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[Continued on next page]

(54) Title: PEPTIDES AND USES THEREOF

(57) Abstract: The present invention relates to purified, isolated or recombinant peptides and pili, and genetically engineered bacteria comprising said peptides or pili, methods for making the peptides, pili and bacteria, pharmaceutical compositions comprising the peptides, pili and bacteria, methods of eliciting immune responses in a subject and methods of vaccinating a subject, uses of the peptides, pili and bacteria for the same, and uses of the peptides, pili and bacteria in the manufacture of medicaments for the same.

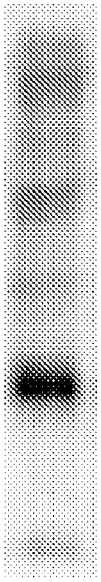

Figure 10

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SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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PEPTIDES AND USES THEREOF

FIELD OF THE INVENTION

[0001] The present invention relates to isolated, purified or recombinant peptides or pili, genetically engineered bacteria comprising recombinant peptides or pili, methods for making the peptides, pili or bacteria, pharmaceutical compositions comprising the peptides, pili or bacteria, methods of eliciting immune responses in a subject and methods of vaccinating a subject, uses of the peptides, pili or bacteria for the same, and uses of the peptides, pili or bacteria in the manufacture of medicaments for the same.

BACKGROUND TO THE INVENTION

[0002] Vaccines remain the most cost effective and feasible means of infectious disease control in the community.

[0003] Modern vaccines have progressed from the crude mixes of killed or attenuated microorganisms that were developed decades ago, towards carefully designed vaccines based on individual peptides. Synthetic peptide vaccines generally comprise a synthetic copy of an immunogenic part of protein antigens.

[0004] Peptide vaccines have a number of advantages, including ease of synthesis, avoidance of potentially toxic biological by-products, reduced response to irrelevant antigens, and straightforward characterisation. However, peptides by themselves are often poorly immunogenic and require administration with adjuvants or as amplified (multimeric) peptides. Additionally, peptides are often sensitive to proteolytic degradation.

[0005] Mucosal surfaces of external body cavities, such as the lungs and gastro-intestinal tract, are a common entry site of many pathogens. The development of vaccines that induce efficient mucosal immune responses through delivery of protective antigens to mucosal sites is thus highly desirable.

[0006] There is an ongoing need for new peptide vaccines and new methods of making peptide vaccines. There is also an ongoing need for new vaccines that are effective when delivered mucosally.

[0007] It is an object of the present invention to provide a method that meets one or more of these needs, or to at least provide the public with a useful choice.

SUMMARY OF THE INVENTION

[0008] In one aspect the invention relates to an isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising a backbone pilin (BP) protein or fragment thereof and one or more peptides of interest, wherein the peptide of interest is heterologous to the BP protein.

[0009] In one aspect the invention relates to an isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising a backbone pilin (BP) protein or fragment thereof and one or more peptides of interest, wherein the peptide of interest is not a BP protein or fragment thereof.

[0010] In one aspect the invention relates to an isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising a backbone pilin (BP) protein or fragment thereof and one or more peptides of interest, wherein the peptide of interest is not a pilus protein or fragment thereof.

[0011] In one aspect the invention relates to an isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising a backbone pilin (BP) protein or fragment thereof and one or more peptides of interest, wherein the peptide of interest is not a *Streptococcus* protein or fragment thereof.

[0012] In one aspect the invention relates to an isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising one or more peptides of interest inserted into or fused to a backbone pilin (BP) protein or fragment thereof and, wherein the peptide of interest is heterologous to the BP protein.

[0013] In one embodiment, the BP protein comprises a BP protein derived from a Gram positive bacterium.

[0014] In one embodiment, the BP protein comprises a *Streptococcus* BP protein.

[0015] In one embodiment, the BP protein comprises a Group A *Streptococcus* BP protein.

[0016] In one embodiment, the BP protein is encoded within the FCT-1, FCT-2, FCT-3, FCT-4, FCT-5, FCT-6 or FCT-9 nucleotide region of a Group A *Streptococcus* genome.

[0017] In one embodiment, the BP protein is encoded within the FCT-2, FCT-3 or FCT-4 nucleotide region of a Group A *Streptococcus* genome.

[0018] In one embodiment, the BP protein comprises a *Streptococcus pyogenes* BP protein.

[0019] In one embodiment, the BP protein is derived from Group A *Streptococcus* serotype M1, M2, M3, M4, M5, M6, M9, M11, M12, M18, M22, M23, M28, M33, M44, M49 (2), M50, M53, M75, M77, M78 or M89.

[0020] In one embodiment, the BP protein is derived from Group A *Streptococcus* serotype M1, M3, M5, M9, M11, M12, M18, M22, M28, M33, M44, M49 (2), M50, M53, M77, M78, or M89.

[0021] In one embodiment, the BP protein has at least about 80% amino acid sequence identity to the polypeptide sequence of SEQ ID No 1.

[0022] In one embodiment, the BP protein is encoded by a nucleotide coding sequence selected from the group consisting of

a. Genbank Accession Number EU725506.1 (GI:198417284)

b. any one of Genbank Accession Numbers KJ816940 (KJ816940.1 GI:692334132) –KJ817040 (KJ817040.1 GI:692334331),

c. Genbank Accession Number 2940764

d. Genbank Accession Number NP_268517 (NP_268517.1 GI:15674343)

e. Genbank Accession Number KJ816977 (KJ816977.1 GI:692334206)

f. Genbank Accession Number KJ817010 (KJ817010.1 GI:692334272)

g. Genbank Accession Number KJ817040 (KJ817040.1 GI:692334331)

h. Genbank Accession Number KJ816969 (KJ816969.1 GI:692334190)

i. Genbank Accession Number 3573111

j. Genbank Accession Number KJ816976 (KJ816976.1 GI:692334204)

k. Genbank Accession Number KJ816959 (KJ816959.1 GI:692334170)

l. Genbank Accession Number KJ816971 (KJ816971.1 GI:692334194)

m. Genbank Accession Number KJ816965 (KJ816965.1 GI:692334182)

n. Genbank Accession Number KJ816984 (KJ816984.1 GI:692334220)

o. Genbank Accession Number KJ816948 (KJ816948.1 GI:692334148)

p. Genbank Accession Number KJ816943 (KJ816943.1 GI:692334138)

q. Genbank Accession Number KJ816940 (KJ816940.1 GI:692334132)

r. Genbank Accession Number KJ816995 (KJ816995.1 GI:692334242)

s. Genbank Accession Number 4067256, and

t. Genbank Accession Number 4063969.

[0023] In one embodiment, the BP protein comprises, consists of, or consists essentially of, an amino acid sequence selected from the group consisting of

a. Genbank Accession Number ACH87870.1 (GI:198417285),

b. any one of Genbank Accession Numbers KJ816940 –KJ817040,

c. Genbank Accession Number 2940764

d. Genbank Accession Number NP_268517

e. Genbank Accession Number KJ816977

f. Genbank Accession Number KJ817010

g. Genbank Accession Number KJ817040

h. Genbank Accession Number KJ816969

i. Genbank Accession Number 3573111

j. Genbank Accession Number KJ816976

k. Genbank Accession Number KJ816959

l. Genbank Accession Number KJ816971

m. Genbank Accession Number KJ816965

n. Genbank Accession Number KJ816984

o. Genbank Accession Number KJ816948

p. Genbank Accession Number KJ816943

q. Genbank Accession Number KJ816940

r. Genbank Accession Number KJ816995

s. Genbank Accession Number 4067256, and

t. Genbank Accession Number 4063969.

[0024] In one embodiment, the BP protein comprises

a. first domain having at least about 80% amino acid sequence identity to the polypeptide sequence of the C-terminal domain of Spy0128 (amino acids 174–340 of SEQ ID No. 1), and/or

b. a second domain having at least about 80% amino acid sequence identity to the polypeptide sequence of the N-terminal domain of Spy0128 (amino acids 1–171 of SEQ ID No. 1).

[0025] In one embodiment, the amino acid sequence comprises the at least one peptide inserted at a site within the C-terminal domain of the BP protein.

[0026] In one embodiment, the amino acid sequence comprises the at least one peptide inserted at a site within the N-terminal domain of the BP protein.

[0027] In one embodiment, the at least one peptide of interest is inserted:

a. in a region between the βE and βF loop regions of the BP protein, for example, corresponding to amino acids 119–124 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1, and/or

b. in a region between the β9 and β10 loop regions of the BP protein, for example, corresponding to amino acids 276–279 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1,

c. in a region between the βB and βC1 loop regions of the BP protein, for example, corresponding to amino acids 61–65 of SEQ ID No: 1, and/or an

analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1,

d. in a region between the β3 and β4 loop regions of the BP protein, for example, corresponding to amino acids 217–221 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1, and/or

e. in a region between the β2 and β3 loop regions of the BP protein, for example, corresponding to amino acids 201-206 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1, and/or

f. in a region between the βD and βE loop regions of the BP protein, for example, corresponding to amino acids 101–108 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1.

[0028] In one embodiment, the amino acid sequence comprises the polypeptide of interest inserted:

a. in a region between the βE and βF loop regions of the BP protein, for example, corresponding to amino acids 119–124 of SEQ ID No: 1, and

b. in a region between the β9 and β10 loop regions of the BP protein, for example, corresponding to amino acids 276–279 of SEQ ID No: 1.

[0029] In one embodiment, the amino acid sequence comprises:

a. a first peptide of interest inserted in a region between the βE and βF loop regions of the BP protein, for example, corresponding to amino acids 119–124 of SEQ ID No: 1, and

b. a second peptide of interest inserted in a region between the β9 and β10 loop regions of the BP protein, for example, corresponding to amino acids 276–279 of SEQ ID No: 1.

[0030] In one embodiment, the polypeptide further comprises an amino acid sequence encoding a pilin tip protein (AP1 protein), an AP2 protein, a Sip protein, a sortase or a combination of any two or more thereof.

[0031] In one embodiment, the polypeptide further comprises amino acid sequences encoding a pilin tip protein (AP1 protein), an AP2 protein, a Sip protein, and a sortase.

[0032] In one embodiment, the polypeptide further comprises amino acid sequences encoding proteins required to form a pilus when the polypeptide is expressed in a bacterium.

[0033] In one embodiment, the one or more peptides of interest comprise an antigenic peptide, an enzyme, or an antibody or fragment thereof.

[0034] In one embodiment, the one or more peptides of interest comprise a T cell antigenic peptide or epitope/fragment thereof or a B cell antigenic peptide or epitope/fragment thereof.

[0035] In one embodiment, the one or more peptides of interest comprises two or more antigenic peptides or epitopes.

[0036] In one embodiment, the one or more peptides of interest are derived from a microorganism, for example, a virus, a bacterium, or a parasite.

[0037] In one embodiment, the one or more peptides of interest are capable of eliciting an IgA response.

[0038] In one embodiment, the one or more peptides of interest comprise a tumour antigenic peptide.

[0039] In one embodiment, the one or more peptides of interest comprise 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, or 10 or more contiguous amino acids that do not encode a BP protein or fragment thereof.

[0040] In one embodiment, the peptide of interest comprises, consists of, or consists essentially of an amino acid sequence selected from the group consisting of

a. 8 or more contiguous amino acids from the sequence SQAVHAAHAEINEAGRI [SEQ ID No. 30], or

b. 8 or more contiguous amino acids from the sequence KQAEDKVKASREAKKQVEKALEQLEDKVQ [SEQ ID No. 31].

[0041] In one embodiment, the peptide of interest comprises, consists of, or consists essentially of an amino acid sequence selected from the group consisting of

a. 8 or more contiguous amino acids from the sequence SIINFEKL [SEQ ID No. 35],

b. 8 or more contiguous amino acids from the sequence TEWTSSNVMEERKIKV [SEQ ID No. 36], or

c. 8 or more contiguous amino acids from the sequence SPSYVYHQF [SEQ ID No. 37].

[0042] In one embodiment, the peptide of interest comprises, consists of, or consists essentially of an amino acid sequence selected from the group consisting of

a. 8 or more contiguous amino acids from the amino acid sequence EKANPVNDLCYPGDFNDYEELKH [SEQ ID no. 38] (HA1

b. 8 or more contiguous amino acids from the amino acid sequence LGHHAVP NGTLVKTITNDQIEVTNATELVQSSSTGRICDSPHRILDGKNCTLIDAL [SEQ ID No. 39],

c. 8 or more contiguous amino acids from the amino acid sequence KRGL FGAIAGFIEGGWQ [SEQ ID No. 40], or

d. 8 or more contiguous amino acids from the amino acid sequence SLLTEVETPIRNEWGCRCNDSSD [SEQ ID No. 41],

[0043] In one embodiment, the peptide of interest comprises, consists of, or consists essentially of an amino acid sequence selected from the group consisting of

a. 8 or more contiguous amino acid residues from the Mtb antigen 85B precursor peptide,

b. 8 or more contiguous amino acids from the amino acid sequence FQDAYNAAGGHNAVF [SEQ ID No: 32, I-A(b) Mtb antigen 85B precursor peptide amino acids 280–294],

c. 8 or more contiguous amino acid residues from the Mtb antigen ESAT-6,

d. 8 or more contiguous amino acids from the amino acid sequence MTEQQWNFAGIEAAASAIQG [SEQ ID No: 33, I-A(b) Mtb ESAT-6 amino acids 1-20],

e. 8 or more contiguous amino acids from the amino acid sequence GAPINSATAM [SEQ ID No: 34, I-A(b) Mtb ESAT-6 amino acids 309 - 318],

f. 8 or more contiguous amino acids from the amino acid sequence YQGVQQKWDATATELNNALQ [SEQ ID No. 42] I-A(b) Mtb ESAT-6 amino acids 51-70], or

g. 8 or more contiguous amino acids from the amino acid sequence SEFAYGSFVRTVSLPVGADE [SEQ ID No. 43].

[0044] In one embodiment, the peptide of interest comprises, consists of, or consists essentially of an amino acid sequence of

a. 8 or more contiguous amino acid residues from amino acid sequence TEWTSSNVMEERKIKV [SEQ ID No 44].

[0045] In one embodiment, the polypeptide, when expressed in a bacterium, assembles to form a pilus on the surface of the bacterium.

[0046] In one aspect the invention relates to a recombinant polypeptide comprising the amino acid sequence of SEQ ID No. 1 wherein one or more, for example, 1–15 contiguous amino acids have been deleted at one or more of the following sites:

a. from amino acid position 61,

b. from amino acid position 101,

c. from amino acid position 119,

d. from amino acid position 201,

e. from amino acid position 217, and/or

f. from amino acid position 276;

and/or

wherein one or more of the following sequences of contiguous amino acids have been deleted:

g. amino acids 61–65,

h. amino acids 101–108,

i. amino acids 119–124,

j. amino acids 201–206,

k. amino acids 217–221, and/or

l. amino acids 276–279;

and, optionally, wherein two or more contiguous amino acids are inserted at the site or sites of the deleted contiguous amino acids.

[0047] In one aspect the invention relates to a recombinant polypeptide comprising an amino acid sequence having at least 90% sequence identity with SEQ ID No. 1 wherein one or more, for example, 1–15 contiguous amino acids have been deleted at one or more of the following sites:

a. from an amino acid position corresponding to amino acid 61 of SEQ ID No. 1,

b. from an amino acid position corresponding to amino acid 101 of SEQ ID No. 1,

c. from an amino acid position corresponding to amino acid 119 of SEQ ID No. 1,

d. from an amino acid position corresponding to amino acid 201 of SEQ ID No. 1,

e. from an amino acid position corresponding to amino acid 217 of SEQ ID No. 1, and/or

f. from an amino acid position corresponding to amino acid 276 of SEQ ID No. 1;

g. and/or wherein one or more of the following sequences of contiguous amino acids have been deleted:

h. an amino acid sequence corresponding to amino acids 61–65 of SEQ ID No. 1,

i. an amino acid sequence corresponding to amino acids 101–108 of SEQ ID No. 1,

j. an amino acid sequence corresponding to amino acids 119–124 of SEQ ID No. 1,

k. an amino acid sequence corresponding to amino acids 201–206 of SEQ ID No. 1,

l. an amino acid sequence corresponding to amino acids 217–221 of SEQ ID No. 1, and/or

m. an amino acid sequence corresponding to amino acids 276–279 of SEQ ID No. 1;

and, optionally, wherein two or more contiguous amino acids are inserted at the site or sites of the deleted contiguous amino acids.

[0048] In one embodiment, the one or more inserted amino acids results from the formation of a restriction enzyme cleavage site.]

[0049] In one embodiment, the recombinant polypeptide further comprising one or more peptides of interest inserted at the site or sites of the deleted amino acids.

[0050] In one aspect the invention relates to a recombinant polypeptide comprising the amino acid sequence of SEQ ID No. 1 and comprising a substitution of 1 to 6, for example, 4 contiguous amino acids for a sequence of 1 to 15 contiguous amino acids of the native sequence at one or more of the following sites:

a. from an amino acid position corresponding to amino acid 61 of SEQ ID No. 1,

b. from an amino acid position corresponding to amino acid 101 of SEQ ID No. 1,

c. from an amino acid position corresponding to amino acid 119 of SEQ ID No. 1,

d. from an amino acid position corresponding to amino acid 201 of SEQ ID No. 1,

e. from an amino acid position corresponding to amino acid 217 of SEQ ID No. 1, and/or

f. from an amino acid position corresponding to amino acid 276 of SEQ ID No. 1;

and/or
comprising a substitution of 1 to 6, for example, 4 contiguous amino acids for one or more of the following sequences of contiguous amino acids in SEQ ID No.1:

g. amino acids 61–65,

h. amino acids 101–108,

i. amino acids 119–124,

j. amino acids 201–206,

k. amino acids 217–221, and/or

l. amino acids 276–279.

[0051] In one aspect the invention relates to a recombinant polypeptide comprising an amino acid sequence having at least 90% sequence identity with SEQ ID No. 1 and comprising a substitution of 1 to 6, for example, 4 contiguous amino acids for a sequence of 1 to 15 contiguous amino acids of the native sequence at one or more of the following sites:

a. from amino acid position 61,

b. from amino acid position 101,

c. from amino acid position 119,

d. from amino acid position 201,

e. from amino acid position 217, and/or

f. from amino acid position 276;

and/or
comprising a substitution of 1 to 6, for example, 4 contiguous amino acids for one or more of the following sequences of contiguous amino acids in SEQ ID No.1:

g. an amino acid sequence corresponding to amino acids 61–65 of SEQ ID No. 1,

h. an amino acid sequence corresponding to amino acids 101–108 of SEQ ID No. 1,

i. an amino acid sequence corresponding to amino acids 119–124 of SEQ ID No. 1,

j. an amino acid sequence corresponding to amino acids 201–206 of SEQ ID No. 1,

k. an amino acid sequence corresponding to amino acids 217–221 of SEQ ID No. 1, and/or

l. an amino acid sequence corresponding to amino acids 276–279 of SEQ ID No. 1.

[0052] In one aspect the invention relates to a recombinant polypeptide of the abovementioned aspect comprising one or more of the following amino acid substitutions:

a. amino acids LELD for amino acids 61–65,

b. amino acids LELD for amino acids 101–108,

c. amino acids LELD for amino acids 119–124,

d. amino acids LELD for amino acids 201–206,

e. amino acids LELD for amino acids 217–221, and/or

f. amino acids LELD for amino acids 276–279.

[0053] In one embodiment, the recombinant polypeptide further comprises one or more peptides of interest inserted at the substitution site or sites between the LE and LD residues.

[0054] In one aspect the invention relates to an isolated, purified, or recombinant pilus comprising a plurality of covalently attached peptides of interest wherein the peptides of interest are heterologous to the pilus.

[0055] In one aspect the invention relates to an isolated, purified, or recombinant pilus comprising one or more repeating/polymerised polypeptide subunits each subunit comprising a backbone pilin (BP) protein and one or more peptides of interest, wherein the peptides of interest are heterologous to the BP protein.

[0056] In one aspect the invention relates to a genetically engineered bacterium comprising at least one recombinant pilus, each pilus comprising a plurality of covalently attached peptides of interest, wherein the peptides of interest are heterologous to the pilus [and the bacterium].

[0057] In one aspect the invention relates to a genetically engineered bacterium comprising at least one recombinant pilus, each pilus comprising one or more repeating/polymerised polypeptide subunits each subunit comprising a backbone pilin (BP) protein and one or more peptides of interest, wherein the peptides of interest are heterologous to the BP protein, or heterologous to the bacterium.

[0058] In one aspect the invention relates to a genetically engineered bacterium comprising a recombinant nucleic acid capable of encoding a polypeptide capable of forming one or more pili on the surface of the bacterium, the nucleic acid encoding one or more

repeating polypeptide subunits each subunit comprising a backbone pilin (BP) protein and one or more peptides of interest, wherein the peptides of interest are heterologous to the BP protein, or to the bacterium.

[0059] In one embodiment, the bacterium is a Gram positive bacterium.

[0060] In one embodiment, the bacterium belongs to a genus selected from the group comprising *Bifidobacterium, Lactobacillus, Lactococcus,* and *Streptococcus*.

[0061] In one embodiment, the bacterium is an attenuated pathogenic bacterium, a non-pathogenic bacterium or a generally regarded as safe (GRAS) bacterium.

[0062] In one aspect the invention relates to a pharmaceutical composition comprising an effective amount of one or more polypeptides, pili or bacteria described herein.

[0063] In one embodiment, the composition is an immunogenic composition.

[0064] In one aspect the invention relates to a vaccine composition comprising an effective amount of one or more polypeptides, pili or bacteria described herein.

[0065] In one embodiment, the composition additionally comprising an adjuvant.

[0066] In one embodiment, the composition does not comprise an extrinsic adjuvant.

[0067] In one aspect the invention relates to a method of vaccinating or eliciting an immune response in a subject comprising administering to the subject an effective amount of a pharmaceutical composition or vaccine composition described herein.

[0068] In one aspect the invention relates to a method of vaccinating or eliciting an immune response in a subject comprising administering to the subject an effective amount of a polypeptide, pilus or bacterium described herein.

[0069] In one aspect the invention relates to use of a polypeptide, pilus or bacterium described herein in the manufacture of a medicament for vaccinating or eliciting an immune response in a subject in a subject in need thereof.

[0070] In one aspect the invention relates to a polypeptide, pilus or bacterium described herein for use in vaccinating or eliciting an immune response in a subject in a subject in need thereof.

[0071] In one aspect the invention relates to a method for producing a genetically engineered bacterium comprising at least one pilus, each pilus comprising a plurality of covalently attached peptides of interest, the method comprising:

a. introducing into said bacterium a polynucleotide comprising a polynucleotide sequence comprising a nucleotide sequence encoding a backbone pilin (BP) protein and one or more peptides of interest, wherein the peptide of interest is heterologous to the BP protein; and

b. growing said bacterium under conditions wherein said polypeptide is expressed and said pilus is formed.

[0072] In one embodiment, the peptide of interest is heterologous to the bacterium.

[0073] In one embodiment, the polynucleotide further comprises a nucleotide sequence encoding a pilin tip protein (AP1 protein), an AP2 protein, a sortase or a combination of any two or more thereof.

[0074] In one embodiment, the polynucleotide further comprises a promoter that is active in the bacterium, and wherein the promoter is operably linked to the polynucleotide encoding said polypeptide.

[0075] In one embodiment, the promoter drives constitutive expression of the polypeptide in the bacterium.

[0076] Any of the embodiments described herein relate to any of the aspects herein.

[0077] It is intended that reference to a range of numbers disclosed herein (for example, 1 to 10) also incorporates reference to all rational numbers within that range (for example, 1, 1.1, 2, 3, 3.9, 4, 5, 6, 6.5, 7, 8, 9 and 10) and also any range of rational numbers within that range (for example, 2 to 8, 1.5 to 5.5 and 3.1 to 4.7).

[0078] To those skilled in the art to which the invention relates, many changes in construction and widely differing embodiments and applications of the invention will suggest themselves without departing from the scope of the invention as defined in the appended claims. The disclosures and the descriptions herein are purely illustrative and are not intended to be in any sense limiting.

[0079] This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more of said parts, elements or

features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

[0080] In this specification, where reference has been made to external sources of information, including patent specifications and other documents, this is generally for the purpose of providing a context for discussing the features of the present invention. Unless stated otherwise, reference to such sources of information is not to be construed, in any jurisdiction, as an admission that such sources of information are prior art or form part of the common general knowledge in the art.

BRIEF DESCRIPTION OF THE DRAWINGS

[0081] The invention will now be described by way of example only and with reference to the drawings in which:

[0082] Figure 1 shows a Western blot analysis using A) an anti-Spy0128 antibody of cell wall extracts obtained from (1) untransformed *L. lactis*; (2) *L. lactis* transformed with a construct expressing native PilM1; and (3) *L. lactis* transformed with a construct expressing PilM1 comprising ovalbumin peptide 323-339 at the N-terminus of the Spy0128 (M1) protein; and B) an anti-OVA antibody of (1) a cell wall extract obtained from *L. lactis* transformed with a construct expressing PilM1 comprising ovalbumin peptide 323-339 at the N-terminus of the Spy0128 (M1) protein, and (2) ovalbumin;

[0083] Figure 2 shows serum IgG response in mice vaccinated with *L. lactis* comprising the OVA peptide at the N-terminus of the Spy0128 (M1) protein (○), *L. lactis* expressing the native M1 pilus (□), and the OVA peptide alone administered subcutaneously (Δ);

[0084] Figure 3 shows a Western blot analysis using A) an anti-Spy0128 antibody of cell wall extracts obtained from (1) untransformed *L. lactis*; (2) *L. lactis* transformed with a construct expressing native PilM1; and (3) *L. lactis* transformed with a construct expressing PilM1 comprising ovalbumin peptide 323-339 at the βE/βF loop of the Spy0128 protein; and B) an anti-OVA antibody of (1) untransformed *L. lactis*; (2) a construct expressing PilM1 expressing ovalbumin peptide 323-339 at the βE/βF loop of the Spy0128 protein, and (3) ovalbumin;

[0085] Figure 4 shows serum IgG response in mice vaccinated with *L. lactis* expressing (1) the OVA peptide at the pilus tip, (2) *L. lactis* transformed with a construct expressing PilM1 comprising ovalbumin peptide 323-339 at the βE/βF loop of the Spy0128 protein, (3)

L. lactis transformed with a construct expressing native PilM1, (4) OVA peptide alone administered intranasally; and (5) OVA peptide alone administered subcutaneously;

[0086] Figure 5 shows bronchoalveolar lavage (BAL) IgA in mice vaccinated with *L. lactis* expressing (1) the OVA peptide at the βE/βF loop of the Spy0128 protein, (2) *L. lactis* transformed with a construct expressing native PilM1, (3) OVA peptide alone administered intranasally; and (4) OVA peptide alone administered subcutaneously;

[0087] Figure 6 shows the nucleotide coding sequence and derived amino acid sequence of the GAS M1 Spy0128 BP protein; and

[0088] Figure 7 shows Western blot analyses using A) an anti-Spy0128 antibody or B) an anti-OVA antibody of cell wall extracts obtained from *L. lactis* transformed with a construct expressing PilM1 comprising ovalbumin peptide 323-339 at the (1) βE/βF loop region, and (2) β9/β10 loop region of the Spy0128 (M1) protein;

[0089] Figure 8 shows Western blot analyses using an anti-Spy0128 antibody of cell wall extracts obtained from *L. lactis* transformed with constructs expressing PilM1 comprising A) the HA2 peptide, B) the HA3 peptide; C) two copies of the HA3 peptide (HA3-HA3 peptide), D) the M2e peptide, and E) two copies of the M2e peptide (M2e-M2e peptide) in the βE/βF loop region of the Spy0128 (M1) protein;

[0090] Figure 9 shows Western blot analyses using an anti-Spy0128 antibody of cell wall extracts obtained from *L. lactis* transformed with constructs expressing PilM1 comprising A) the ESAT-6_{1-20} peptide in the βE/βF loop region of the Spy0128 (M1) protein, B) the ESAT-6_{51-70} peptide in the β9/β10 loop region of the Spy0128 (M1) protein (both lanes), and C) the TBA61 peptide in the βE/βF loop region of the Spy0128 (M1) protein;

[0091] Figure 10 shows a Western blot analysis using an anti-Spy0128 antibody of a cell wall extract obtained from *L. lactis* transformed with a construct expressing PilM1 comprising a 41 amino acid peptide in the βE/βF loop region of the Spy0128 (M1) protein;

[0092] Figure 11 shows a Western blot analysis using an anti-Spy0128 antibody of a cell wall extract obtained from *L. lactis* transformed with a construct expressing PilM1 comprising a J14-$Ova_{323=327}$ fusion peptide in the βE/βF loop region of the Spy0128 (M1) protein;

[0093] Figure 12 shows a Western blot analysis using an anti-Spy0128 antibody of a cell wall extract obtained from *L. lactis* transformed with a construct expressing PilM1 comprising a $Ova_{323=327}$ peptide in the βG_1region of the Spy0128 (M1) protein;

[0094] Figure 13 shows a multiple sequence alignment of the M1 and M18 pilus operon sequences prepared using Clustal W;Figure 14 shows Western blot analyses using an anti-M18 Spy0128 antibody of cell wall extracts obtained from *L. lactis* transformed with a construct expressing PilM18 comprising a single ovalbumin peptide 323-339 (lanes 1 and 2) or a double ovalbumin peptide 323-339 (lanes 3 and 4) at the βE/βF loop region;

[0095] Figure 15 shows flow cytometry analysis using an antibody against M18_Spy0128 of 1) untransformed, wildtype *L. lactis* cells; 2) *L. lactis* cells transformed with a construct expressing the M18 pilus with an OVA-OVA peptide insert; and 3) *L. lactis* cells transformed with a construct expressing the M18 pilus with an OVA peptide insert;

[0096] Figure 16 shows using an α-spy0128 antibody of L. lactis cells transformed with 1) Pilvax-βG1_Ova construct (Ex 8); 2) PilVax-βE/F-AP construct (Ex 5); 3) PilVax-βE/F-M2eM2e construct (Ex 3); 4) PilVax-βE/F-M2e construct (Ex 3); 5) PilVax-βE/F-HA3HA3 construct (Ex 3); 6) PilVax-βE/F-HA3 construct (Ex 3); 7) PilVax-βE/F-HA2 construct (Ex 3); 8) PilVax-βE/F-J14Ova construct (Ex 5); 9) PilVax-βE/F-Ova construct (Ex 2); 10) Pilvax (no peptide insert); or 11) untransformed, wildtype L. lactis; 12) secondary antibody only control; and 13) unstained control.

[0097] Figure 17 shows A) serum 1gG response to M2e antigen; B) IgA response to M2e antigen in bronchoalveolar lavage (BAL) fluid; and C) serum IgG response to Spy0128 in in mice vaccinated with *L. lactis* expressing M1 pili displaying an M2e peptide;

[0098] Figure 18 shows A) serum 1gG response to M2e antigen; B) IgA response to M2e antigen in bronchoalveolar lavage (BAL) fluid; and C) serum IgG response to Spy0128 in in five mice vaccinated with *L. lactis* expressing M1 pili displaying an M2eM2e peptide; and

[0099] Figure 19 shows a Western blot analysis using an anti-Spy0128 antibody of a cell wall extract obtained from *L. lactis* transformed with a construct expressing PilM1 comprising the B16 peptide in the βE/βF loop region of the Spy0128 (M1) protein.

DETAILED DESCRIPTION OF THE INVENTION

[0100] The present invention relates to isolated, purified or recombinant polypeptides comprising a backbone pilin protein or fragment thereof and one or more heterologous polypeptides of interest. The present invention further relates to pili comprising a plurality of covalently attached peptides of interest wherein the peptides of interest are heterologous to the pilus, and a genetically engineered bacterium comprising one or more pili of the invention, and methods of preparing the genetically engineered bacterium.

[0101] The invention further relates to a pharmaceutical composition or vaccine composition comprising polypeptides, pili or bacteria of the invention, and the use of such composition for eliciting an immune response in a subject or vaccinating a subject.

[0102] The polypeptides, pili, and bacteria described herein provide for the presentation of a plurality of proteins of interest on the surface of cells. In particular, the polypeptides, pili, and bacteria are useful for the display of a plurality of antigens or fragments thereof (epitopes). In some embodiments, compositions described herein comprising polypeptides, pili, and bacteria of the invention are useful for eliciting a T cell or B cell immune response in a subject. In various embodiments, compositions of the invention are vaccine compositions, suitable for administration via various routes, for example, certain compositions described herein provide such a response when delivered to a subject mucosally, for example, intranasally.

[0103] Advantages of the invention include:

a. enhanced peptide immunogenicity,

b. increased peptide stability,

c. ease and efficiency of peptide manufacture

d. no requirement for chemical coupling or use of potentially toxic adjuvants, and

e. low production costs.

1. Definitions

[0104] The term "and/or" can mean "and" or "or".

[0105] The term "comprising" as used in this specification means "consisting at least in part of". When interpreting statements in this specification which include that term, the features, prefaced by that term in each statement, all need to be present but other features can also be present. Related terms such as "comprise" and "comprised" are to be interpreted in the same manner.

[0106] The term "heterologous peptide" as used herein with reference to a peptide of interest present in a polypeptide or pilus of the invention that additionally comprises a backbone pilin (BP) protein, including a polypeptide or pilus of the invention when present in or on a genetically engineered bacterium, means a peptide sequence that is not part of the native BP protein or is not encoded or expressed naturally by the gene encoding that BP protein.

[0107] As used herein "purified" does not require absolute purity; rather, it is intended as a relative term where the material in question is more pure than in the environment it was in previously. In practice the material has typically, for example, been subjected to fractionation to remove various other components, and the resultant material has substantially retained its desired biological activity or activities. The term "substantially purified" refers to materials that are at least about 60% free, preferably at least about 75% free, and most preferably at least about 90% free, at least about 95% free, at least about 98% free, or more, from other components with which they may be associated during manufacture.

[0108] The term "α-amino acid" or "amino acid" refers to a molecule containing both an amino group and a carboxyl group bound to a carbon which is designated the α-carbon. Suitable amino acids include, without limitation, both the D- and L-isomers of the naturally-occurring amino acids, as well as non-naturally occurring amino acids prepared by organic synthesis or other metabolic routes. Unless the context specifically indicates otherwise, the term amino acid, as used herein, is intended to include amino acid analogs.

[0109] In certain embodiments the peptide or pilus comprises only natural amino acids. The term "naturally occurring amino acid" refers to any one of the twenty amino acids commonly found in peptides synthesized in nature, and known by the one letter abbreviations A, R, N, C, D, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y and V.

[0110] The term "amino acid analog" or "non-naturally occurring amino acid" refers to a molecule which is structurally similar to an amino acid and which can be substituted for an amino acid. Amino acid analogs include, without limitation, compounds which are structurally identical to an amino acid, as defined herein, except for the inclusion of one or more additional methylene groups between the amino and carboxyl group (e.g., a-amino β-carboxy acids), or for the substitution of the amino or carboxy group by a similarly reactive group (e.g., substitution of the primary amine with a secondary or tertiary amine, or substitution or the carboxy group with an ester).

[0111] Unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology, biochemistry and immunology, which are within the skill of the art may be employed in practicing the methods described herein. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, second edition (Sambrook *et al.*, 1989); Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Animal Cell Culture (R.I. Freshney, ed., 1987); Handbook of Experimental Immunology (D.M. Weir & C.C. Blackwell, eds.); Gene Transfer Vectors for Mammalian Cells (J.M. Miller & M.P. Calos, eds., 1987); Current Protocols in Molecular Biology (F.M. Ausubel *et al.*, eds., 1987);

PCR: The Polymerase Chain Reaction, (Mullis *et al.*, eds., 1994); Current Protocols in Immunology (J.E. Coligan *et al.*, eds., 1991); The Immunoassay Handbook (David Wild, ed., Stockton Press NY, 1994); Antibodies: A Laboratory Manual (Harlow *et al.*, eds., 1987); and Methods of Immunological Analysis (R. Masseyeff, W.H. Albert, and N.A. Staines, eds., Weinheim: VCH Verlags gesellschaft mbH, 1993).

[0112] The term "peptide" and the like is used herein to refer to any polymer of amino acid residues of any length. The polymer can be linear or non-linear (*e.g.*, branched), it can comprise modified amino acids or amino acid analogs. The term also encompasses amino acid polymers that have been modified naturally or by intervention, for example, by disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other modification or manipulation, for example conjugation with labelling or bioactive component.

[0113] The term "(s)" following a noun contemplates the singular or plural form, or both.

[0114] The term "subject" is intended to refer to an animal, preferably a mammal, more preferably a human. Mammalian subjects include cats, dogs and horses. Other mammalian subjects include an agricultural animal, including a horse, a pig, a sheep, a goat, a cow, a deer, or a fowl, or a laboratory animal, including a monkey, a rat, a mouse, a rabbit or a guinea pig.

[0115] The term "treat" and its derivatives should be interpreted in their broadest possible context. The term should not be taken to imply that a subject is treated until total recovery. Accordingly, "treat" broadly includes maintaining a subject's disease progression, symptoms or burn or wound healing at a substantially static level, increasing a subject's rate of recovery, amelioration and/or prevention of the onset of the symptoms or severity of a particular condition, burn, wound or other injury, or extending a patient's quality of life. The term "treat" also broadly includes the maintenance of good health for sensitive individuals and building stamina for disease, infection or infestation prevention.

[0116] The invention consists in the foregoing and also envisages constructions of which the following gives examples only and in no way limit the scope thereof.

2. Pili and pili polypeptides

[0117] Pili are long, filamentous, hair-like protrusions that are attached to, and extend from, the surface of bacterial cells. In particular, the present invention takes advantage of

pili derived from Gram positive bacteria, such as Group A *Streptococcus* (GAS). In an exemplary embodiment of the invention, proteins derived from *Streptococcus pyogenes* are used.

[0118] GAS pili are important for bacterial cell adherence and aggregation. In particular, GAS pili have been shown to mediate cell adhesion to human tonsil epithelium and primary human keratinocytes – two of the major infection sites of GAS (Abbot *et al*. Cell Microbiol. 2007;9(7):1822-33.).

[0119] GAS pili comprise a repeating backbone pilin subunit (a "BP protein") and other minor subunits that are covalently assembled on the cell surface by pilus-associated sortases. The elongated BP protein subunits are stacked head to tail and linked covalently to form the shaft of the pilus. Approximately 50-100 BP subunits are assembled in this way, before a cell wall adaptor molecule ancillary pilin 2 (AP2) protein stops assembly and covalently attaches the pilus to peptidoglycan in the bacterial cell wall. Repeating BP protein subunits extend from the AP2 protein linked by intermolecular isopeptide bonds. An ancillary pilin 1 (AP1) protein is located at the terminus of the pilus.

[0120] The present invention provides for the insertion of one or more peptides into the BP protein monomer, resulting in the expression of potentially hundreds of repeated copies of the one or more peptides along the pilus shaft, and thus potentially thousands of copies on the surface of the bacterium.

[0121] Without wishing to be bound by any theory or mechanism, it is believed that, in embodiments where the peptide of interest is an antigen or epitope, the presence of thousands of copies of the peptide of interest on the pili of the invention enhances the immunological effects of pili and bacteria of the invention. The concentrated display of a large number of antigens provides a greater opportunity for the peptides to encounter cells of the immune system.

[0122] The pilus operon is located within a variable region of the bacterial chromosome known as fibronectin-binding, collagen-binding, T antigen (FCT) region.

[0123] In an exemplary embodiment of the invention, the serotype M1 GAS pilus structure is utilized.

[0124] The M1 pilus comprises the BP protein Spy0128. The complete amino acid sequence of Spy0128 is provided as SEQ ID No. 1. The complete nucleotide coding sequence of Spy0128 is provided as SEQ ID No. 2.

[0125] The M1 pilus further comprises a collagen-binding protein or tip adhesion molecule (Spy0125, an AP1 protein) and an AP2 protein Spy0130.

[0126] The present inventors have identified a number of regions of the Spy0128 suitable for insertion of a peptide of interest. Suitable sites for insertion of a peptide of interest are those sites that preserve the correct assembly of the pilus on the surface of bacteria when expressed. Furthermore, it is desirable that the peptide of interest does not undergo substantial degradation when expressed.

[0127] In various embodiments, the BP protein into which one or more heterologous peptides is inserted is or comprises at least one domain of the Spy0128 protein of SEQ ID No: 1. For example, the BP protein is the N-terminal domain of the Spy0128 protein of SEQ ID No: 1, comprising amino acids 1 to 171 of SEQ ID No: 1. In another example, the BP protein is the C-terminal domain of the Spy0128 protein of SEQ ID No: 1, comprising amino acids 174 to 340 of SEQ ID No: 1.

[0128] In other embodiments, the BP protein into which one or more heterologous peptides is inserted is or comprises at least 150 contiguous amino acids of the Spy0128 protein of SEQ ID No: 1. For example, the BP protein comprises at least about 100 contiguous amino acids from the N-terminal domain of the Spy0128 protein of SEQ ID No: 1 (amino acids 1 to 171 of SEQ ID No: 1). In another example, the BP protein comprises at least about 100 contiguous amino acids from the C-terminal domain of the Spy0128 protein of SEQ ID No: 1 (amino acids 174 to 340 of SEQ ID No: 1).

[0129] In other embodiments, the BP is selected from the group consisting of one or more of the GAS T antigen sequences presented in Table 1 below

Table 1 – Representative BP proteins.

FCT-region	Tee type	emm/M type	strain	NCBI Accession #
FCT-1	Tee6	M6	MGAS10394	2940764
FCT-2	Tee1	M1	SF370	NP_268517
FCT-3/4/7/8	Tee8	emm59	10303	KJ816977
	Tee10	emm89	96/364	KJ817010
	Tee9	M5	90/306	KJ817040
	Tee11	emm44/61	11070	KJ816969
	Tee28.1	M28	MGAS6180	3573111
	Tee28.2	emm56	9779	KJ816976
	Tee18.1	emm217	11574	KJ816959
	Tee18.2	emm49	GAS1042	KJ816971
	Tee13	emm41	10028	KJ816965
	Tee3	emm65	13637	KJ816984
	Tee12	emm12	GAS10150	KJ816948
	Tee7	emm114	GAS10143	KJ816943
	Tee14	emm101	GAS09426	KJ816940

	Tee5	emm82	9893	KJ816995
FCT-5	Tee4	M4	MGAS10750	4067256
FCT-6	Tee2	M2	MGAS10270	4063969

[0130] Other BP proteins suitable for use in the invention are available from Genbank under the accession numbers KJ816940 – KJ817040.

[0131] The immunogenic pilus proteins have also been shown to play a role in bacterial pathogenesis in several studies using animal models. The genes encoding the pilus proteins and assembly enzymes are clustered in the **F**ibronectin-binding, **C**ollagen-binding, **T** antigen (FCT) region, of which 9 variants with diverse gene organisation and sequences have been reported, but only a few types have been studied in depth.

[0132] The inventors have found that certain polypeptides, pili and bacteria of the present invention are suitable vehicles for eliciting immunological responses to heterologous peptides presented therein.

[0133] The recombinant BP, for example, the recombinant M1 pilus, can be expressed and assembled on the surface of a recombinant bacteria, for example a recombinant gram positive bacteria, including non-pathogenic bacteria such as *L. lactis*, a non-pathogenic relative of GAS. In other embodiments, the recombinant BP, for example, the recombinant M1 pilus, is expressed from an operon comprising the recombinant gene encoding the one or more heterologous peptides of interest inserted into the BP together with the other pilus-forming genes (e.g., the FCT region) incorporated into a bacterial host, whether or not the native, non-recombinant host is capable of natively expressing or presenting pili.

3. Antigens

[0134] It will be appreciated that a great many antigens, for example tumour antigens or antigens from various pathogenic organisms, have been characterised and are suitable for use in the present invention, for example in the polypeptides, pili or genetically engineered bacteria of the present invention. All antigens, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

[0135] The polypeptides, pili or bacteria of the present invention find application in a wide range of immunotherapies, including but not limited to the treatment and prevention of conditions or diseases caused by pathogenic bacteria or viruses, for example including but not limited to the treatment and prevention of tuberculosis, and of cancer and neoplastic conditions including but not limited to colorectal cancer.

[0136] Also contemplated are antigens comprising one or more amino acid substitutions, such as one or more conservative amino acid substitutions.

[0137] A "conservative amino acid substitution" is one in which an amino acid residue is replaced with another residue having a chemically similar or derivatised side chain. Families of amino acid residues having similar side chains, for example, have been defined in the art. These families include, for example, amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Amino acid analogs (e.g., phosphorylated or glycosylated amino acids) are also contemplated in the present invention, as are peptides substituted with non-naturally occurring amino acids, including but not limited to N-alkylated amino acids (e.g. N-methyl amino acids), D-amino acids, β-amino acids, and γ-amino acids.

[0138] Fragments and variants of antigens are also specifically contemplated.

[0139] A "fragment" of a peptide, is a subsequence of the peptide that performs a function that is required for the enzymatic or binding activity and/or provides three dimensional structure of the peptide, such as the three dimensional structure of a polypeptide.

[0140] The term "variant" as used herein refers to peptide sequences, including for example peptide sequences different from the specifically identified sequences, wherein one or more amino acid residues is deleted, substituted, or added. Variants are naturally-occurring variants, or non-naturally occurring variants. Variants are from the same or from other species and may encompass homologues, paralogues and orthologues. In certain embodiments, variants of peptides including peptides possess biological activities that are the same or similar to those of the wild type peptides. The term "variant" with reference to peptides encompasses all forms of peptides as defined herein.

[0141] Those of skill in the art will appreciate that the polypeptides, pili, bacteria and compositions of the present invention are in certain embodiments suited for stimulating T-cell responses or B-cell responses or both T-cell and B-cell responses.

[0142] Many different antigens or fragments thereof may be suitable for use in the invention. For example, suitable antigens to target a particular disease or condition may be

identified using the Immune Epitope Database and Analysis Resource (accessible at http://www.iedb.org/home_v3.php).

[0143] Those of skill in the art will appreciate that the polypeptides, pili, bacteria and compositions of the present invention are in certain embodiments suited for stimulating T-cell responses, for example in the treatment of neoplastic diseases, including cancer, or in the treatment of infectious disease.

[0144] Those of skill in the art will appreciate that the polypeptides, pili and bacteria of the present invention are in certain embodiments suited for stimulating T-cell responses, for example in the treatment of neoplastic diseases, including cancer. Compositions and vaccines of the present invention comprising one or more tumour antigens are contemplated. It will be appreciated that tumour antigens contemplated for use in the preparation of compositions, vaccines, and/or polypeptides, pili or bacteria of the invention will generally comprise one or more peptides. In certain embodiments of the invention, including for example pharmaceutical compositions of the invention, one or more additional tumour antigens may be present, including tumour antigens wherein the one or more tumour antigens does not comprise a peptide. Tumour antigens are typically classified as either unique antigens, or shared antigens, with the latter group including differentiation antigens, cancer-specific antigens, and over-expressed antigens. Examples of each class of antigens are amenable to use in the present invention. Representative tumour antigens for use in the treatment, for example immunotherapeutic treatment, or vaccination against neoplastic diseases including cancer, are discussed below. Polypeptides, pili, bacteria, vaccines and compositions comprising one or more antigens prepared using those methods of immunisation are specifically contemplated.

[0145] In certain embodiments, the tumour antigen is a polypeptide tumour antigen or glycoprotein tumour antigens. In certain embodiments, the tumour antigen is a saccharide-containing tumour antigen, such as a glycolipid tumour antigen or a ganglioside tumour antigen.

[0146] Tumour antigens appropriate for the use in the present invention encompass a wide variety of molecules, such as (a) peptide-containing tumour antigens, including peptide epitopes (which can range, for example, from 8-20 amino acids in length, although lengths outside this range are also common), lipopolypeptides and glycoproteins, (b) saccharide-containing tumour antigens, including poly-saccharides, mucins, gangliosides, glycolipids and glycoproteins, including and (c) polynucleotides that express antigenic polypeptides. Again, those skilled in the art will recognise that a tumour antigen present in a conjugate or composition of the present invention will typically comprise peptide.

[0147] In certain embodiments, the tumour antigens are, for example, (a) full length molecules associated with cancer cells, (b) homologues and modified forms of the same, including molecules with deleted, added and/or substituted portions, and (c) fragments of the same, provided said fragments remain antigenic or immunogenic. In certain embodiments, the tumour antigens include, for example, class I-restricted antigens recognized by CD8+ lymphocytes or class II-restricted antigens recognized by CD4+ lymphocytes.

[0148] Shared tumour antigens are generally considered to be native, unmutated sequences that are expressed by tumours due to epigenetic changes that allow de-repression of developmentally-repressed genes. Accordingly, shared antigens are typically considered preferable to over-expressed or differentiation-associated antigens because there is no expression in normal tissues. Also, the same antigens can be targeted in a number of cancer patients. For example, the cancer-testis antigen NY-ESO-1 is present in the majority of patients with many tumours, and a sizeable minority of patients with other tumours. In another example, breast differentiation tumour antigens NYBR-1 and NYBR-1.1 are found in a proportion of breast cancer sufferers. Shared tumour antigens thus represent an attractive target for development.

[0149] The use of shared tumour antigens, such as cancer-testis antigens including NY-ESO-1, CTSP-1, CTSP-2, CTSP-3, CTSP-4, SSX2, and SCP1, and breast cancer antigens NYBR-1 and NYBR-1.1, in combination with peptides or conjugates of the present invention is specifically contemplated herein.

[0150] In one specifically contemplated embodiment, the polypeptide of the invention, for example, the isolated, purified, or recombinant polypeptide or the pili or bacterium comprising a polypeptide of the invention comprises an amino acid sequence selected from the group consisting of 8 or more contiguous, 10 or more contiguous, 12 or more contiguous, 15 or more contiguous, 20 or more contiguous, or 25 or more contiguous amino acids of a heterologous peptide epitope or peptide antigen.

[0151] In various embodiments, the heterologous peptide comprises more than one peptide epitope or antigen, for example two or more amino acid sequences comprising peptide epitopes or peptide antigens.

[0152] Unique antigens are considered to be those antigens that are unique to an individual or are shared by a small proportion of cancer patients, and typically result from mutations leading to unique protein sequences. Representative examples of unique tumour antigens include mutated Ras antigens, and mutated p53 antigens. As will be appreciated by those skilled in the art having read this specification, the methods of the present

invention enable the ready preparation of polypeptides, pili or bacteria comprising one or more unique tumour antigens, for example to elicit specific T-cell responses to one or more unique tumour antigens, for example in the preparation of patient-specific therapies.

[0153] Accordingly, representative tumour antigens include, but are not limited to, (a) antigens such as RAGE, BAGE, GAGE and MAGE family polypeptides, for example, GAGE-1, GAGE-2, MAGE-1, MAGE-2, MAGE-3, MAGE-4, MAGE-5, MAGE-6, and MAGE-12 (which can be used, for example, to address melanoma, lung, head and neck, NSCLC, breast, gastrointestinal, and bladder tumours), (b) mutated antigens, for example, p53 (associated with various solid tumours, for example, colorectal, lung, head and neck cancer), p21/Ras (associated with, for example, melanoma, pancreatic cancer and colorectal cancer), CDK4 (associated with, for example, melanoma), MUM1 (associated with, for example, melanoma), caspase-8 (associated with, for example, head and neck cancer), CIA 0205 (associated with, for example, bladder cancer), HLA-A2-R1701, beta catenin (associated with, for example, melanoma), TCR (associated with, for example, T-cell non-Hodgkins lymphoma), BCR-abl (associated with, for example, chronic myelogenous leukemia), triosephosphate isomerase, MA 0205, CDC-27, and LDLR-FUT, (c) over-expressed antigens, for example, Galectin 4 (associated with, for example, colorectal cancer), Galectin 9 (associated with, for example, Hodgkin's disease), proteinase 3 (associated with, for example, chronic myelogenous leukemia), Wilm's tumour antigen-1 (WT 1, associated with, for example, various leukemias), carbonic anhydrase (associated with, for example, renal cancer), aldolase A (associated with, for example, lung cancer), PRAME (associated with, for example, melanoma), HER-2/neu (associated with, for example, breast, colon, lung and ovarian cancer), alpha-fetoprotein (associated with, for example, hepatoma), KSA (associated with, for example, colorectal cancer), gastrin (associated with, for example, pancreatic and gastric cancer), telomerase catalytic protein, MUC-1 (associated with, for example, breast and ovarian cancer), G-250 (associated with, for example, renal cell carcinoma), p53 (associated with, for example, breast, colon cancer), and carcinoembryonic antigen (associated with, for example, breast cancer, lung cancer, and cancers of the gastrointestinal tract such as colorectal cancer), (d) shared antigens, for example, melanoma-melanocyte differentiation antigens such as MART-1/Melan A, gp100, MC1R, melanocyte-stimulating hormone receptor, tyrosinase, tyrosinase related protein-1/TRP1 and tyrosinase related protein-2/TRP2 (associated with, for example, melanoma), (e) prostate associated antigens such as PAP, prostatic serum antigen (PSA), PSMA, PSH-P1, PSM-P1, PSM-P2, associated with for example, prostate cancer, (f) immunoglobulin idiotypes (associated with myeloma and B cell lymphomas, for example), and (g) other tumour antigens, such as polypeptide- and saccharide-containing antigens including (i) glycoproteins such as sialyl Tn and sialyl Le.sup.x (associated with, for example, breast and

colorectal cancer) as well as various mucins; glycoproteins are coupled to a carrier protein (for example, MUC-1 are coupled to KLH); (ii) lipopolypeptides (for example, MUC-1 linked to a lipid moiety); (iii) polysaccharides (for example, Globo H synthetic hexasaccharide), which are coupled to a carrier proteins (for example, to KLH), (iv) gangliosides such as GM2, GM12, GD2, GD3 (associated with, for example, brain, lung cancer, melanoma), which also are coupled to carrier proteins (for example, KLH).

[0154] Other representative tumour antigens amenable to use in the present invention include TAG-72, (See, e.g., U.S. Pat. No. 5,892,020; human carcinoma antigen (See, e.g., U.S. Pat. No. 5,808,005); TP1 and TP3 antigens from osteocarcinoma cells (See, e.g., U.S. Pat. No. 5,855,866); Thomsen-Friedenreich (TF) antigen from adenocarcinoma cells (See, e.g., U.S. Pat. No. 5,110,911); KC-4 antigen from human prostrate adenocarcinoma (See, e.g., U.S. Pat. No. 4,743,543); a human colorectal cancer antigen (See, e.g., U.S. Pat. No. 4,921,789); CA125 antigen from cystadenocarcinoma (See, e.g., U.S. Pat. No. 4,921,790); DF3 antigen from human breast carcinoma (See, e.g., U.S. Pat. Nos. 4,963,484 and 5,053,489); a human breast tumour antigen (See, e.g., U.S. Pat. No. 4,939,240); p97 antigen of human melanoma (See, e.g., U.S. Pat. No. 4,918,164); carcinoma or orosomucoid-related antigen (CORA) (See, e.g., U.S. Pat. No. 4,914,021); T and Tn haptens in glycoproteins of human breast carcinoma, MSA breast carcinoma glycoprotein; MFGM breast carcinoma antigen; DU-PAN-2 pancreatic carcinoma antigen; CA125 ovarian carcinoma antigen; YH206 lung carcinoma antigen, Alphafetoprotein (AFP), hepatocellular carcinoma antigen; Carcinoembryonic antigen (CEA); bowel cancer antigen; Epithelial tumour antigen (ETA); breast cancer antigen; Tyrosinase; the raf oncogene product; gp75; gp100; EBV-LMP 1 & 2; EBV-EBNA 1, 2 & 3C; HPV-E4, 6, 7; CO17-1A; GA733; gp72; p53; proteinase 3; telomerase; and melanoma gangliosides. These and other tumour antigens, whether or not presently characterized, are contemplated for use in the present invention.

[0155] In certain embodiments, the tumour antigens are derived from mutated or altered cellular components. Representative examples of altered cellular components include, but are not limited to ras, p53, Rb, altered protein encoded by the Wilms' tumour gene, ubiquitin, mucin, protein encoded by the DCC, APC, and MCC genes, as well as receptors or receptor-like structures such as neu, thyroid hormone receptor, platelet derived growth factor (PDGF) receptor, insulin receptor, epidermal growth factor (EGF) receptor, and the colony stimulating factor (CSF) receptor.

[0156] The present invention also contemplates the preparation of polypeptides, pili and bacteria comprising viral antigens that are capable of stimulating T-cell to elicit effective anti-viral immunity in patients who are or have been immunosuppressed, for

example patients who have had bone marrow transplants, haematopoietic stem cell transplants, or are otherwise undergoing immunosuppression.

[0157] Similarly, antigens derived from viruses associated with increased incidence of cancer, or that are reported to be cancer-causing, such as human papillomavirus, hepatitis A virus, and hepatitis B virus, are contemplated for use in the present invention.

[0158] For example, in certain embodiments, the tumour antigens include, but are not limited to, p15, Hom/Mel-40, H-Ras, E2A-PRL, H4-RET, IGH-IGK, MYL-RAR, Epstein Barr virus antigens, human papillomavirus (HPV) antigens, including E6 and E7, hepatitis B and C virus antigens, human T-cell lymphotropic virus antigens, TSP-180, p185erbB2, p180erbB-3, c-met, mn-23H1, TAG-72-4, CA 19-9, CA 72-4, CAM 17.1, NuMa, K-ras, p16, TAGE, PSCA, CT7, 43-9F, 5T4, 791 Tgp72, beta-HCG, BCA225, BTAA, CA 125, CA 15-3 (CA 27.29\BCAA), CA 195, CA 242, CA-50, CAM43, CD68\KP1, CO-029, FGF-5, Ga733 (EpCAM), HTgp-175, M344, MA-50, MG7-Ag, MOV18, NB/70K, NY-CO-1, RCAS1, SDCCAG16, TA-90 (Mac-2 binding protein\cyclophilin C-associated protein), TAAL6, TAG72, TLP, TPS, and the like.

[0159] It will likewise be appreciated in light of the teaching of this specification that antigens derived from agents causing or associated with infectious diseases are contemplated for use as described herein.

[0160] Representative antigens for use in vaccination against pathogenic organisms are discussed below. Compounds, vaccines and compositions comprising one or more antigens prepared using those methods of immunisation are specifically contemplated.

Tuberculosis antigens

[0161] It will be appreciated that a great many *M. tuberculosis* antigens have been characterised and are suitable for use in the present invention. All *M. tuberculosis* antigens, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

[0162] Exemplary *M. tuberculosis* antigens suitable for use include early secretary antigen target (ESAT) -6, Ag85A, Ag85B (MPT59), Ag85B, Ag85C, MPT32, MPT51, MPT59, MPT63, MPT64, MPT83, MPB5, MPB59, MPB64, MTC28, Mtb2, Mtb8.4, Mtb9.9, Mtb32A, Mtb39, Mtb41, TB10.4, TB10C, TB11B, TB12.5, TB13A, TB14, TB15, TB15A, TB16, TB16A, TB17, TB18, TB21, TB20.6, TB24, TB27B, TB32, TB32A, TB33, TB38, TB40.8, TB51, TB54, TB64, CFP6, CFP7, CFP7A, CFP7B, CFP8A, CFP8B, CFP9, CFP10, CFP11, CFP16, CFP17, CFP19, CFP19A, CFP19B, CFP20, CFP21, CFP22, CFP22A, CFP23, CFP23A, CFP23B, CFP25,

CFP25A, CFP27, CFP28, CFP28B, CFP29, CFP30A, CFP30B, CFP50, CWP32, hspX (alpha-crystalline), APA, Tuberculin purified protein derivative (PPD), ST-CF, PPE68, LppX, PstS-1, PstS-2, PstS-3, HBHA, GroEL, GroEL2, GrpES, LHP, 19kDa lipoprotein, 71kDa, RD1-ORF2, RD1-ORF3, RD1-ORF4, RD1-ORF5, RD1-ORF8, RD1-ORF9A, RD1-ORF9B, Rv1984c, Rv0577, Rv1827, BfrB, Tpx. Rv1352, Rv1810, PpiA, Cut2, FbpB, FbpA, FbpC, DnaK, FecB, Ssb, RplL, FixA, FixB, AhpC2, Rv2626c, Rv1211, Mdh, Rv1626, Adk, ClpP, SucD (Belisle et al, 2005; US 7,037,510; US 2004/0057963; US 2008/0199493; US 2008/0267990), or at least one antigenic portion or T-cell epitope of any of the above mentioned antigens.

Hepatitis antigens

[0163] A number of hepatitis antigens have been characterised and are suitable for use in the present invention. Exemplary hepatitis C antigens include C – p22, E1 – gp35, E2 - gp70, NS1 – p7, NS2 – p23, NS3 – p70, NS4A – p8, NS4B – p27, NS5A – p56/58, and NS5B – p68, and together with one or more antigenic portions or epitopes derived therefrom are each (whether alone or in combination) suitable for application in the present invention. All hepatitis antigens, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

Influenza antigens

[0164] Many influenza antigens have been characterised and are suitable for use as described herein. Exemplary influenza antigens suitable for use include PB, PB2, PA, any of the hemagglutinin (HA) or neuramimidase (NA) proteins, NP, M, and NS, and together with one or more antigenic portions or epitopes derived therefrom are each (whether alone or in combination) suitable for application in the present invention. All influenza antigens, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

Respiratory Syncytial Virus

[0165] Antigens from Respiratory Syncytial Virus (RSV) have been identified, for example those used in screening methodologies to identify infection, and are suitable as potential candidates for use as described herein. Exemplary RSV antigens include those derived from RSV F protein, such as antigens derived from the A, B, C or AB antigenic regions, antigens derived from RSV G protein, or antigens bound by commercially available anti-RSV antibodies such as palivizumab (Synagis™). All RSV antigens, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

Anthrax antigens

[0166] A number of *B. anthracis* antigens have been identified as potential candidates for vaccine development and are useful in the present invention. For example, PA83 is one such antigen for vaccine development. Currently, only one FDA licensed vaccine for anthrax is available called "Anthrax Vaccine Adsorbed" (AVA) or BioThrax®. This vaccine is derived from the cell-free supernatant of a non-encapsulated strain of *B. anthracis* adsorbed to aluminum adjuvant. PA is the primary immunogen in AVA. Other exemplary anthrax antigens suitable for use in the present invention include Protective antigen (PA or PA63), LF and EF (proteins), poly-gamma-(D-glutamate) capsule, spore antigen (endospore specific components), BclA (exosporium specific protein), BxpB (spore-associated protein), and secreted proteins. All anthrax antigens together with one or more antigenic portions or epitopes derived therefrom, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

Tularemia antigens

[0167] A number of *F. tularensis* antigens have been identified as potential candidates for vaccine development and are useful in the present invention. For example, AcpA and IglC are antigens suitable for vaccine development. Other exemplary Tularemia antigens suitable for use in the present invention include O-antigen, CPS, outer membrane proteins (e.g. FopA), lipoproteins (e.g. Tul4), secreted proteins and lipopolysaccharide. All tularemia antigens together with one or more antigenic portions or epitopes derived therefrom, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

Brucellosis antigens

[0168] A number of *B. abortusis* antigens have been identified as potential candidates for vaccine development and are useful in the present invention. For example, Omp16 is one such antigen for vaccine development. Other exemplary Brucellosis antigens suitable for use in the present invention include O-antigen, lipopolysaccharide, outer membrane proteins (e.g. Omp16), secreted proteins, ribosomal proteins (e.g. L7 and L12), bacterioferritin, p39 (a putative periplasmic binding protein), groEL(heat-shock protein), lumazine synthase, BCSP31 surface protein, PAL16.5 OM lipoprotein, catalase, 26 kDa periplasmic protein, 3l kDa Omp31, 28 kDa Omp, 25 kDa Omp, and 10 kDA Om lipoprotein. All brucellosis antigens together with one or more antigenic portions or epitopes derived therefrom, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

Meningitis antigens

[0169] A number of *N. meningitidis* antigens have been identified as potential candidates for vaccine development and are useful in the present invention. For example, Cys6, PorA, PorB, FetA, and ZnuD are antigens suitable for vaccine development. Other exemplary Meningitis antigens suitable for use in the present invention include O-antigen, factor H binding protein (fHbp), TbpB, NspA, NadA, outer membrane proteins, group B CPS, secreted proteins and lipopolysaccharide. All menigitis antigens together with one or more antigenic portions or epitopes derived therefrom, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

Dengue antigens

[0170] A number of Flavivirus antigens have been identified as potential candidates for vaccine development to treat dengue fever and are useful in the present invention. For example, dengue virus envelope proteins E1 – E4 and the membrane proteins M1 – M4 are antigens suitable for vaccine development. Other exemplary dengue antigens suitable for use in the present invention include C, preM, 1, 2A, 2B, 3, 4A, 4B and 5. All dengue antigens together with one or more antigenic portions or epitopes derived therefrom, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

Ebola antigens

[0171] A number of ebola virus antigens have been identified as potential candidates for vaccine development to treat ebola infection and are useful in the present invention. For example, *Filoviridae* Zaire ebolavirus and Sudan ebolavirus virion spike glycoprotein precursor antigens ZEBOV-GP, and SEBOV-GP, respectively, are suitable for vaccine development. Other exemplary ebola antigens suitable for use in the present invention include NP, vp35, vp40, GP, vp30, vp24 and L. All ebola antigens together with one or more antigenic portions or epitopes derived therefrom, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

West Nile antigens

[0172] A number of West Nile virus antigens have been identified as potential candidates for vaccine development to treat infection and are useful in the present invention. For example, *Flavivirus* envelope antigen (E) from West Nile virus (WNV) is a non-toxic protein expressed on the surface of WNV virions (WNVE) and are suitable for

vaccine development. Other exemplary WNV antigens suitable for use in the present invention include Cp, Prm, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.

[0173] All West Nile antigens together with one or more antigenic portions or epitopes derived therefrom, whether or not presently characterized, that are capable of eliciting an immune response are contemplated.

[0174] The above-listed or referenced antigens are exemplary, not limiting, of the present invention.

4. Methods

[0175] The inventors have found that certain polypeptides, pili and bacteria of the present invention have immunological activity.

[0176] Cell-mediated immunity is primarily mediated by T-lymphocytes. Pathogenic antigens are expressed on the surface of antigen presenting cells (such as macrophages, B-lymphocytes, and dendritic cells), bound to either major histocompatibility MHC Class I or MHC Class II molecules. Presentation of pathogenic antigen coupled to MHC Class II activates a helper (CD4+) T-cell response. Upon binding of the T-cell to the antigen-MHC II complex, CD4+ T-cells, release cytokines and proliferate.

[0177] Presentation of pathogenic antigens bound to MHC Class I molecules activates a cytotoxic (CD8+) T-cell response. Upon binding of the T-cell to the antigen-MHC I complex, CD8+ cells secrete perforin and other mediators, resulting in target cell death.

[0178] Methods to assess and monitor the onset or progression of a cell-mediated response in a subject are well known in the art. Convenient exemplary methods include those in which the presence of or the level of one or more cytokines associated with a cell-mediated response, such as those identified herein, is assessed. Similarly, cell-based methods to assess or monitor the onset and progression of a cell-mediated response are amenable to use in the present invention, and may include cell proliferation or activation assays, including assays targeted at identifying activation or expansion of one or more populations of immune cells, such as T-lymphocytes.

[0179] In various embodiments, the polypeptides, pili or bacteria of the invention elicit a humoral immune response.

[0180] The humoral immune response is mediated by secreted antibodies produced by specific B cells. Generally, full B-cell activation and antibody production occurs following

interaction of the B-cell with a T helper cell (CD4+). The secreted antibodies bind to antigens presented on the surface of invading pathogens, flagging them for destruction.

[0181] Again, methods to assess and monitor the onset or progression of a humoral response are well known in the art. These include antibody binding assays, ELISA, skin-prick tests and the like. Representative methods are employed herein in the Examples.

[0182] In certain embodiments, methods of the invention elicit both a cell-mediated immune response and a humoral response.

[0183] The compositions of the invention are amenable for administration via various routes. Certain embodiments are particularly suited for mucosal delivery, for example, intranasal delivery. As can be seen herein, the antigen presentation provided by the invention enables a robust IgA production, rendering the invention particularly suited to eliciting a mucosal immune response in subjects to whom the polypeptides, pili, bacteria compositions, and vaccines of the invention are administrated.

[0184] In some embodiments, the recombinant proteins, and compositions comprising same, can be administered via oral immunisation methods, such as those discussed in Robinson et al., Oral vaccination of mice against tetanus with recombinant Lactococcus lactis, Nature Biotechnology (1997) vol 15 pp 653-7, Ahmed et al., Oral immunisation with *Lactococcus lactis*-expressing EspB induces protective immune responses against *Escherichia coli* O157:H7 in a murine model of colonization, Vaccine 32 (2014) 3909–3916, and Zhang X, et al., Heterologous expression of carcinoembryonic antigen in *Lactococcus lactis* via LcsB mediated surface displaying system for oral vaccine development, Journal of Microbiology, Immunology and Infection (2014), http://dx.doi.org/10.1016/j.jmii.2014.11.009.

[0185] Other methods of administration, and formulations particularly suited to such administration methods, are well known in the art.

EXAMPLES

EXAMPLE 1

1. Cloning of the complete M1 pilus operon

[0186] The pilus operon [SEQ ID No: 29] was PCR amplified from GAS strain SF370 (serotype M1) using the primers listed in Table 2.

Table 2: List of primers used to amplify M1 pilus operon from GAS strain SF370 (serotype M1).

Primer name	Sequence	SEQ ID
PilM1_Bam.fw	gcggatccgatatgatgtcacattgagag	3
PilM1_Bam.rev	gcggatccgtcgtggggcaataaaaaattc	4

[0187] After digest with BamHI, the operon was cloned into plasmid pLZ12KmP23R for expression in *L. lactis*. The pLZ12Km vector (Okada, N., et al., (1998). *Streptococcus pyogenes* protein F promotes invasion of HeLa cells. Microbiology 144, 3079–3086) and modified to add the *L. lactis* promoter P23.

[0188] The recombinant plasmid was first introduced into *E. coli* by heat-shock. Purified plasmid DNA was then electroporated into *Lactococcus lactis* strain MG1363.

2. Expression of the M1 pilus in *L. lactis*

[0189] The P23 promoter constitutively expresses the pilus. This was confirmed by Western blot analysis of *L. lactis* cell wall extract. Cell wall extracts were obtained by enzymatically removing the cell wall (using mutanolysin and lysozyme) and removing the insoluble fraction by centrifugation. The soluble fraction (cell wall extract with pili) was loaded onto an SDS-PAGE gel, blotted onto nitrocellulose and probed with specific anti-Spy0128 serum (generated in a rabbit after immunization with purified recombinant Spy0128). Blots were developed using the Amersham ECL Western Blotting System (GE Healthcare) and the resulting chemiluminescence was detected using a Fujifilm LAS-3000 Scanner (Alphatech).

3. Introduction of the XhoI site within the DNA encoding for loop regions.

[0190] To introduce a XhoI cleavage site into the Spy0128 nucleotide region of the M1 pilus operon at specific sites, the plasmid pLZ12KmP23R comprising the pilus operon was PCR amplified using the primer sets listed in Table 3.

[0191] Each PCR product was digested with XhoI and re-cloned into pLZ12km_P23R.

Table 3: List of primers used to introduce XhoI restriction sites.

Position for insertion of XhoI cleavage (SEQ ID No. 1)	Amino acids deleted	Primer sequences	SEQ ID

N-terminus aa28 (Cpa tip protein)	None	F: CCCG**CTCGAG**GGAGCCAAACTAACAGTTAC	5
		R: CCCG**CTCGAG**GTTTACAACAGTCTCCCC	6
βB-C1 loop amino acid 61	61–65	F: GAGAGAGA**CTCGAG**GGAAATAAGTTTAAA GGTGTAGC	7
		R: GAGAGAGA **CTCGAG**AGTATCAGGTTC GATTTTAAATG	8
βD-E loop amino acid 101	101–108	F: GAGAGAGA**CTCGAG**GGTGTTTATTATTAC AAAG	9
		R: GAGAGAGA**CTCGAG**AAAATCAAATTCTGC AG	10
β_E/β_F loop amino acid 119	119–124	F: GAGAGAGA**CTCGAG**GGTGTTTCTTATGAT ACAAC	11
		R: GAGAGAGA**CTCGAG**CTCCTCAGTTACTTT G	12
β2-3 loop amino acid 201	201–206	F: GAGAGAGA**CTCGAG**TCAGAAAAAGTCATG ATTGAG	13
		R: GAGAGAGA**CTCGAG**TGCTTTTAAAGTCAG ACC	14
β3-4 loop amino acid 217	217–221	F: GAGAGAGA**CTCGAG**CCTGTTCAAACAGAG GCTAG	15
		R: GAGAGAGA**CTCGAG**AGTTGTCTTCTCAAT CATGAC	16
β_9/β_{10} loop amino acid 276	276–279	F: GAGAGAGA**CTCGAG**AAAAATATCGCAGGT AAT TC	17
		R: GAGAGAGA**CTCGAG**TTGAGGACTAACTTC CACG	18

4. Introduction of OVA peptide sequence.

[0192] The peptide sequence for Ova323-339 was reverse transcribed to DNA using the *L. lactis* codon usage as shown in Table 4. A 5'- XhoI site and a 3' SalI site were added to the peptide.

Table 4: Ova323-339 nucleotide and peptide sequences.

10 20 30 40 TCA CAA GCT GTT CAT GCT GCA CAT GCA GAA ATT AAT GAA GCA GGT AGA	SEQ ID 19
Ser Gln Ala Val His Ala Ala His Ala Glu Ile Asn Glu Ala Gly Arg S Q A V H A A H A E I N E A G R	20

[0193] The OVA peptide DNA was digested with XhoI/SalI and cloned into the respective XhoI sites in the Spy0128 region (see Table 2) of the pLZ12km_P23R plasmid.

[0194] The amino acid sequences encoding the recombinant Spy0128 protein region of each construct are listed in Table 5, in which the heterologous peptide (OVA peptide or J14 peptide) is indicated in uppercase and bold.

[0195] The recombinant plasmids were electroporated into *Lactococcus lactis* strain MG1363.

Table 5: Sequence of recombinant polypeptides in each construct.

Position of Ova peptide insertion	Sequence	SEQ ID
N-terminus amino acid 28	mklrhllltg aaltsfaatt vhgetvvn**LESQAVHAAHAEINEAGRLD**ga kltvtknldl vnsnalipnt dftfkiepdtt vnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	21
	mklrhllltg aaltsfaatt vhgetvvn **LEKQAEDKVKASREAKKQVEKALEQLEDKVQLDg**a kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	22
βB-C1 loop amino acid 61	mklrhllltg aaltsfaatt vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt **LESQAVHAAHAEINEAGRLD**gnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk	23

	dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	
βD-E loop amino acid 101	mklrhllltg aaltsfaatt vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf **LESQAVHAAHAEINEAGRLD**gv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	24
βE/βF loop amino acid 119	mklrhllltg aaltsfaatt vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee**LESQAVHAAHAEINEAGRLD**gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	25
β2-3 loop amino acid 201	mklrhllltg aaltsfaatt vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka **LESQAVHAAHAEINEAGRLD**sekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	26
β3-4 loop amino acid 217	mklrhllltg aaltsfaatt vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miektt**LESQAVHAAHAEINEAGRLD**pvqteasid qlyhftlkdg	27

	esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	
β9/β10 loop amino acid 276	mklrhllltg aaltsfaatt vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspq**LEKQAEDKVKASREAKKQVEKALEQLEDKVQLD**k niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	28

5. Mouse studies

[0196] The various *L. lactis* PilM1 strains with and without heterologous peptide were grown in GM17 media/Kan200 until an OD of 0.5 was reached. Cells were centrifuged and resuspended in 10% glycerol/PBS at an OD of 20. 0.5 ml aliquots were frozen at -80 ºC. Multiple samples were tested for bacterial enumeration. Western blot of cell wall extracts confirmed pilus expression.

[0197] Female Balb/c mice were vaccinated intranasally (i.n.) by administration of 20 µl of cell suspension (10^9 CFU) into the nostril. The mice were vaccinated with a dose of 10^9 CFU on days 1, 14 and 27. Blood samples collected on day 39 were analyzed. The mice were sacrificed on day 39, and lung lavage fluids were obtained postmortem by injecting and withdrawing 1 ml of PBS into the exposed trachea. The supernatants were then stored at -80°C.

6. Detection of specific antibodies in serum and bronchoalveolar lavage (BAL) fluid.

[0198] A 96-well microplate (Nunc; Thermoscientific) was coated overnight at 4°C with 10 µg ovalbumin per well. The coated plate was blocked with 3% BSA in PBS-Tween for 15 minutes to prevent nonspecific binding. Serum (1:200 dilution) or lung fluid was reacted with the coated wells for 3 hours. Antibody production was detected using anti-mouse IgG or anti-mouse IgA secondary antibodies coupled to alkaline phosphatase (Sigma). Absorbance was measured at 450 nm after 15 min with the addition of the (TMB). Endpoint (day 39) antibody titres were determined in serum samples by using a similar enzyme-linked immunosorbent assay (ELISA), except that the mouse samples were applied from a

serial dilution to the plate. Antibody titers were calculated as the dilution producing the same 450 nm of more than three standard deviations above the mean OD of control wells containing control mouse saliva or sera (obtained from mice immunised with MG1363/PilM1 only).

7. Results

L. lactis comprising M1 pili expressing OVA peptide at the N-terminus of pilus protein Spy0128 [SEQ ID No: 21]

[0199] When the OVA peptide was expressed at the N-terminus of the Spy0128 protein, the pilus was correctly assembled on the cell surface. This is indicated by a high molecular protein ladder due to the covalent attachment of individual pilin monomers as shown in Figure 1A. However, the Ova peptide was barely detectable as shown in Figure 1B.

[0200] No significant serum IgG response was detected in vaccinated mice as shown in Figure 2.

L. lactis comprising M1 pili expressing OVA peptide in βE/βF loop of Spy0128 protein [SEQ ID No: 25]

[0201] When the OVA peptide was expressed at the βE/βF loop of the Spy0128 protein in the M1 pilus, the pilus was correctly assembled on the cell surface as shown in Figure 3A. The OVA peptide was strongly detected by Western blot as shown in Figure 3B, suggesting high expression and little degradation of the OVA peptide when expressed at this site in the Spy0128 protein.

[0202] A significant serum IgG response was detected in vaccinated mice as shown in Figure 4.

[0203] A significant bronchoalveolar lavage (BAL) IgA response was detected in mice vaccinated with the peptide of SEQ ID No: 25 carrying the OVA peptide in the βE/βF loop, as shown in Figure 5.

EXAMPLE 2

[0204] This example demonstrates assembly on the surface of *L. lactis* of M1 pili comprising the OVA peptide inserted in the βE/βF loop or in the β9/β10 loop of the Spy0128 protein.

1. Method

[0205] Ova323-339 was introduced into the βE/βF loop or β9/β10 loop of the Spy0128 protein of the M1 pilus as described for Example 1.

[0206] The amino acid sequences encoding the recombinant Spy0128 protein region of each construct are listed in Table 6, in which the heterologous peptide Ova peptide is indicated in uppercase and bold.

[0207] The recombinant plasmids were electroporated into *Lactococcus lactis* strain MG1363.

Table 6: Sequence of recombinant polypeptides in each construct.

Position of Ova peptide insertion	Sequence	SEQ ID
βE/βF loop amino acid 119	mklrhllltg aaltsfaatt vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeLESQAVHAAHAEINEAGRLDgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	25
β9/β10 loop amino acid 276	mklrhllltg aaltsfaatt vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqLESQAVHAAHAEINEAGRVEk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	45

[0208] Assembly of the pili on the surface of *L. lactis* was determined by Western blot as described for Example 1.

2. Result

[0209] The M1 pilus correctly assembled on the surface of *L. lactis* when the OVA peptide was expressed at either the βE/βF loop or β9/β10 loop of the Spy0128 protein as shown in Figure 7.

EXAMPLE 3

[0210] This example demonstrates assembly on the surface of *L. lactis* of M1 pili comprising antigenic peptides from the influenza virus inserted in the βE/βF loop region of the Spy0128 protein.

1. Method

[0211] The peptide sequences for the following influenza peptides was reverse transcribed to DNA using *L. lactis* codon usage. A 5'- XhoI site and a 3' SalI site were added to the peptide.

- hemagglutinin HA2 peptide (Xu et al. 2005. Comprehensive serological profiling of human populations using a synthetic human virome, *Science* 348:6239),
- hemagglutinin HA3 peptide (CD4 epitope, database 129144 IEDB database at www.iedb.org; Terajima et al., 2013. Cross-reactive human B cell and T cell epitopes between influenza A and B viruses, *Virol J.* 10:244), and
- hemagglutinin M2e (Stepanova et al., 2015. Protection against Multiple Influenza A Virus Strains Induced by Candidate Recombinant Vaccine Based on Heterologous M2e Peptides Linked to Flagellin, *PLoS One* 10(3): e0119520. doi:10.1371/journal.pone.019520).

[0212] The amino acid and nucleotide sequences of the peptides are listed in Tale 6A below.

Table 6A: Insertion peptide nucleotide and peptide sequences.

Peptide and sequence	SEQ ID
HA2 Cttggtcatcacgctgttcctaatggaactttggtaaaaacaattactaatgatcaaattgaagttact Aatgctacagaacttgtacaatcatctagtacaggaagaatttgtgattcaccacatagaattcttgat ggtaaaaattgtactttgatcgatgctctt	46
L G H H A V P N G T L V K T I T N D Q I E V T	39

N A T E L V Q S S S T G R I C D S P H R I L D G K N C T L I D A L	
HA3 Aaaagaggtcttttt<u>ggagctattgcaggtttt</u>atcgaaggaggttggcaa	47
K R G L F G A I A G F I E G G W Q	40
M2e tcacttttaactgaagttgaaaca<u>cctattagaaatgaatgggg</u>ttgtagatgtaatgattcatctgat	48
S L L T E V E T P I R N E W G C R C N D S S D	41

[0213] The peptide DNA was digested with XhoI/SalI and cloned into the respective XhoI sites in the Spy0128 region of the M1 pilus of the pLZ12km_P23R plasmid as described for Example 1.

[0214] The amino acid sequences encoding the recombinant Spy0128 protein region of each construct are listed in Table 7, in which the heterologous peptide is indicated in uppercase and bold. Additional amino acids introduced during cloning as an artefact are underlined. A point mutation in the M2e peptide is indicated in large font.

[0215] The recombinant plasmids were electroporated into *Lactococcus lactis* strain MG1363.

Table 7: Sequence of recombinant polypeptides in each construct.

Peptide and position of insertion	**Sequence**	**SEQ ID**
HA2 βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee**LELGHHAVP NGTLVKTITNDQIEVTNATELVQSSSTGRICDSPHRILDGKNCTLIDALLE**gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	49
HA3 βE/βF loop	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee	50

amino acid 119	**LEKRGLFGAIAGFIEGGWQLEEFLE**gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	
HA3-HA3 βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee **LEKRGL FGAIAGFIEGGWQLEKRGLFGAIAGFIEGGWQLE**gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	51
M2e βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee **LENSGGSLESLL TEVETPIRNEWGCRCNDSSDLEEFRRILE** gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	52
M2e-M2e βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee **LESHL TEVETPIRNEWGCRCNDSSDLKESVGSLESLLTEVETPIRNEWGCRCNDSSDLE** gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	53

[0216] Assembly of the recombinant pili expressing the antigenic peptides on the surface of *L. lactis* was determined by Western blot as described for Example 1.

2. Results

[0217] M1 pili expressing the HA2 peptide, the M2e peptide and the M2e-M2e peptide assembled effectively on the surface of *L. lactis* as shown in Figures 8A, D and E, respectively.

[0218] M1 pili expressing the HA3 peptide and the HA3-HA3 peptide assembled, but somewhat less effectively as shown in Figures 8 B and C, respectively.

EXAMPLE 4

[0219] This example demonstrates assembly on the surface of *L. lactis* of M1 pili comprising antigenic peptides from the *Mycobacterium tuberculosis* inserted in the βE/βF loop or the β9/β10 loop regions of the Spy0128 protein.

1. Method

[0220] The peptide sequences for the following tuberculosis peptides was reverse transcribed using *L. lactis* codon usage. A 5'- XhoI site and a 3' SalI site were added to the peptide.

- ESAT-6, amino acids 1-20,
- ESAT-6, amino acids 51-70 (Olsen *et al,* 2000. Efficient protection against *Mycobacterium tuberculosis* by vaccination with a single subdominant epitope from the ESAT-6 antigen. *Eur. J. Immunol*. 30:1724-32), and
- TBA61 (Williams et al., 2004. Passive protection with immunoglobulin A antibodies against tuberculous early infection of the lungs. *Immunology* 111(3):328-33).

[0221] The amino acid and nucleotide sequences of the peptides are listed in Tale 6A below.

Table 7A: Insertion peptide nucleotide and peptide sequences.

Peptide and sequence	SEQ ID
ESAT-6 1-20 ATGACAGAACAACAATGGAATTTTGCTGGAATTGAAGCTGCAGCTTCAGCTATTCAAGGA	54
M T E Q Q W N F A G I E A A A S A I Q G	33
ESAT-6 51-70	55

TATCAAGGTGTTCAACAAAAATGGGATGCTACTGCAACAGAACTTAATAACGCTTTACAA	
Y Q G V Q Q K W D A T A T E L N N A L Q	42
TBA1 TCTGAATTTGCATATGGTTCATTTGTTCGTACAGTTTCTCTTCCAGTCGGAGCAGATGAA	56
S E F A Y G S F V R T V S L P V G A D E	43

[0222] The peptide DNA was digested with XhoI/SalI and cloned into the respective XhoI sites in the Spy0128 region of the M1 pilus of the pLZ12km_P23R plasmid as described for Example 1.

[0223] The amino acid sequences encoding the recombinant Spy0128 protein region of each construct are listed in Table 8, in which the heterologous peptide is indicated in uppercase and bold.

[0224] The recombinant plasmids were electroporated into *Lactococcus lactis* strain MG1363.

Table 8: Sequence of recombinant polypeptides in each construct.

Peptide and position of insertion	**Sequence**	**SEQ ID**
ESAT-6$_{1-20}$ βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee **LEMTEQQWNFAGIEAAASAIQGLE** gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	57
ESAT-6$_{51-70}$ B9/β10 loop amino acid 276	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspq **LDYQGVQQKWDATATELNNALQVE** k niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	58

TBA61 βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt sntktaefdf dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sevtfekpgv yyykvtee**LESEF AYGSFVRTVSLPVGADELE**gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	59

[0225] Assembly of recombinant pili expressing the antigenic peptides on the surface of *L. lactis* was determined by Western blot as described for Example 1.

2. Results

[0226] The M1 pilus expressing the ESAT-6_{1-20} peptide, ESAT-6_{51-70} peptide and TBA61 peptide assembled on the surface of *L. lactis* as shown in Figures 9A–9C, respectively.

EXAMPLE 5

[0227] This example demonstrates assembly on the surface of *L. lactis* of M1 pili comprising an artificial 41 amino acid peptide.

1. Method

[0228] The peptide sequence for the 41 amino acid peptide [SEQ ID No. X] was reverse transcribed to DNA using *L. lactis* codon usage.

[0229] A 5'- XhoI site and a 3' SalI site were added to the peptide.

[0230] The peptide DNA was cloned into the βE/βF loop region of Spy0128 of the M1 pilus of the pLZ12km_P23R plasmid as described for Example 1.

[0231] The amino acid sequence encoding the recombinant Spy0128 protein region of the construct is outlined in Table 9. The heterologous peptide is indicated in uppercase and bold.

[0232] The recombinant plasmid was electroporated into *Lactococcus lactis* strain MG1363.

Table 9: Sequence of recombinant polypeptide in construct.

Peptide and position of insertion	Sequence	SEQ ID
41 amino acid peptide βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee**LE** **GSVPSEFLEEFRSDPSRKEVFLELLQVLSKEVGNSRNSGRI** **LE**gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	60

[0233] Assembly of recombinant pili expressing the 41 amino acid peptide on the surface of *L. lactis* was determined by Western blot as described for Example 1.

2. Results

[0234] The M1 pilus expressing the 41 amino acid peptide assembled on the surface of *L. lactis* as shown in Figure 10.

EXAMPLE 6

[0235] This example demonstrates assembly on the surface of *L. lactis* of M1 pili comprising a peptide fusion.

1. Preparation of pili construct

[0236] The cloning strategy used to produce a construct that expresses the M1 pilus having comprising a peptide fusion in the βE/βF loop region is shown below.

[0237] A XhoI cleavage site was introduced into the Spy0128 nucleotide region of the M1 pilus operon at the βE/βF loop region according to the method described in Example.

[0238] The peptide sequences for the J14 peptide [SEQ ID No. 31] and the $Ova_{323-339}$ peptide [SEQ ID No. 30] were reverse transcribed to DNA using *L. lactis* codon usage and introducing a 5'- XhoI site and a 3' SalI site.

[0239] The J14 PCR product was digested with XhoI and cloned into pLZ12km P23R to produce a construct expressing the M1 pilus comprising the J14 antigenic peptide at the βE/βF loop region.

[0240] The XhoI restriction site present at the 5' end of the J14 sequence in the construct was exploited to produce a construct expressing the M1 pilus comprising a fusion peptide comprising two antigens.

[0241] The $Ova_{323-337}$ PCR product was digested with XhoI and cloned into the construct to produce the fusion construct.

[0242] The amino acid sequence encoding the recombinant Spy0128 protein region of the construct is listed in Table 10, in which the heterologous peptide is indicated in uppercase and bold. Each antigen is underlined.

[0243] The recombinant plasmid was electroporated into *Lactococcus lactis* strain MG1363.

Cloning strategy used to prepare construct expressing M1 pilus comprising a double peptide fusion.

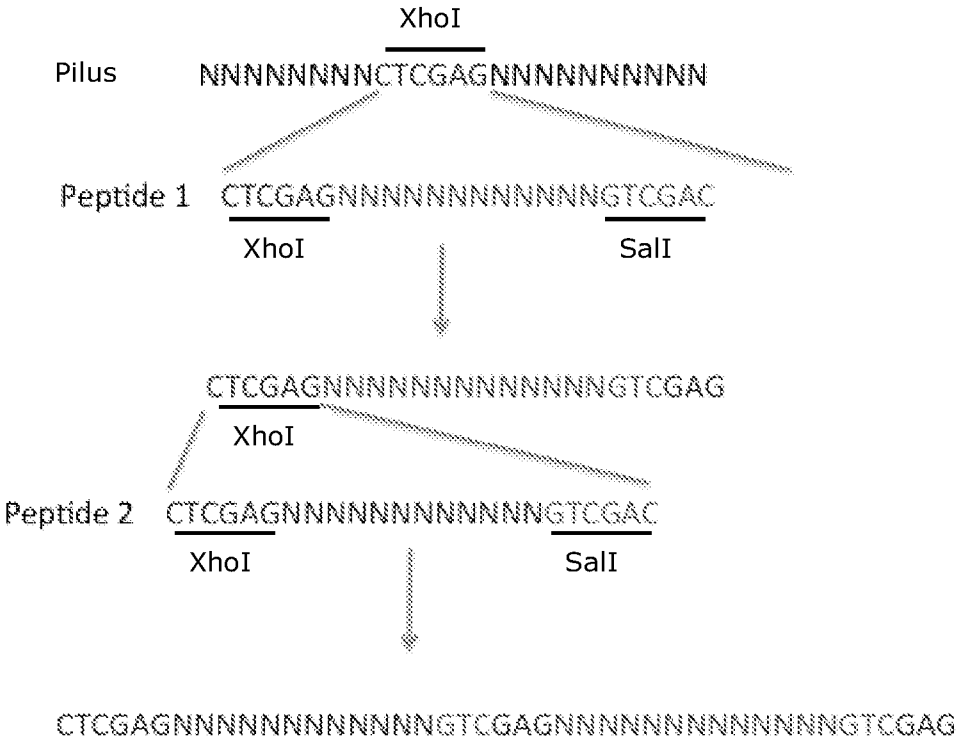

Table 10: Sequence of recombinant polypeptide in construct.

Position of peptide insertion	Sequence	SEQ ID
βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv **yyykvteeLESQAVH AAHAEINEAGRVEKQAEDKVKASREAKKQVEKALEQLEDKVQVEgvsydt tsytvqvhvl wneeqqkpva tyivgykegs** kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	61

[0244] Assembly of recombinant pili expressing the 41 amino acid peptide on the surface of *L. lactis* was determined by Western blot as described for Example 1.

2. Results

[0245] The M1 pilus expressing the fusion peptide assembled on the surface of *L. lactis* as shown in Figure 11.

EXAMPLE 7

[0246] This example describes a cloning strategy for producing a construct expressing peptides of interest at distinct regions of the BG1 protein in the M1 pilus.

[0247] The J14 peptide was introduced into the β9/β10 loop region of Spy0128 in the pLZ12km P23R by introducing a XhoI site according to the method described in Example 1 to produce the construct encoded by SEQ ID No. 28.

[0248] A ClaI cleavage site was then introduced into the βE/βF loop region of the Spy0128 nucleotide region by PCR amplification. Introduction of the ClaI site enables cloning into the βE/βF loop of a PCR product encoding a second peptide that has been digested by ClaI.

[0249] The amino acid sequence encoding the recombinant Spy0128 protein region of the construct is listed in Table 11. The heterologous J14 peptide is indicated in uppercase and bold. The ClaI site is underlined.

Table 11: Sequence of recombinant polypeptide in construct.

Position of peptide insertion	Sequence	SEQ ID
J14 peptide at β9/β10 loop Cla I site at βE/βF loop	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee**ID** gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspq**LEKQAEDKVKASREAKKQVEKALEQLEDKVQLD**k niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	62

EXAMPLE 8

[0250] This example demonstrates assembly on the surface of *L. lactis* of M1 pili comprising a peptide inserted in the βG_1 region of the Spy0128 BP protein.

1. Method

[0251] A XhoI restriction site was introduced between the βG and β1 strand regions of the Spy0128 nucleotide sequence in plasmid pLZ12KmP23R. The XhoI site replaced amino acids 172 and 173 located between the βG and β1 strand regions of the Spy0128 sequence [SEQ ID No. 1].

[0252] A PCR product encoding the $OVA_{323-339}$ peptide sequence was cloned into the plasmid at the XhoI restriction site as described for Example 1.

[0253] The amino acid sequence encoding the recombinant Spy0128 protein region of the construct is listed in Table 12. The heterologous peptide is indicated in uppercase and bold.

Table 12: Sequence of recombinant polypeptide in construct.

Position of peptide insertion	Sequence	SEQ ID
$OVA_{323-339}$ peptide at BG_1 region	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt **tvnedgn**kfk gvalntpmtk	63

	vtytnsdkgg sntktaefdf sevtfekpgv yyykvteeki dkvpgvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl d **LESQAVHAAHAEINEAGRVE** tltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp t**gvamtvapy ialgivavgg alyfvkkkna**	

[0254] Assembly of recombinant pili on the surface of *L. lactis* was determined by Western blot as described for Example 1.

2. Results

[0255] The M1 pilus expressing the $OVA_{323-339}$ peptide in the BG_1 region assembled on the surface of *L. lactis* as shown in Figure 12.

EXAMPLE 9

[0256] This example demonstrates assembly on the surface of *L. lactis* of M18 pili expressing a heterologous peptide.

1. Cloning of the M18 pilus operon

[0257] The protein sequence of the BP pilus protein of GAS strain MGAS8232 (serotype M18, GenBank: AAL96938.1) was used to generate a structure model. This was achieved by using the Swiss-PDB modeller (swissmodel.expasy.org) and the crystal structure of Spy0128 (3B2M) as a template. The βEF loop region in M18_Spy0128 was identified by superimposing the model structure with the Spy0128 crystal structure and by aa sequence alignment using ClustalW (genome.jp/tools/clustalw/)

[0258] The nucleotide sequence of the M18 pilus operon is provided as SEQ ID No. 64.

[0259] The sequence of the M18 pilus BP protein was aligned with the M1 pilus operon using Clustal W software. The sequence alignment is shown in Figure 12.

[0260] The pilus operon [SEQ ID No: 64] was PCR amplified from GAS strain MGAS8232 (serotype M18) using the primers listed in Table 12A.

Table 12A: List of primers used to amplify M18 pilus operon from GAS strain MGAS8232 (serotype M18).

Primer name	Sequence	SEQ ID
PilM18_Bam.fw	gcggatcc gaccatatacacggttgcagga	65
PilM18_Pst.rev	cggctgcag tcttatatggtacaaaaacctatcagc	66

2. Preparation of a construct encoding the M18 pilus operon comprising the $OVA_{323-339}$ peptide

[0261] The recombinant plasmid was first introduced into *E. coli* by heat-shock. Purified plasmid DNA was then electroporated into *Lactococcus lactis* strain MG1363.

[0262] To introduce a XhoI cleavage site into the βE/βF loop region of the M18 Spy0128 nucleotide region of the M18 pilus operon, the plasmid pLZ12KmP23R comprising the pilus operon was PCR amplified using the primers listed in Table 12B.

Primer name	Sequence	SEQ ID
M18_βEF_Xho.fw	5′ gagagaga ctcgagTAGCTTATGATTCTCAAC 3′	67
M18_βEF_Xho.rev	5′ gagagaga ctcgag TACTTCAGAAACCGTATAAC 3′	68

[0263] The PCR product was digested with XhoI and re-cloned into pLZ12km_P23R.

[0264] $OVA_{323-339}$ peptide DNA was digested with XhoI/SalI and cloned into the XhoI sites in the M18 pilus of the pLZ12km_P23R plasmid as described for Example 1.

[0265] The amino acid sequences encoding the recombinant M18 Spy0128 protein region of each construct are listed in Table 13, in which the heterologous peptide is indicated in uppercase and bold. Clones were prepared encoding a single $OVA_{323-339}$ and double $OVA_{323-339}$ peptide.

[0266] The recombinant plasmids were electroporated into *Lactococcus lactis* strain MG1363.

Table 13: Sequence of recombinant polypeptides in each construct.

Peptide and position of insertion	Sequence	SEQ ID
$OVA_{323-339}$	MKKNKLLLATAILATALGTASLNQNVKAETAGVIDGSTLVVKKTFPSYTD DKVLMPKADYTFKVEADDNAKGKTKDGLDIKPGVIDGLENTKTIHYGNSD	69

βE/βF loop	KTTAKEKSVNFDFANVKFPGVGVYRYTVSEV**LESQAVHAAHAEINEAGRVE** IAYDSQQWTVDVYVVNREDGGFEAKYIVSTEGGQSDKKPVLFKNFFDTTS LKVTKKVTGNTGEHQRSFSFTLLLTPNECFEKGQVVNILQGGETKKVVIGE EYSFTLKDKESVTLSQLPVGIEYKVTEEDVTKDGYKTSATLKDGDVTDGYNL GDSKTTDKSTDEIVVTNKRDTQVPTGVVGTLAPFAVLSIVAIGGVIYITKRKK	
$OVA_{323-339}$ double peptide βE/βF loop	MKKNKLLLATAILATALGTASLNQNVKAETAGVIDGSTLVVKKTFPSYTD DKVLMPKADYTFKVEADDNAKGKTKDGLDIKPGVIDGLENTKTIHYGNSD KTTAKEKSVNFDFANVKFPGVGVYRYTVSEV**LESQAVHAAHAEINEAGRVE** **SQAVHAAHAEINEAGRVE**IAYDSQQWTVDVY VVNREDGGFEAKYIVSTEGGQSDKKPVLFKNFFDTTSLKVTKKVTGNTGE HQRSFSFTLLLTPNECFEKGQVVNILQGGETKKVVIGEEYSFTLKDKESV TLSQLPVGIEYKVTEEDVTKDGYKTSATLKDGDVTDGYNLGDSKTTDKST DEIVVTNKRDTQVPTGVVGTLAPFAVLSIVAIGGVIYITKRKK	70

3. Assembly of the M18 pilus on the surface of *L. lactis*

[0267] Assembly of the recombinant pili on the surface of *L. lactis* was determined by Western blotting according to the protocol described in Example 1. Blots were probed with specific anti-M18 Spy0128 serum (generated in a rabbit after immunization with purified recombinant Spy0128).

[0268] The M18 pilus expressing the $OVA_{323-339}$ peptide and the pilus expressing the double peptide both assembled on the surface of *L. lactis* as demonstrated by the Western blot shown in Figure 14.

[0269] Expression of the pilus on the surface of *L. lactis* was quantified by flow cytometry of transformed *L. lactis* using an antibody against M18_Spy0128 using the method described in Example 11.

[0270] The results are shown in Figure 15 and Table 14 below. The results show a significant shift in cells transformed with the constructs described above compared with untransformed *L. lactis* indicating assembly of the pilus on the cell surface.

Table 14: Flow cytometry using an anti-M18_Spy0128 antibody

	Single cell MFI	**Pilus positive %**	**Pilus positive MFI**
L lactis (untransformed)	19.4	0.055 %	n/a
PilVax-M18-Ova-OVa	224	26.0 %	561
PilVax-M18-Ova	638	58.9 %	995
Unstained	19.7	0 %	n/a

2nd only	13.2	0 %	n/a

EXAMPLE 10

[0271] This example quantifies the amount of recombinant M1 pili described herein on the surface of *L. lactis*.

1. Method

[0272] L. lactis were transformed as described above with constructs expressing the recombinant pili set out in Table 15.

[0273] *L. lactis* culture was diluted to OD_{600nm} = 0.4 and incubated in blocking buffer (PBS/3% FBS/5 mM EDTA) on ice for 30 min. The cells were washed with FACS buffer (PBS/1% FBS/ 5mM EDTA) and incubated with anti-Spy0128 polyclonal antibodies on ice for 30 min. The cells were washed again in FACS buffer, then incubated with FITC-conjugated anti-rabbit IgG antibody on ice for 30 min. After extensive washing with FACS buffer, the cells were fixed in 2% paraformaldehyde (in PBS) at 37°C for 10 min. The FITC signals were analysed on a LSRII flow cytometer (Becton Dickinson), and 10,000 events were collected for each experiment. Controls of unstained cells or cells only treated with secondary antibody were used to define positive FITC signals. MFI (mean fluorescence intensity) and percentage of pilus-positive population were calculated by FlowJo software.

2. Results

[0274] The results are shown in Figure 16 and Table 15 below.

[0275] The results show that the transformed *L. lactis* expressed recombinant pili on the cell surface.

Table 15: Flow cytometry using an α-spy0128 antibody

#	Sample/strain Example ref	Bacteria single cell MFI	Pilus positive percentage	Pilus positive population MFI
1	Pilvax-βG1_Ova Example 8	189	29%	3845
2	PilVax-βE/F-AP Example 5	256	34.4%	622
3	PilVax-βE/F-M2eM2e Example 3	635	65.7 %	868

4	PilVax-βE/F-M2e Example 3	681	74.4 %	862
5	PilVax-βE/F-HA3HA3 Example 3	384	52.0 %	632
6	PilVax-βE/F-HA3 Example 3	14.6	0.072 %	502
7	PilVax-βE/F-HA2 Example 3	130	15.1 %	492
8	PilVax-βE/F-J14Ova Example 5	629	69.9 %	839
9	PilVax-βE/F-Ova Example 2	1291	97.7 %	1318
10	L. lactis_PilM1 (no peptide insert)	3267	99.1 %	3297
11	L lactis WT (untransformed)	46.4	0.20 %	2509
12	2nd antibody only control	29.3	0.31 %	2701
13	Unstained control	19.7	0.021 %	251

EXAMPLE 11

[0276] This example demonstrates serum IgG and BAL IgA in mice vaccinated with transformed *L. lactis* described herein.

1. Method

[0277] Mice were vaccinated with *L. lactis* PilM1 strains displaying the M2e or M2eM2e peptides (described in Example 3) using the protocol described in Example 1.

[0278] Purified GST-M2e recombinant protein diluted in PBS to a concentration of 1 µg/ml was used to coat a 96-well Maxisorb plate (Nunc) plate overnight at 4°C (100 µl/ well). The plate was washed in PBS-T (PBS/0.05% Tween-20) to remove excess antigens, then blocked with 3% BSA (w/v) in PBS-T at room temperature for 15 min. A 4-fold serial dilution of mouse sera (starting from 1:200) or BAL fluid (starting from neat) was prepared in PBS, added to the plate and incubated at room temperature for 3 h. The plate was washed extensively in PBS-T then probed with HRP-conjugated anti rabbit IgG or IgA secondary antibody. The bound complexes were detected with TMB substrate (Pierce) and

the colorimetric reaction was developed in the dark. The reaction was stopped by adding 1 M HCl, and the absorbance at 450 nm was measured on an EnSpire Multimode plate reader (Perkin Elmer).

[0279] Control wells with no protein added, or with pre-immune sera were used to define background absorbance, which was subtracted from the measurements of experiment wells. The cut-off value was defined as mean + 3 standard deviations of the absorbance of control wells.

[0280] The endpoint titre was determined as the dilution that produced an absorbance higher than the cut-off value.

2. Result

[0281] The results for the M2e and M2eM2e strains are shown in Figures 17 and 18, respectively.

[0282] For both strains, a strong serum IgG response was observed against Spy0128 antigen, indicating successful delivery of the vaccine. An endpoint titre against Spy0128 in serum IgG of 1:204,800 was achieved with both strains.

[0283] For the M2e strain, endpoint titres of 1:800 in serum IgG and 1:256 against M2e antigen were achieved.

[0284] For the M2eM2e strain, endpoint titres of 1:51,200 in serum IgG and 1:4,096 against M2e antigen were achieved.

EXAMPLE 12

[0285] This example demonstrates assembly on the surface of *L. lactis* of M1 pili comprising a B16 peptide.

1. Method

[0286] The peptide sequence for the B16 peptide was reverse transcribed to DNA using *L. lactis* codon usage.

[0287] The amino acid and nucleotide sequences of the B-16 peptide is listed in Tale 15A below.

Table 15A: Insertion peptide nucleotide and peptide sequence.

Peptide and sequence	SEQ ID
B16 ACAGAATGGACTTCATCTAATGTTATGGAAGAGCGTAAAATTAAAGTT	71
T E W T S S N V M E E R K I K V	72

[0288] A 5′- XhoI site and a 3′ SalI site were added to the peptide.

[0289] The peptide DNA was cloned into the βE/βF loop region of Spy0128 of the M1 pilus of the pLZ12km_P23R plasmid as described for Example 1.

[0290] The amino acid sequence encoding the recombinant Spy0128 protein region of the construct is outlined in Table 16. The heterologous peptide is indicated in uppercase and bold.

[0291] The recombinant plasmid was electroporated into *Lactococcus lactis* strain MG1363.

Table 16: Sequence of recombinant polypeptide in construct.

Peptide and position of insertion	**Sequence**	**SEQ ID**
B16 peptide βE/βF loop amino acid 119	**mklrhllltg aaltsfa**att vhgetvvnga kltvtknldl vnsnalipnt dftfkiepdt tvnedgnkfk gvalntpmtk vtytnsdkgg sntktaefdf sevtfekpgv yyykvtee**LETEW TSSNVMEERKIKVLE**gvsydt tsytvqvhvl wneeqqkpva tyivgykegs kvpiqfknsl dsttltvkkk vsgtggdrsk dfnfgltlka nqyykasekv miekttkggq apvqteasid qlyhftlkdg esikvtnlpv gvdyvvtedd yksekyttnv evspqdgavk niagnsteqe tstdkdmtit ftnkkdfevp tgvamtvapy ialgivavgg alyfvkkkna	73

[0292] Assembly of recombinant pili expressing the B16 peptide on the surface of *L. lactis* was determined by Western blot as described for Example 1.

2. Results

[0293] The M1 pilus expressing the B16 peptide assembled on the surface of *L. lactis* as shown in Figure 19.

INDUSTRIAL APPLICATION

[0294] The apparatus and methods of the invention have utility for therapeutic applications, particularly vaccination, including vaccination against pathogenic agents such as viruses and bacteria, and immunological treatments of conditions such as cancers.

CLAIMS

1. An isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising a backbone pilin (BP) protein or fragment thereof and one or more peptides of interest, wherein the peptide of interest is heterologous to the BP protein.

2. An isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising a backbone pilin (BP) protein or fragment thereof and one or more peptides of interest, wherein the peptide of interest is not a BP protein or fragment thereof.

3. An isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising a backbone pilin (BP) protein or fragment thereof and one or more peptides of interest, wherein the peptide of interest is not a pilus protein or fragment thereof.

4. An isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising a backbone pilin (BP) protein or fragment thereof and one or more peptides of interest, wherein the peptide of interest is not a *Streptococcus* protein or fragment thereof.

5. An isolated, purified or recombinant polypeptide comprising one or more amino acid sequences comprising one or more peptides of interest inserted into or fused to a backbone pilin (BP) protein or fragment thereof and, wherein the peptide of interest is heterologous to the BP protein.

6. The polypeptide of any one of the preceding claims wherein the BP protein is a BP protein derived from a Gram positive bacterium.

7. The polypeptide of any one of the preceding claims wherein the BP protein is a *Streptococcus* BP protein.

8. The polypeptide of any one of the preceding claims wherein the BP protein is a Group A *Streptococcus* BP protein.

9. The polypeptide of any one of the preceding claims wherein the BP protein is encoded within the FCT-1, FCT-2, FCT-3, FCT-4, FCT-5, FCT-6 or FCT-9 nucleotide region of a Group A *Streptococcus* genome.

10. The polypeptide of any one of the preceding claims wherein the BP protein is encoded within the FCT-2, FCT-3 or FCT-4 nucleotide region of a Group A *Streptococcus* genome.

11. The polypeptide of any one of the preceding claims wherein the BP protein is a *Streptococcus pyogenes* BP protein.

12. The polypeptide of any one of the preceding claims wherein the BP protein is derived from Group A *Streptococcus* serotype M1, M2, M3, M4, M5, M6, M9, M11, M12, M18, M22, M23, M28, M33, M44, M49 (2), M50, M53, M75, M77, M78, M89.

13. The polypeptide of any one of the preceding claims wherein the BP protein is derived from Group A *Streptococcus* serotype M1, M3, M5, M9, M11, M12, M18, M22, M28, M33, M44, M49 (2), M50, M53, M77, M78, M89.

14. The polypeptide of any one of the preceding claims wherein the BP protein has at least about 80% amino acid sequence identity to the polypeptide sequence of SEQ ID No 1.

15. The polypeptide of any one of the preceding claims wherein the BP protein is encoded by a nucleotide coding sequence selected from the group consisting of
    a. Genbank Accession Number EU725506.1 (GI:198417284)
    b. any one of Genbank Accession Numbers KJ816940 –KJ817040,
    c. Genbank Accession Number 2940764
    d. Genbank Accession Number NP_268517
    e. Genbank Accession Number KJ816977
    f. Genbank Accession Number KJ817010
    g. Genbank Accession Number KJ817040
    h. Genbank Accession Number KJ816969
    i. Genbank Accession Number 3573111
    j. Genbank Accession Number KJ816976
    k. Genbank Accession Number KJ816959
    l. Genbank Accession Number KJ816971
    m. Genbank Accession Number KJ816965
    n. Genbank Accession Number KJ816984

o. Genbank Accession Number KJ816948
p. Genbank Accession Number KJ816943
q. Genbank Accession Number KJ816940
r. Genbank Accession Number KJ816995
s. Genbank Accession Number 4067256, and
t. Genbank Accession Number 4063969.

16. The polypeptide of any one of the preceding claims wherein the BP protein comprises, consists of, or consists essentially of, an amino acid sequence selected from the group consisting of
a. Genbank Accession Number ACH87870.1 (GI:198417285),
b. any one of Genbank Accession Numbers KJ816940 –KJ817040,
c. Genbank Accession Number 2940764
d. Genbank Accession Number NP_268517
e. Genbank Accession Number KJ816977
f. Genbank Accession Number KJ817010
g. Genbank Accession Number KJ817040
h. Genbank Accession Number KJ816969
i. Genbank Accession Number 3573111
j. Genbank Accession Number KJ816976
k. Genbank Accession Number KJ816959
l. Genbank Accession Number KJ816971
m. Genbank Accession Number KJ816965
n. Genbank Accession Number KJ816984
o. Genbank Accession Number KJ816948
p. Genbank Accession Number KJ816943
q. Genbank Accession Number KJ816940
r. Genbank Accession Number KJ816995
s. Genbank Accession Number 4067256, and
t. Genbank Accession Number 4063969.

17. The polypeptide of any one of the preceding claims wherein the BP protein comprises
a. a first domain having at least about 80% amino acid sequence identity to the polypeptide sequence of the C-terminal domain of Spy0128 (amino acids 174– 340 of SEQ ID No. 1), and/or
b. a second domain having at least about 80% amino acid sequence identity to the polypeptide sequence of the N-terminal domain of Spy0128 (amino acids 1–171 of SEQ ID No. 1).

18. The polypeptide of any one of the preceding claims wherein the amino acid sequence comprises the at least one peptide inserted at a site within the C-terminal domain of the BP protein.

19. The polypeptide of any one of the preceding claims wherein the amino acid sequence comprises the at least one peptide inserted at a site within the N-terminal domain of the BP protein.

20. The polypeptide of any one of the preceding claims wherein the at least one peptide of interest is inserted:
    a. in a region between the βE and βF loop regions of the BP protein, for example, corresponding to amino acids 119–124 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1, and/or
    b. in a region between the β9 and β10 loop regions of the BP protein, for example, corresponding to amino acids 276–279 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1,
    c. in a region between the βB and βC1 loop regions of the BP protein, for example, corresponding to amino acids 61–65 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1,
    d. in a region between the β3 and β4 loop regions of the BP protein, for example, corresponding to amino acids 217–221 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1, and/or
    e. in a region between the β2 and β3 loop regions of the BP protein, for example, corresponding to amino acids 201-206 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1, and/or
    f. in a region between the βD and βE loop regions of the BP protein, for example, corresponding to amino acids 101–108 of SEQ ID No: 1, and/or an analogous region in a BP protein having greater than 90% identity to the amino acid sequence of SEQ ID No: 1.

21. The polypeptide of any one of the preceding claims wherein the amino acid sequence comprises the polypeptide of interest inserted:
    a. in a region between the βE and βF loop regions of the BP protein, for example, corresponding to amino acids 119–124 of SEQ ID No: 1, and

b. in a region between the β9 and β10 loop regions of the BP protein, for example, corresponding to amino acids 276–279 of SEQ ID No: 1,

22. The polypeptide of any one of the preceding claims wherein the amino acid sequence comprises:
    a. a first peptide of interest inserted in a region between the βE and βF loop regions of the BP protein, for example, corresponding to amino acids 119–124 of SEQ ID No: 1, and
    b. a second peptide of interest inserted in a region between the β9 and β10 loop regions of the BP protein, for example, corresponding to amino acids 276–279 of SEQ ID No: 1.

23. The polypeptide of any one of the preceding claims wherein the polypeptide further comprises an amino acid sequence encoding a pilin tip protein (AP1 protein), an AP2 protein, a Sip protein, a sortase or a combination of any two or more thereof.

24. The polypeptide of any one of the preceding claims wherein the polypeptide further comprises amino acid sequences encoding a pilin tip protein (AP1 protein), an AP2 protein, a Sip protein, and a sortase.

25. The polypeptide of any one of the preceding claims wherein the polypeptide further comprises amino acid sequences encoding proteins required to form a pilus when the polypeptide is expressed in a bacterium.

26. The polypeptide of any one of the preceding claims wherein the one or more peptides of interest comprise an antigenic peptide, an enzyme, or an antibody or fragment thereof.

27. The polypeptide of any one of the preceding claims wherein the one or more peptides of interest comprise a T cell antigenic peptide or epitope/fragment thereof or a B cell antigenic peptide or epitope/fragment thereof.

28. The polypeptide of any one of the preceding claims wherein the one or more peptides of interest comprises two or more antigenic peptides or epitopes.

29. The polypeptide of any one of the preceding claims wherein the one or more peptides of interest are derived from a microorganism, for example, a virus, a bacterium, a parasite…

30. The polypeptide of any one of the preceding claims wherein the one or more peptides of interest are capable of eliciting an IgA response.

31. The polypeptide of any one of the preceding claims wherein the one or more peptides of interest comprise a tumour antigenic peptide.

32. The polypeptide of any one of the preceding claims wherein the one or more peptides of interest comprise 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, or 10 or more contiguous amino acids that do not encode a BP protein or fragment thereof.

33. The polypeptide of any one of the preceding claims wherein the peptide of interest comprises, consists of, or consists essentially of an amino acid sequence selected from the group consisting of
    a. 8 or more contiguous amino acids from the sequence SQAVHAAHAEINEAGRI [SEQ ID No. 30], or
    b. 8 or more contiguous amino acids from the sequence KQAEDKVKASREAKKQVEKALEQLEDKVQ [SEQ ID No. 31].

34. The polypeptide of any one of the preceding claims wherein the peptide of interest comprises, consists of, or consists essentially of an amino acid sequence selected from the group consisting of
    a. 8 or more contiguous amino acid residues from the Mtb antigen 85B precursor peptide,
    b. 8 or more contiguous amino acids from the amino acid sequence FQDAYNAAGGHNAVF [SEQ ID No: 32, I-A(b) Mtb antigen 85B precursor peptide amino acids 280–294],
    c. 8 or more contiguous amino acid residues from the Mtb antigen ESAT-6,
    d. 8 or more contiguous amino acids from the amino acid sequence MTEQQWNFAGIEAAASAIQG [SEQ ID No: 33, I-A(b) Mtb ESAT-6 amino acids 1-20],
    e. 8 or more contiguous amino acids from the amino acid sequence GAPINSATAM [SEQ ID No: 34, I-A(b) Mtb ESAT-6 amino acids 309 - 318].

35. The polypeptide of any one of the preceding claims wherein the peptide of interest comprises, consists of, or consists essentially of an amino acid sequence selected from the group consisting of
    a. 8 or more contiguous amino acids from the sequence SIINFEKL [SEQ ID No. 35],

b. 8 or more contiguous amino acids from the sequence TEWTSSNVMEERKIKV [SEQ ID No. 36], or
c. 8 or more contiguous amino acids from the sequence SPSYVYHQF [SEQ ID No. 37].

36. The polypeptide of any one of the preceding claims wherein the polypeptide, when expressed in a bacterium, assembles to form a pilus on the surface of the bacterium.

37. A recombinant polypeptide comprising the amino acid sequence of SEQ ID No. 1 wherein one or more, for example, 1–15 contiguous amino acids have been deleted at one or more of the following sites:
a. from amino acid position 61,
b. from amino acid position 101,
c. from amino acid position 119,
d. from amino acid position 201,
e. from amino acid position 217, and/or
f. from amino acid position 276;
and/or
wherein one or more of the following sequences of contiguous amino acids have been deleted:
g. amino acids 61–65,
h. amino acids 101–108,
i. amino acids 119–124,
j. amino acids 201–206,
k. amino acids 217–221, and/or
l. amino acids 276–279;
and, optionally, wherein two or more contiguous amino acids are inserted at the site or sites of the deleted contiguous amino acids.

38. A recombinant polypeptide comprising an amino acid sequence having at least 90% sequence identity with SEQ ID No. 1 wherein one or more, for example, 1–15 contiguous amino acids have been deleted at one or more of the following sites:
a. from an amino acid position corresponding to amino acid 61 of SEQ ID No. 1,
b. from an amino acid position corresponding to amino acid 101 of SEQ ID No. 1,
c. from an amino acid position corresponding to amino acid 119 of SEQ ID No. 1,
d. from an amino acid position corresponding to amino acid 201 of SEQ ID No. 1,
e. from an amino acid position corresponding to amino acid 217 of SEQ ID No. 1, and/or

f. from an amino acid position corresponding to amino acid 276 of SEQ ID No. 1;

and/or

wherein one or more of the following sequences of contiguous amino acids have been deleted:

g. an amino acid sequence corresponding to amino acids 61–65 of SEQ ID No. 1,
h. an amino acid sequence corresponding to amino acids 101–108 of SEQ ID No. 1,
i. an amino acid sequence corresponding to amino acids 119–124 of SEQ ID No. 1,
j. an amino acid sequence corresponding to amino acids 201–206 of SEQ ID No. 1,
k. an amino acid sequence corresponding to amino acids 217–221 of SEQ ID No. 1, and/or
l. an amino acid sequence corresponding to amino acids 276–279 of SEQ ID No. 1;

and, optionally, wherein two or more contiguous amino acids are inserted at the site or sites of the deleted contiguous amino acids.

39. The recombinant polypeptide of claim 38 wherein the one or more inserted amino acids result from the formation of a restriction enzyme cleavage site.]

40. The recombinant polypeptide of claim 39 further comprising one or more peptides of interest inserted at the site or sites of the deleted amino acids.

41. A recombinant polypeptide comprising the amino acid sequence of SEQ ID No. 1 and comprising a substitution of 1 to 6, for example, 4 contiguous amino acids for a sequence of 1 to 15 contiguous amino acids of the native sequence at one or more of the following sites:

a. from an amino acid position corresponding to amino acid 61 of SEQ ID No. 1,
b. from an amino acid position corresponding to amino acid 101 of SEQ ID No. 1,
c. from an amino acid position corresponding to amino acid 119 of SEQ ID No. 1,
d. from an amino acid position corresponding to amino acid 201 of SEQ ID No. 1,
e. from an amino acid position corresponding to amino acid 217 of SEQ ID No. 1, and/or
f. from an amino acid position corresponding to amino acid 276 of SEQ ID No. 1;

and/or

comprising a substitution of 1 to 6, for example, 4 contiguous amino acids for one or more of the following sequences of contiguous amino acids in SEQ ID No.1:

a. amino acids 61–65,
b. amino acids 101–108,
c. amino acids 119–124,
d. amino acids 201–206,
e. amino acids 217–221, and/or
f. amino acids 276–279.

42. A recombinant polypeptide comprising an amino acid sequence having at least 90% sequence identity with SEQ ID No. 1 and comprising a substitution of 1 to 6, for example, 4 contiguous amino acids for a sequence of 1 to 15 contiguous amino acids of the native sequence at one or more of the following sites:

a. from amino acid position 61,
b. from amino acid position 101,
c. from amino acid position 119,
d. from amino acid position 201,
e. from amino acid position 217, and/or
f. from amino acid position 276;

and/or

comprising a substitution of 1 to 6, for example, 4 contiguous amino acids for one or more of the following sequences of contiguous amino acids in SEQ ID No.1:

a. an amino acid sequence corresponding to amino acids 61–65 of SEQ ID No. 1,
b. an amino acid sequence corresponding to amino acids 101–108 of SEQ ID No. 1,
c. an amino acid sequence corresponding to amino acids 119–124 of SEQ ID No. 1,
d. an amino acid sequence corresponding to amino acids 201–206 of SEQ ID No. 1,
e. an amino acid sequence corresponding to amino acids 217–221 of SEQ ID No. 1, and/or
f. an amino acid sequence corresponding to amino acids 276–279 of SEQ ID No. 1.

43. A recombinant polypeptide of any one of the preceding claims comprising one or more of the following amino acid substitutions:

a. amino acids LELD for amino acids 61–65,
b. amino acids LELD for amino acids 101–108,
c. amino acids LELD for amino acids 119–124,
d. amino acids LELD for amino acids 201–206,
e. amino acids LELD for amino acids 217–221, and/or
f. amino acids LELD for amino acids 276–279.

44. The recombinant polypeptide of any one of the preceding claims further comprising one or more peptides of interest inserted at the substitution site or sites between the LE and LD residues.

45. An isolated, purified, or recombinant pilus comprising a plurality of covalently attached peptides of interest wherein the peptides of interest are heterologous to the pilus.

46. An isolated, purified, or recombinant pilus comprising one or more repeating/polymerised polypeptide subunits each subunit comprising a backbone pilin (BP) protein and one or more peptides of interest, wherein the peptides of interest are heterologous to the BP protein.

47. A genetically engineered bacterium comprising at least one recombinant pilus, each pilus comprising a plurality of covalently attached peptides of interest, wherein the peptides of interest are heterologous to the pilus [and the bacterium].

48. A genetically engineered bacterium comprising at least one recombinant pilus, each pilus comprising one or more repeating/polymerised polypeptide subunits each subunit comprising a backbone pilin (BP) protein and one or more peptides of interest, wherein the peptides of interest are heterologous to the BP protein, or heterologous to the bacterium.

49. A genetically engineered bacterium comprising a recombinant nucleic acid capable of encoding a polypeptide capable of forming one or more pili on the surface of the bacterium, the nucleic acid encoding one or more repeating polypeptide subunits each subunit comprising a backbone pilin (BP) protein and one or more peptides of interest, wherein the peptides of interest are heterologous to the BP protein [and the bacterium].

50. The pilus or bacterium of any of the preceding claims wherein the bacterium is a Gram positive bacterium.

51. The pilus or bacterium of any of the preceding claims wherein the bacterium belongs to a genus selected from the group comprising *Bifidobacterium, Lactobacillus, Lactococcus,* and *Streptococcus*.

52. The pilus or bacterium of any of the preceding claims wherein the bacterium is an attenuated pathogenic bacterium, a non-pathogenic bacterium or a generally regarded as safe (GRAS) bacterium.

53. A pharmaceutical composition comprising an effective amount of one or more polypeptides, pili or bacteria of any one of the preceding claims.

54. The composition of claim 53 wherein the composition is an immunogenic composition.

55. A vaccine composition comprising an effective amount of one or more polypeptides, pili or bacteria of any one of the preceding claims.

56. The composition of claim 55 additionally comprising an adjuvant.

57. A method of vaccinating or eliciting an immune response in a subject comprising administering to the subject an effective amount of a pharmaceutical composition or vaccine composition of any one of the preceding claims.

58. A method of vaccinating or eliciting an immune response in a subject comprising administering to the subject an effective amount of a polypeptide, pilus or bacterium of any one of the preceding claims.

59. Use of a polypeptide, pilus or bacterium of any of the preceding claims in the manufacture of a medicament for vaccinating or eliciting an immune response in a subject in a subject in need thereof.

60. A polypeptide, pilus or bacterium of any of the preceding claims for use in vaccinating or eliciting an immune response in a subject in a subject in need thereof.

61. A method for producing a genetically engineered bacterium comprising at least one pilus, each pilus comprising a plurality of covalently attached peptides of interest, the method comprising
    a) introducing into said bacterium a polynucleotide comprising a polynucleotide sequence comprising a nucleotide sequence encoding a backbone pilin (BP) protein and one or more peptides of interest, wherein the peptide of interest is heterologous to the BP protein; and
    b) growing said bacterium under conditions wherein said polypeptide is expressed and said pilus is formed.

62. The method of any of the preceding claims wherein the peptide of interest is heterologous to the bacterium.

63. The method of any of the preceding claims wherein the polynucleotide further comprises a nucleotide sequence encoding a pilin tip protein (AP1 protein), an AP2 protein, a sortase or a combination of any two or more thereof.

64. The method of any of the preceding claims wherein the polynucleotide further comprises a promoter that is active in the bacterium, and wherein the promoter is operably linked to the polynucleotide encoding said polypeptide.

65. The method of any of the preceding claims wherein said promoter drives constitutive expression of the polypeptide in the bacterium.

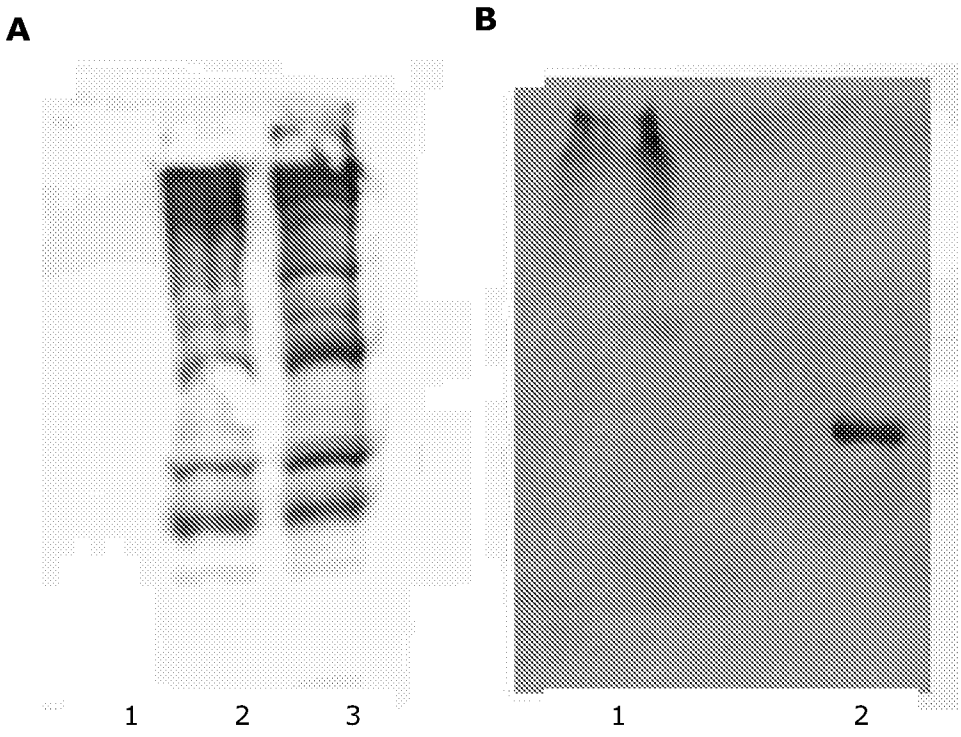

Figure 1

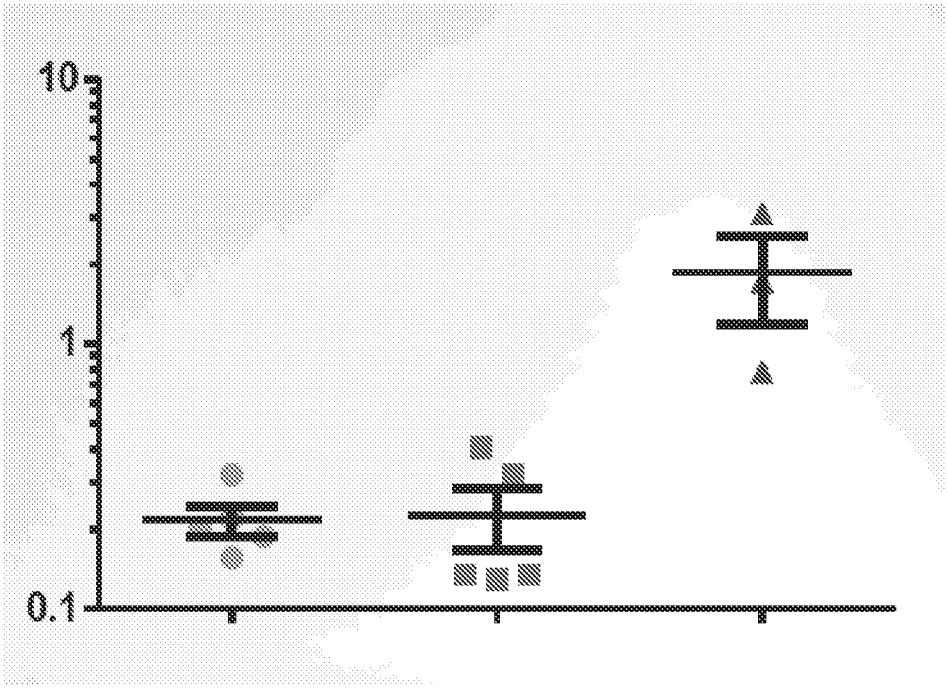

Figure 2

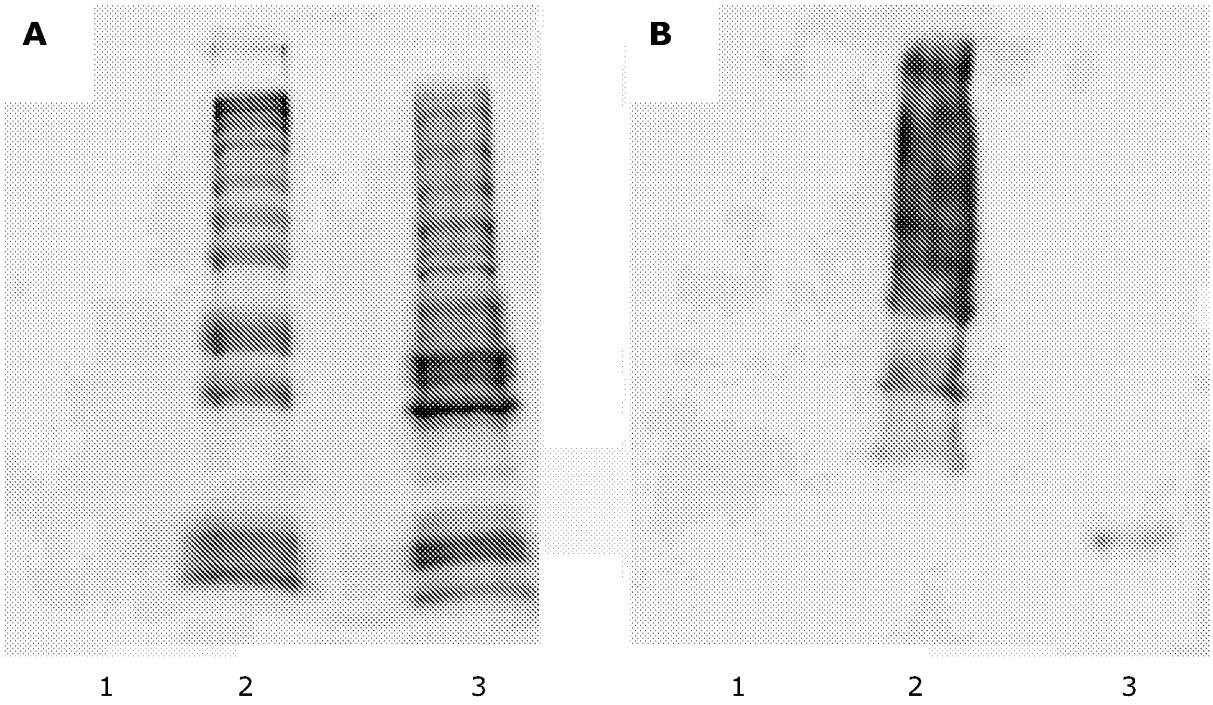

Figure 3

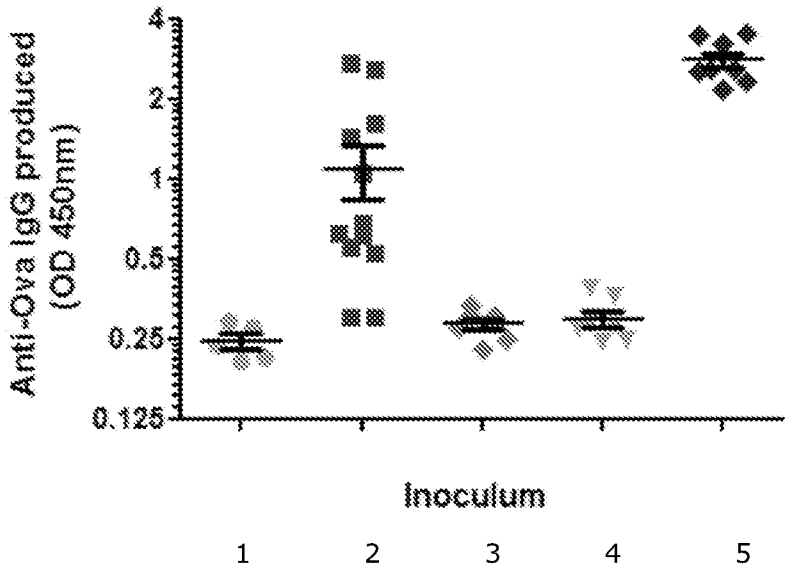

Figure 4

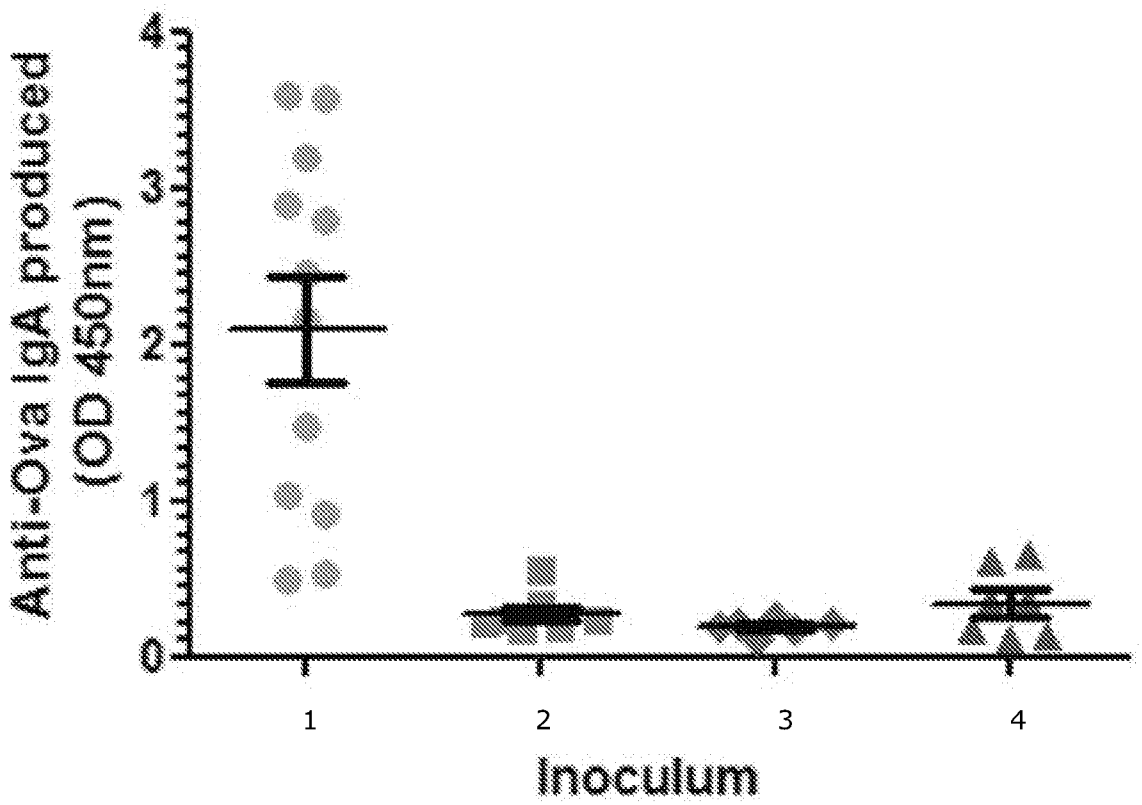

Figure 5

```
          10          20          30          40          50          60
ATG AAA TTA CGT CAC TTA CTA TTA ACG GGA GCA GCC CTA ACT AGT TTT GCT GCT ACA ACA
M   K   L   R   H   L   L   L   T   G   A   A   L   T   S   F   A   A   T   T

          70          80          90         100         110         120
GTT CAC GGG GAG ACT GTT GTA AAC GGA GCC AAA CTA ACA GTT ACA AAA AAC CTT GAT TTA
V   H   G   E   T   V   V   N   G   A   K   L   T   V   T   K   N   L   D   L

         130         140         150         160         170         180
GTT AAT AGC AAT GCA TTA ATT CCA AAT ACA GAT TTT ACA TTT AAA ATC GAA CCT GAT ACT
V   N   S   N   A   L   I   P   N   T   D   F   T   F   K   I   E   P   D   T

         190         200         210         220         230         240
ACT GTC AAC GAA GAC GGA AAT AAG TTT AAA GGT GTA GCT TTG AAC ACA CCG ATG ACT AAA
T   V   N   E   D   G   N   K   F   K   G   V   A   L   N   T   P   M   T   K

         250         260         270         280         290         300
GTC ACT TAC ACC AAT TCA GAT AAA GGT GGA TCA AAT ACG AAA ACT GCA GAA TTT GAT TTT
V   T   Y   T   N   S   D   K   G   G   S   N   T   K   T   A   E   F   D   F
                                                        PstI
         310         320         330         340         350         360
TCA GAA GTT ACT TTT GAA AAA CCA GGT GTT TAT TAT TAC AAA GTA ACT GAG GAG AAG ATA
S   E   V   T   F   E   K   P   G   V   Y   Y   Y   K   V   T   E   E   K   I

         370         380         390         400         410         420
GAT AAA GTT CCT GGT GTT TCT TAT GAT ACA ACA TCT TAC ACT GTT CAA GTT CAT GTC TTG
D   K   V   P   G   V   S   Y   D   T   T   S   Y   T   V   Q   V   H   V   L

         430         440         450         460         470         480
TGG AAT GAA GAG CAA CAA AAA CCA GTA GCT ACT TAT ATT GTT GGT TAT AAA GAA GGT AGT
W   N   E   E   Q   Q   K   P   V   A   T   Y   I   V   G   Y   K   E   G   S

         490         500         510         520         530         540
AAG GTG CCA ATT CAG TTC AAA AAT AGC TTA GAT TCT ACT ACA TTA ACG GTG AAG AAA AAA
K   V   P   I   Q   F   K   N   S   L   D   S   T   T   L   T   V   K   K   K

         550         560         570         580         590         600
GTT TCA GGT ACC GGT GGA GAT CGC TCT AAA GAT TTT AAT TTT GGT CTG ACT TTA AAA GCA
V   S   G   T   G   G   D   R   S   K   D   F   N   F   G   L   T   L   K   A

         610         620         630         640         650         660
AAT CAG TAT TAT AAG GCG TCA GAA AAA GTC ATG ATT GAG AAG ACA ACT AAA GGT GGT CAA
N   Q   Y   Y   K   A   S   E   K   V   M   I   E   K   T   T   K   G   G   Q

         670         680         690         700         710         720
GCT CCT GTT CAA ACA GAG GCT AGT ATA GAT CAA CTC TAT CAT TTT ACC TTG AAA GAT GGT
A   P   V   Q   T   E   A   S   I   D   Q   L   Y   H   F   T   L   K   D   G

         730         740         750         760         770         780
GAA TCA ATC AAA GTC ACA AAT CTT CCA GTA GGT GTG GAT TAT GTT GTC ACT GAA GAC GAT
E   S   I   K   V   T   N   L   P   V   G   V   D   Y   V   V   T   E   D   D

         790         800         810         820         830         840
TAC AAA TCA GAA AAA TAT ACA ACC AAC GTG GAA GTT AGT CCT CAA GAT GGA GCT GTA AAA
Y   K   S   E   K   Y   T   T   N   V   E   V   S   P   Q   D   G   A   V   K

         850         860         870         880         890         900
AAT ATC GCA GGT AAT TCA ACT GAA CAA GAG ACA TCT ACT GAT AAA GAT ATG ACC ATT ACT
N   I   A   G   N   S   T   E   Q   E   T   S   T   D   K   D   M   T   I   T

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TTT ACA AAT AAA AAA GAC TTT GAA GTG CCA ACA GGA GTA GCA ATG ACT GTG GCA CCA TAT
F   T   N   K   K   D   F   E   V   P   T   G   V   A   M   T   V   A   P   Y

         970         980         990        1000        1010        1020
ATT GCT TTA GGA ATT GTA GCA GTT GGT GGA GCT CTT TAC TTT GTT AAA AAG AAA AAT GCT
I   A   L   G   I   V   A   V   G   G   A   L   Y   F   V   K   K   K   N   A

TAA
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Figure 6

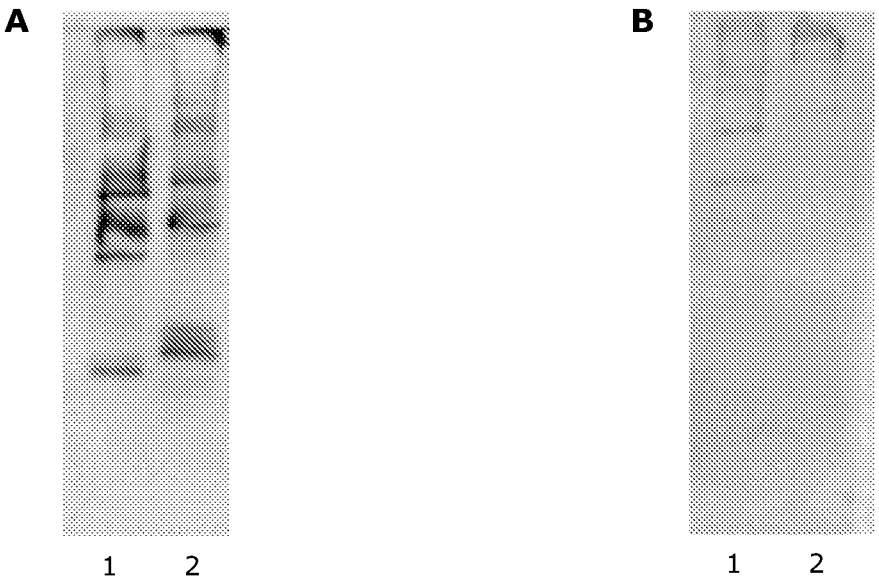
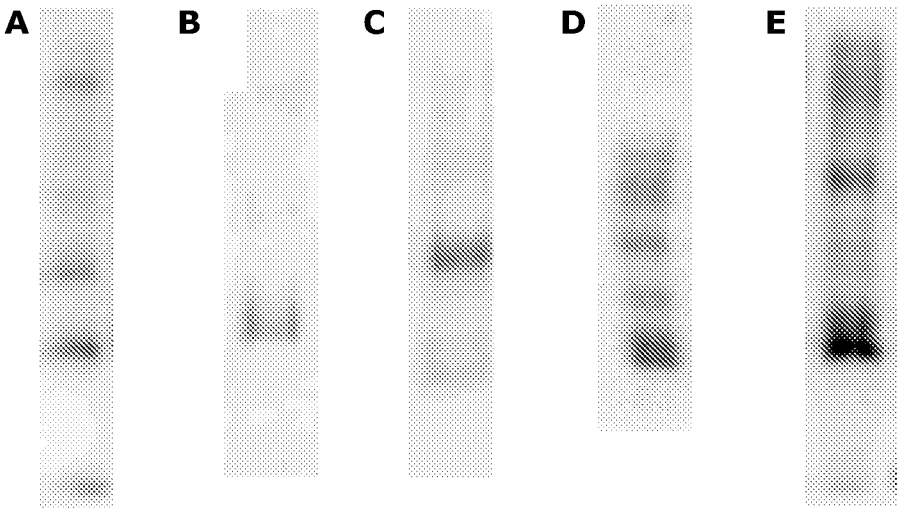


Figure 7



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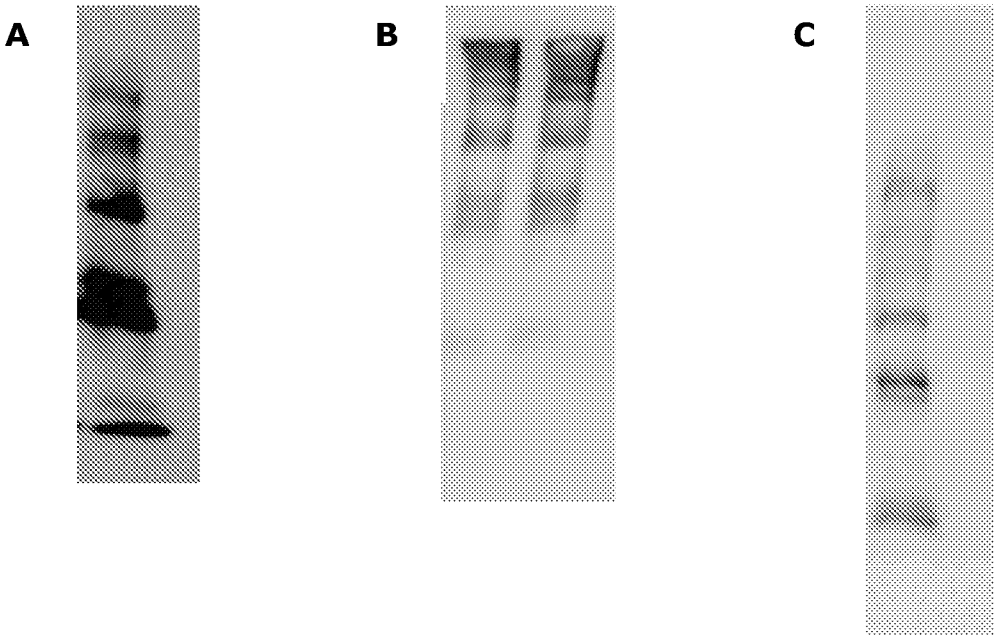


Figure 9

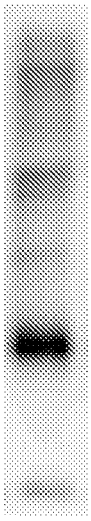
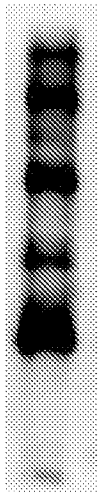


Figure 10



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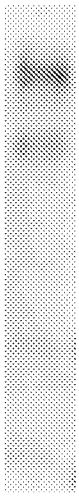


Figure 12

```
M1      MKLRHLLLLIGAALISFAATI-------VHGEIVYNGAKLTVTKNLD-LVNSNALIPNIDF
M18     MKKNKLLLATAILAIALGTASLNQNVKAETAGVIDGSTLVYKKTFPSYTDPKVLMPKADV
        ** .:***: * *:: .*:       ..   *::*:.*.*.*.:   .:.:.*:*::*:

M1      TFKIEPDTIVN---EDGNKFKGVALN--TPMTKVIYTNSDKGGSNTKIAEFDFSEVTFEK
M18     TFKVEADDNAKGKTKDGLDIKPGVIDGLENTKTIHYGNSDKTTAKEKSVNFDFANVKFPG
        ***:*.* ..:   :** .:*  .::     ..: * ****  :: *:.:***::*.*

M1      PGVYYYKVTEEKTDKVRGVSYDTTSYTVQVHVLNNEEQQKPVAIYIVGYKEGS--KVPIQ
M18     VGVIRYTVSEVNGNKAG-IAYDSQQWIVDVYVYNREDGG-FEAKYIVSTEGGQSDKKPVL
         *** *.*:* : :*.  ::**: .:**:*:*: .*:     *.***. : *.  * *:

M1      FKNSLDSTILTVKKKVSGTGGDRSKDFNFGLTLKANQYYKASEKVMIEKTTKGGQAFVQT
M18     FKNFFDTTSLKVTKKVIGNTGEHQRSFSFILLLTPNECFEKGQVVNILQGGE------TK
        *** :*:*:*.*.***:*. *::.:.*.* * *..*: :: .: * * :  :       .

M1      EASIDQLYHFTLKDGESIKVTNLPVGVDYVVIEDDVKSEKYTINVEVSPQDGAVKNIAGN
M18     KVVIGEEYSFILKDKESVTLSQLPVGIEVKVTEEDVIKDGVKTSATLKDGDVTDGVNLGD
        :. *.: * ***** **:.:::****::* ***:* ..: *.*.. :.  * :     *:

M1      STEQETSIDKDMTITFTNKKDFEVPTGVAMTVAPYIALGIVAVGGALVFVKKKNA
M18     SKTTDKSTD---EIVVTNKRDTQVPIGVVGTLAPFAVLSIVAIGGVIYIIKRKK-
        *.  :.***    *..***:* :*****. *:**: .*.***:**.:*:.*:*:
```

Figure 13

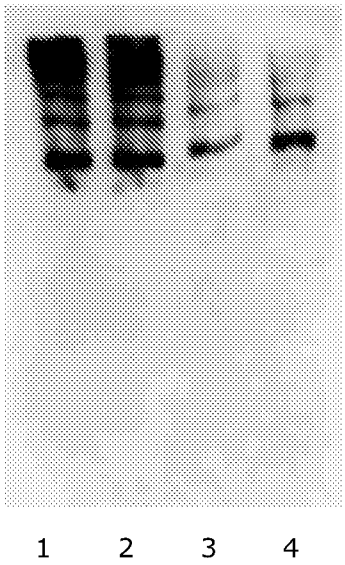


Figure 14

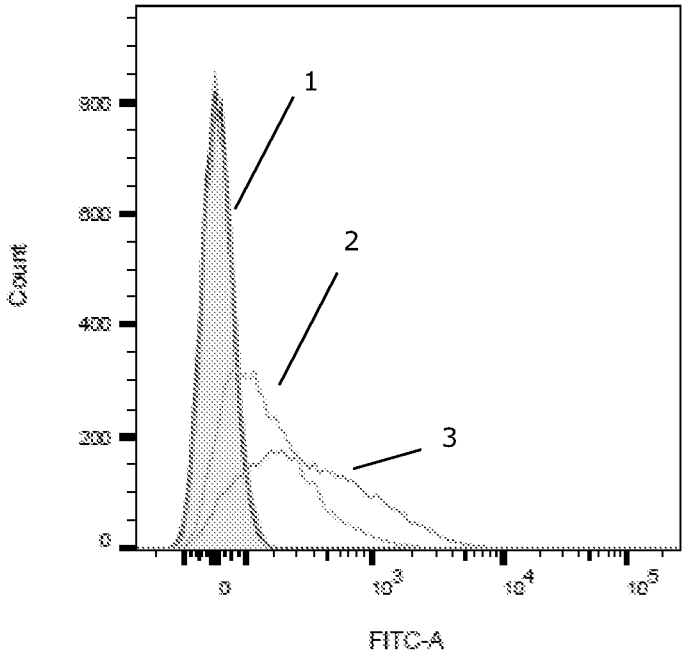


Figure 15

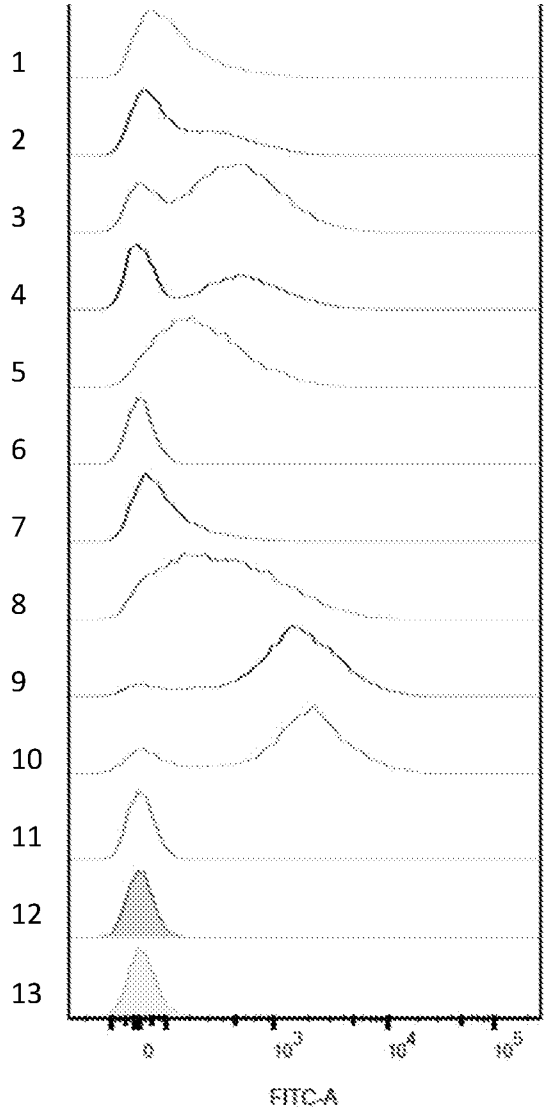
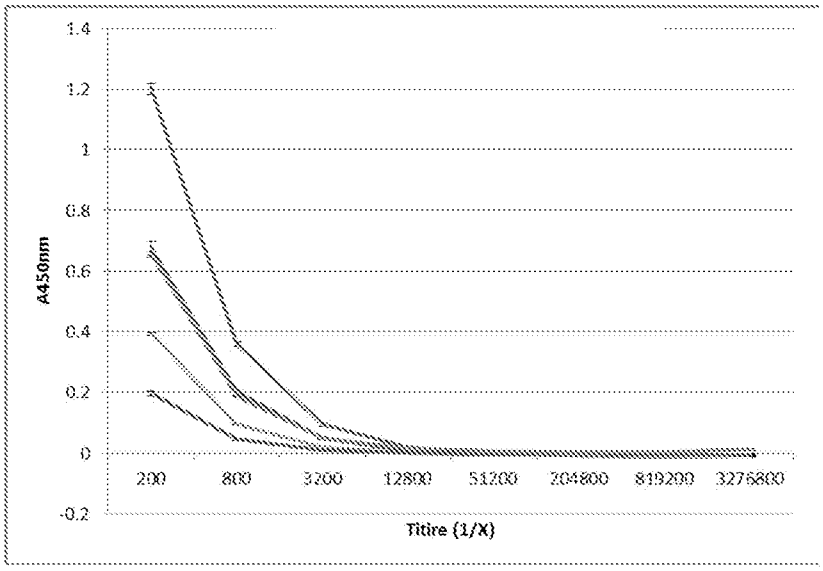
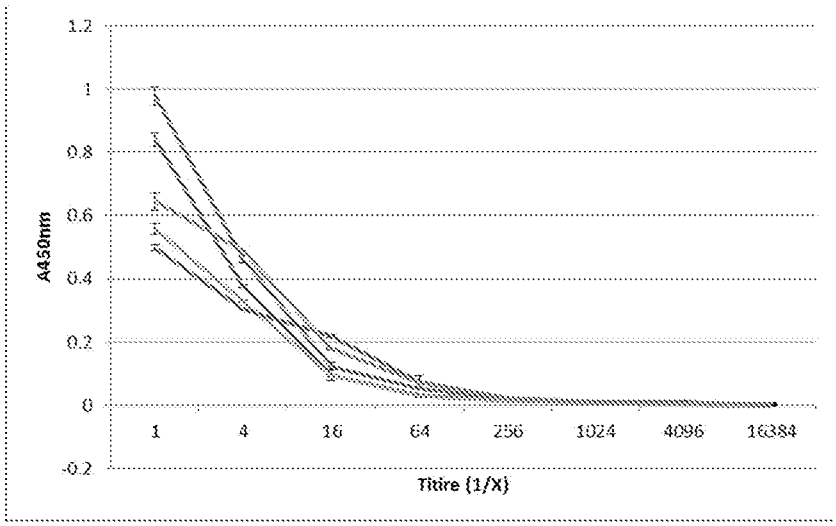


Figure 16

A



B



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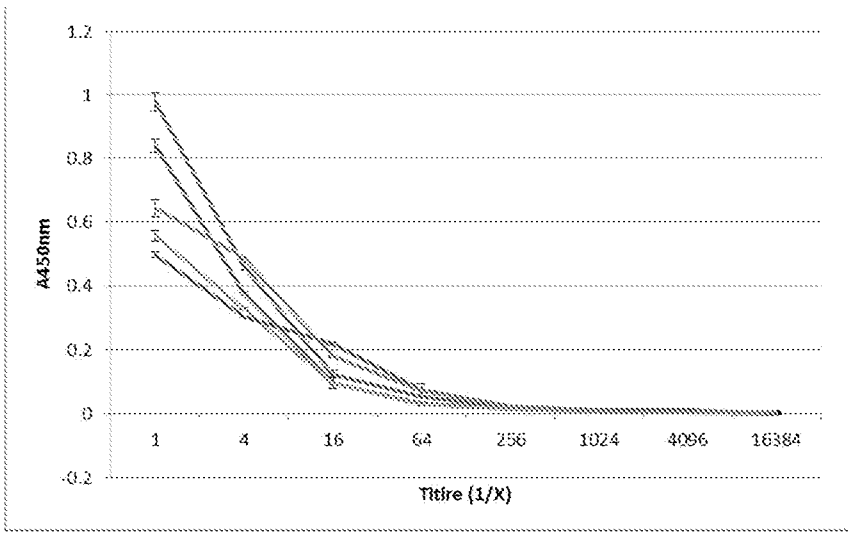
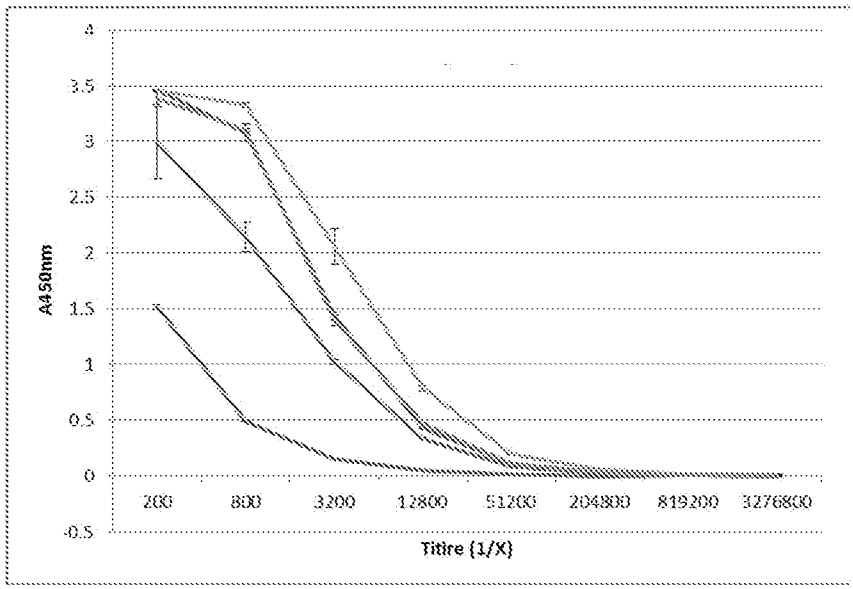


Figure 17

A



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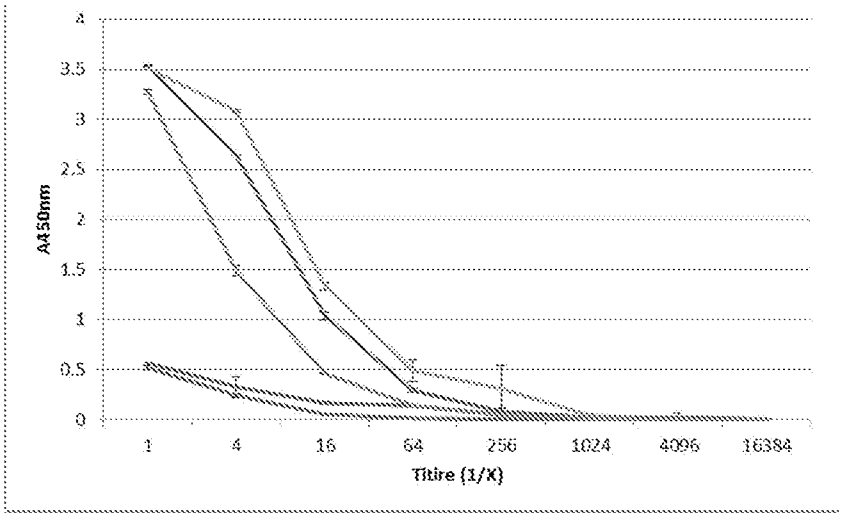

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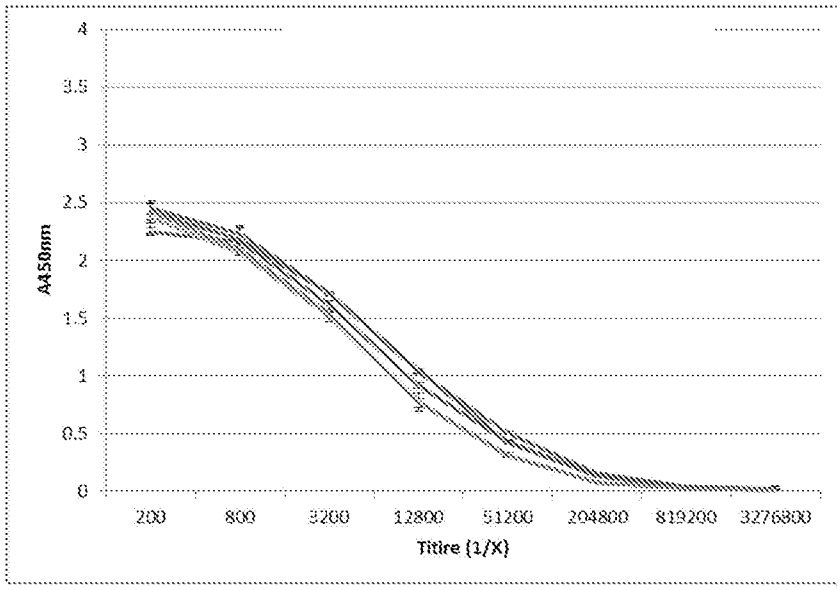

Figure 18

Figure 19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ2016/050111

A. CLASSIFICATION OF SUBJECT MATTER

A61K 39/09 (2006.01) C07K 14/315 (2006.01) C12N 15/00 (2006.01)

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)

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 practicable, search terms used)

Databases: MEDLINE, WPIAP, EPODOC, BIOSIS, EMBASE, GENBANK

Keywords: spy0128, backbone protein, BP protein, gram positive bacteria, GAS, Group A *Streptococcus*, pilin, fimbria, fimbrilin, vaccine, composition, fusion protein, chimeric protein and other related terms

Applicant/inventor search databases: PATENTSCOPE, PUBMED, ESPACENET

Applicant and inventor names searched in internal databases provided by IP Australia

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"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
27 September 2016	27 September 2016
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Kim Lau AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832416

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2011/0189236 A1 (SCOTT J. et al) 04 August 2011 refer to whole document, in particular, abstract; [0003]; [0004]; [0050]; [0053]; [0054]; [0083]; [0102]; [0117]-[0119]; [0124]-[0126]; [0129]-[0132]; [0138]; [0155]; [0156]; [0191]-[0210]; Figure 5; claims	1-17, 23-32, 36, 45-65
X	WO 2011/098772 A1 (ISIS INNOVATION LIMITED) 18 August 2011 refer to whole document, in particular, page 39, lines 16-24; Figure 9; claims; SEQ ID NO: 1	1-8, 11-19, 29, 32, 37, 38
X	LO SAPIO M., *et al*, "A novel strategy to over-express and purify homologous proteins from *Streptococcus pneumonia*", Journal of Biotechnology, 2012, vol 157, pages 279-286 refer to whole document, in particular, results 3.3 "The pMU1328-P96 over-expression system can be used to design homologous pneumococcal protein secretion allowing simple protein recovery from the bacterial supernatant"; Figure 2; Figure 3	1-7, 32
X	NUCCITELLI A., *et al*, "Structure based approach to rationally design a chimeric protein for an effective vaccine against Group B *Streptococcus* infections", Proceedings of the National Academy of Sciences USA, 2011, vol 108, pages 10278-10283 refer to whole document, in particular, abstract; discussion; Figure 3; Table 2	1-7, 26-29, 32, 53-55, 57-60
X	HARFOUCHE C., *et al*, "RrgB321, a fusion protein of the three variants of the Pneumococcal pilus backbone RrgB, is protective *in vivo* and elicits opsonic antibodies", Infection and Immunity, 2012, vol 80, pages 451-460 refer to whole document, in particular, abstract; results: RrgB321 is immunogenic and elicits antibodies against the three RrgB variants; results: RrgB321 protects against challenge with pneumococcal strains expressing each of the three RrgB variants; discussion	1, 5-7, 26-29, 53-60
X	CHEN H., *et al*, "Construction, characterization, and immunogenicity of an attenuated *Salmonella enterica* Serovar Typhimurium *pgtE* vaccine expressing fimbriae with integrated viral epitopes from the *spiC* promoter", Infection and Immunity, 2003, vol 71, pages 4664-4673 refer to whole document, in particular, results: expression of intact chimeric FasA on a pgtE mutant of Salmonella; results: Booster immunization enhanced the immune responses against fimbriae; Figure 1; Figure 6	45, 47, 52-55, 57-60, 62, 64
X	ZALEWSKA B., *et al*, "Chimeric Dr fimbriae with a Herpes Simplex Virus type 1 epitope as a model for a recombinant vaccine", Infection and Immunity, 2003, vol 71, pages 5505-5513 refer to whole document, in particular, abstract; materials and methods: immunization with fimbriae; results: construction of the recombinant plasmid pDraE-HSV3 encoding the DraE-syg adhesin with the HSV-1 epitope; results: immunogenicity of chimeric Dr-HSV fimbriae; Figure 1; Figure 5; Figure 6	45, 47, 52-60, 62, 64
A	STEEMSON J. D., *et al*, "Survey of the *bp/tee* genes from clinical group A streptococcus isolates in New Zealand – implications for vaccine development", Journal of Medical Microbiology, 2014, vol 63, pages 1670-1678 refer to whole document	15, 16
A	FERRETTI J. J., *et al*, "Compete genome sequence of an M1 strain of *Streptococcus pyogenes*", Proceedings of the National Academy of Sciences USA, 2001, vol 98, pages 4658-4663 refer to whole document	15, 16
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.