the result of the ligation of the blunt ended plasmid with the blunt ended insert which was not removed.

Stage 2: The 3.6 Kb EcoRI insert of pLS408 was ligated into the EcoRI site of pBND13.1. Colony-PCR was performed for the selection of a recombinant clone in which the insert is in the desired orientation relative to the beta-gal promoter. The resulting construct was named pBND16.2, whose sequence is set forth in SEQ ID NO:20.

Stage 3: The fliC region of the pBND16.2, which is flanked by HindIII-Kpnl sites, was removed by restriction and replaced by the HindIII-Kpnl restriction fragment of pBVX00, which contains the corresponding modified fliC (containing the Eagl and Bsul sites flanking the EcoRV site). The resulting clone was named pBND18.1 whose polynucleotide sequence is set forth in SEQ ID NO:21.
EXAMPLE 3: Humoral Effect of Vaccination in Rabbits

Experimental Design:

Immunization Phase: 26 female NZW rabbits aged 12 weeks old were assigned randomly to 13 groups. Rabbits were immunized on days 0, 21, and 42. Blood collection was performed on day 0 and 14 days after each immunization, for antibody titration. Table 4 below shows the treatment groups.

On the first immunization, formulations of 600µg flagella in ImI PBS, or emulsified 1:1 (v/v) in CFA (or in IFA on boost immunizations) or in alum were used. When CFA was used as adjuvant, the administration was subcutaneous instead of intramuscular. For the intranasal immunization, the same amount of 600µg vaccine was slowly dripped into the animal's nostrils. On the second and third administration, half of the amount was administered (300µg/rabbit) to all groups.

An ELISA assay was performed to detect specific polyclonal antibodies to the HA 91-108 peptide (figure 7) or to the flagella (figure 8) in serum sampled from immunized rabbits.

Table 4: Treatment Groups

<table>
<thead>
<tr>
<th>Group #</th>
<th>VACCINE PREPARATION</th>
<th>LPS</th>
<th>HEAT TREATMENT</th>
<th>AMMONIUM SULFATE</th>
<th>ADJUVANT</th>
<th>ROUTE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Fla 91-108</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>IN</td>
</tr>
<tr>
<td>2</td>
<td>Fla 91-108</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>IN</td>
</tr>
<tr>
<td>3</td>
<td>Fla 91-108</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>IN</td>
</tr>
<tr>
<td>4</td>
<td>Fla 91-108</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>IN</td>
</tr>
<tr>
<td>5</td>
<td>Fla 91-108</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>IN</td>
</tr>
<tr>
<td>6</td>
<td>Native Flagella</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>IN</td>
</tr>
<tr>
<td>7</td>
<td>Fla 91-108</td>
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<td>+</td>
<td>+</td>
<td>CFA/IFA</td>
<td>IM</td>
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<tr>
<td>8</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>IM</td>
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<td>+</td>
<td>+</td>
<td>Alum</td>
<td>IM</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>IM</td>
</tr>
<tr>
<td>11</td>
<td>Fla 91-108 SN</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>CFA/IFA</td>
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<td>+</td>
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Fla 91-108: recombinant flagellin expressing the HA91-108 epitope prepared from the bacterial pellet. Flia 91-108 SN: recombinant flagellin expressing the HA91-108
Conclusions

Vaccination by both routes of administration resulted in the production of antibodies specific to the flagella and to the peptide. Intramuscular administration is more effective in terms of antibody production than intranasal administration. Addition of Alum as adjuvant is unnecessary since it does not further improve the response. Immunization with the lowest dose of LPS (corresponding to approved values of up to 100µg/dose in human) did not reduce the ability of the rabbits to produce antibodies.

The rabbits responded equally well to flagella prepared from the bacterial medium as to flagella prepared from pellets. In terms of yield, preparation of recombinant flagella from the bacterial growth medium is easier and more efficient.

**EXAMPLE 4: Humoral Effect of Vaccination in Mice**

**Experimental Design:**

Immunization Phase: 88 female C57B1 Female C57B1 mice aged 11-12 week old mice were assigned randomly to 11 groups. Mice were immunized on days 0, 20, and 41. Blood collection was performed on day 0 and 14 days after each immunization. Table 5 below shows the treatment groups.

Mice were immunized intramuscularly or intranasally with formulations of 200µg FlA 91-108 in 50 µl PBS, or subcutaneously when emulsified 1:1 (v/v) in CFA (or in IFA on boost immunizations) or intramuscularly when mixed with alum.

The mice were sacrificed on day 56 and their lungs were removed to follow IgA concentration in their lungs. An ELISA assay was performed to detect specific polyclonal antibodies to the HA 91-108 peptide (figure 9) or to the intact H3N2 virus (figure 10) in pooled sera samples from immunized mice. These antigens were used for coating ELISA plates and the concentration of specific polyclonal antibodies were detected in sera or lung homogenates samples.

Conclusions

Vaccination by both routes of administration resulted in the production of antibodies specific to the virus and to the peptide. Antibodies to the H3N2 virus A/Texas/1/77 were detected even though HA 91-108 is a hidden epitope. Intramuscular administration is more effective in terms of antibody production than intranasal administration. Immunization with the lowest dose of LPS (corresponding to approved values of up to 100u/dose in human) did not reduce the ability of the mice to produce antibodies.

EXAMPLE 4: Cellular Activation Assays

Mice expressing human HLA A2.1 were immunized (200µg/dose, subcutaneously in CFA/IFA) with recombinant flagella expressing different epitopes, or with a mixture of recombinant flagella expressing separately 6 influenza epitopes (HA91-108, HA354-372, HA307-319, NP335-350, NP380-393 and M1 2-12).

Cell lysis assay. Immunization of mice with the mixture of recombinant flagella resulted in induction of killing by NK cells as determined by the classical assay employing YAC-I cells (an NK sensitive cell line) as targets (figure 11). Splenic
lymphocytes from immunized mice or control mice (immunized with native flagellin) were sensitized with a mixture of peptides included in the recombinant flagella vaccine (HA91-108, HA354-372, HA307-319, NP335-350, NP380-393 and M1 2-12) for 5 days, and then incubated with $^{35}$S-Met labeled YAC-I cells. Specific lysis was determined at different EffectoπTarget (E:T) ratios.

**T-cell proliferation** assay. Inguinal and periaortic lymph nodes were aseptically harvested from mice previously immunized with recombinant flagella expressing the CTL epitope NP335-350. A single cell suspension was prepared, after washing; the cells were resuspended in RPMI without serum and counted by trypan blue exclusion with a phase contrast microscope. The cell number was adjusted to a concentration of $3 \times 10^6$ cells/ml in RPMI containing 2% normal mouse serum. The thymidine incorporation assay was used to determine proliferation responses to the H3N2 whole virus (figure 12A) and to the immunizing peptide NP335-350 (figure 12B). Following 3 days of incubation with the antigen (peptide or virus at the concentrations indicated in the graph) the cultures were pulsed for 18 hours with 1 µCi/well of $[^3H]$-thymidine and harvested for counting by liquid scintillation. Thymidine incorporation is expressed as the ratio of mean replicate experimental values minus the mean replicate non-stimulated (background) values to the mean replicate non-stimulated (background) values. CFA Spleen: response detected in splenocytes from mice immunized with PBS in CFA (Complete Freund Adjuvant); F335 Spleen: response detected in splenocytes from mice immunized with recombinant flagella expressing NP335-350 influenza epitope; F335 LN: response detected in lymph nodes cells from mice immunized with recombinant flagella expressing NP335-350 influenza epitope.

**IFN-gamma secretion** assay. IFN-gamma secretion from proliferating splenocytes was measured after two and three immunizations with 200µg recombinant flagella expressing HA91-108 (Fla-91), NP335-350 (Fla-335), NP380-393 (Fla-380), M1 2-12 (Fla-M1(2-12)), or the whole combination of 6 influenza epitopes HA91-108, HA354-372, HA307-319, NP335-350, NP380-393 and M1 2-12 (BiondFlu) emulsified in CFA. A significant IFN-gamma secretion was detected following in vitro incubation with the corresponding peptide (figure 13).

**Conclusions**

Proliferation responses were observed following administration of recombinant flagella expressing one influenza CTL epitope. IFN-gamma secretion from lymphocytes
incubated with the peptides indicates a TH1 response. In addition, innate immune responses were evaluated by the NK cells activation following immunization with the mixture of recombinant flagella with 6 influenza epitopes (HA91-108, HA354-372, HA307-319, NP335-350, NP380-393 and M1 2-12).

The present invention has been described with reference to specific preferred embodiments and examples. It will be appreciated by the skilled artisan that many possible alternatives will be apparent within the scope of the present invention which is not intended to be limited by the specific embodiments exemplified herein but rather by the following claims.
REFERENCES


CLAIMS

1. A polynucleotide comprising a modified *Salmonella* flagellin gene *fliC* comprising a plurality of unique restriction enzyme sites, wherein at least one of the unique restriction enzyme site yields cohesive ends upon restriction with its respective restriction enzyme, and which sites either individually or collectively are useful for the cloning of at least one heterologous peptide epitope of at least 15 nucleotides.

2. The polynucleotide according to claim 1 wherein the two unique restriction enzyme sites are EagI and Bsu36I.

3. The polynucleotide according to claim 1 having SEQ ID NO:3.

4. The polynucleotide according to claim 1 wherein the at least one peptide is up to about 150 nucleotides in length.

5. A polynucleotide construct that encodes a recombinant flagellin protein, the polynucleotide comprising a modified *Salmonella* flagellin gene (fliC) comprising a plurality of unique restriction enzyme sites, wherein at least one of the unique restriction enzyme site yields cohesive ends upon restriction with its respective restriction enzyme, and which sites either individually or collectively are useful for the cloning of at least one heterologous peptide of at least 15 nucleotides.

6. The polynucleotide construct according to claim 5 wherein the modified *fliC* gene is set forth in SEQ ID NO:3.

7. The polynucleotide construct according to claim 5 further comprising a kanamycin resistant gene.

8. The polynucleotide construct according to any one of claims 5 or 6, set forth in SEQ ID NO:1.

9. The polynucleotide construct according to any one of claims 5 or 6, set forth in SEQ ID NO:21.

10. The polynucleotide construct according to claim 5 wherein the peptide epitope is an artificial peptide.

11. The polynucleotide construct according to claim 5 wherein the peptide epitope derives from a heterologous organism.
12. The polynucleotide construct according to claim 11 wherein the heterologous organism is selected from a plant, a bacterium, a virus, a parasite and a fungus.

13. The polynucleotide construct according to claim 5 wherein the peptide epitope derives from an auto-antigen.

14. The polynucleotide construct according to claim 13 wherein the auto-antigen is selected from a tumor associated antigen and an autoimmune related antigen derived from non-human animal or human cells.

15. The polynucleotide construct according to claim 12 wherein the virus is an influenza virus.

16. A host cell comprising the polynucleotide construct according to claim 5.

17. The host cell according to claim 16 wherein the host is a bacterium.

18. The host cell according to claim 17, which is a flagella-deficient strain.

19. The host cell according to claim 18, which is selected from a flagella-deficient strain of bacteria selected from Escherichia coli, Salmonella and Shigella strain of bacteria.

20. A bacterium comprising the polynucleotide set forth in SEQ ID NO:1 deposited at the CNCM, Institut Pasteur, and assigned registration number CNCM 1-3735.

21. A polypeptide encoded by the polynucleotide according to any one of claims 1-15.

22. A vaccine comprising a polypeptide according to claim 21.

23. An immunogenic composition comprising a polypeptide according to claim 21, for eliciting an immune response for treating a disease in an individual in need thereof.

24. The vaccine according to claim 22, wherein the vaccine elicits an immune response selected from a cellular response, a humoral response, and a combination of a cellular and humoral response.

25. A method for inducing an immune response and conferring protection against a heterologous organism in a subject, wherein the method comprises administering to the subject a vaccine according to claim 22.

26. The method according to claim 25, wherein the vaccine protects the subject against a viral disease.
27. The method according to claim 25, wherein the vaccine protects the subject from influenza virus.

28. The method according to claim 25 wherein the route of administration of the vaccine is selected from intramuscular, intranasal, oral, intraperitoneal, intravenous, subcutaneous, topical, mucosal and transdermal delivery.

29. The method according to claim 28 wherein the vaccine is administered intranasally.

30. The method according to claim 28 wherein the vaccine is administered intramuscularly.

31. A method for inducing an immune response and conferring treatment of a disease in a subject, wherein the disease is selected from an autoimmune disease, cancer, allergy, prion disease, neurodegenerative disease, wherein the method comprises administering to the subject an immunogenic composition according to claim 23.

32. The method according to claim 31 wherein the route of administration of the immunogenic composition is selected from intramuscular, intranasal, oral, intraperitoneal, intravenous, subcutaneous, topical, mucosal and transdermal delivery.

33. The method according to claim 32 wherein the immunogenic composition is administered intranasally.

34. The method according to claim 32 wherein the immunogenic composition is administered intramuscularly.
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FIG. 2
FIG. 3
FIG. 5
FIG. 11
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| 0-5-1 | Barry Newman |

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/195

According to International Patent Classification (IPC) and to both national classification and IPC

B. FIELD SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, Sequence Search, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>SBROGIO-ALMEIDA M E ET AL: &quot;Host and bacterial factors affecting induction of immune responses to flagellin expressed by attenuated Salmonella vaccines strains&quot; INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, vol. 72, no. 5, May 2004 (2004-05), pages 2546-2555, XP003009267 ISSN: 0019-9567 page 2546, left-hand column, paragraph 1 - page 2553, left-hand column, paragraph 1; figure 4</td>
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Date of the actual completion of the international search
13 September 2007

Date of mailing of the international search report
24/09/2007

Authorized officer
Stoyanov, Boris

Form PCT/ISA/2.10 (second sheet) (April 2005)
This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos because they relate to subject matter not required to be searched by this Authority, namely

   Although claims 25-34 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

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 64(a).

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

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 an additional fee, this Authority did not invite payment of any additional fee.

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.

Remark on Protest
- [ ] The additional search fees were accompanied by the applicant’s protest
- [ ] No protest accompanied the payment of additional search fees

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