(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 October 2011 (20.10.2011)

PCT

(10) International Publication Number
WO 2011/128892 A2

(51) **International Patent Classification**:
C07K 14/315 (2006.01)

(21) **International Application Number**:
PCT/IL2011/000295

(22) **International Filing Date**:
10 April 2011 (10.04.2011)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/322,926 12 April 2010 (12.04.2010) US

(71) **Applicant** *(for all designated States except US)*: **PROTEA VACCINE TECHNOLOGIES LTD.** [IL/IL]; P.O. Box 4122, 74140 Ness-Ziona (IL).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: **TAL, Michael** [IL/IL]; 24 Ha'Hadrim Street, 76965 Kefar Bilu (IL). **MIZRACHI NEBENZAHL, Yaffa** [IL/IL]; 24 Katzir Aharon Street, 84836 Beer Sheva (IL). **PORTNOI, Maxim** [IL/IL]; 10/6 Oscar Schindler Street, 84718 Beer Sheva (IL). **DAGAN, Ron** [IL/IL]; 11 Haruv Street, 84965 Omer (IL).

(74) **Agents**: **WEBB, Cynthia** et al.; Webb & Co., P.O. Box 2189, 76121 Rehovot (IL).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2011/128892 A2

(54) **Title**: MODIFIED FORMS OF PNEUMOCOCCAL SURFACE IMMUNOGENIC PROTEIN B (PSIPB)

(57) **Abstract**: The present invention relates to modified forms of the protein Pneumococcal surface immunogenic protein B (PsipB) derived from Streptococcus pneumonia (S. pneumoniae). The modified PsipB polypeptides and fragments have enhanced immunogenicity, stability at physiological pH formulation and formulation properties as compared to wild type PsipB. The invention further provides immunogenic compositions and vaccines comprising the modified PsipB polypeptides and fragments, and use thereof in methods for eliciting protective immunity to S. pneumoniae.

MODIFIED FORMS OF PNEUMOCOCCAL SURFACE IMMUNOGENIC PROTEIN B (PSIPB)

FIELD OF THE INVENTION

The present invention relates to modified forms of the protein Pneumococcal surface immunogenic protein B (PsipB) derived from *Streptococcus pneumoniae* (*S. pneumoniae*). The modified PsipB polypeptides have enhanced immunogenicity, stability and formulation properties as compared to wild type PsipB. The invention further provides immunogenic compositions and vaccines comprising the modified PsipB polypeptides, and use thereof in methods for eliciting protective immunity to *S. pneumoniae*.

BACKGROUND OF THE INVENTION

Streptococcus pneumoniae (*S. pneumoniae*) is part of the commensal flora of the human respiratory tract, but can also cause invasive infections. Most children in the developing world become nasopharyngeal carriers of *S. pneumoniae*, and many develop invasive pneumococcal disease which can manifest as bacteremia, sepsis or meningitis, or mucosal infections such as pneumonia or otitis media. *S. pneumoniae* is the leading cause of non-epidemic childhood meningitis in Africa and other regions of the developing world. Approximately one to two million childhood deaths due to pneumococcal pneumonia occur each year, accounting for about 20% of worldwide childhood deaths under the age of five. These high morbidity and mortality rates and the persistent emergence of antibiotic resistant strains of *S. pneumoniae* heighten the need to develop an effective means of prevention, such as vaccination.

The immunologically variant capsular polysaccharides of *S. pneumoniae* are used for the typing of clinical isolates. There are more than 90 capsular serotypes and their prevalence among human isolates varies with age, disease type and geographical origin. A 23-valent capsular polysaccharide-based vaccine is licensed for use in adults (Hutchison et al., Can Fam Physician. 1999;45:2381–93; Wuorimaa et al., Scand J Immunol. 2000;56:111–29), but it does not elicit an efficient antibody response or protection in children below 2 years of age and in immunocompromised patients (Breiman et al., Arch Intern Med. 2000;160:2633–8).

To overcome the lack of responsiveness to the T cell independent polysaccharide antigens in young children, conjugate pneumococcal vaccines have been developed. These seven- to 13-valent vaccines consist of *S. pneumoniae* capsular polysaccharides covalently linked to a protein carrier (for example, Shouval et al., Pediatr Infect Dis J. 2009 Apr;28(4):277-82). In particular, the 7-valent conjugate vaccine has been shown to significantly reduce the rates of invasive antibiotic-resistant pneumococcal diseases in children under two years of age and in adults 65 years of age and older (Kyaw et al., N. Engl. J. Med. 2006, 354, 1455-63). However, carriage and disease incidence resulting from serotypes not included in the vaccine have been shown to increase with vaccine use (for example, Dagan, Clin Microbiol Infect. 2009 Apr;15 Suppl 3:16-20; Huang et al., Pediatrics 2005, 116, e408-13; Byington et al., Clin Infect Dis. 2005 Jul1;41(1):21-9). A 13-valent polysaccharide vaccine has been disclosed to be more effective than 10-valent and 7-valent vaccines in preventing infection caused by antibiotic resistant strains (Shouval et al., Pediatr Infect Dis J. 2009 Apr;28(4):277-82).

In addition to the limited serotype coverage offered by conjugate polysaccharide vaccines, other drawbacks include their high cost and the relative complexity of the manufacturing process. Furthermore, there are geographical and age-related variations in the prevalence of clinically important serotypes of *S. pneumoniae* which render particular vaccines less effective among some groups (for example, Hausdorff Eur J. Pediatr. 2002 Dec, 161 Suppl 2:S135-9; Barricarte et al., An Sist Sanit Navar. 2008 May-Aug:31(2): 171-92). Accordingly, there is a need for improved pneumococcal vaccines that are immunogenic in all age and population groups and broadly cross-protective against all serotypes.

Vaccines comprising various *S. pneumoniae* proteins have been disclosed.

International Patent Application Publication No. WO 98/23631 discloses *S. pneumoniae* genes and gene products, polynucleotides having at least 70% identity to the genes, compositions and vaccination methods.

International Patent Application Publication No. WO 02/077021 discloses the nucleic acid sequences of about 2,500 *S. pneumoniae* genes, and their corresponding amino acid sequences that were identified *in silico*. The use of a subset of 432 of those sequences as antigens for immunization is suggested although no guidance is provided for selecting proteins useful as antigens in the production of vaccines.

International Patent Application Publication No. WO 2006/84467 discloses the sequences of 282 surface-located *S. pneumoniae* polypeptides, and immunogenic compositions and vaccines comprising such polypeptides.

Biochemical and proteomic analyses have been used for identification of proteins in *S. pneumoniae* cell wall extracts which exhibit age-dependent antigenicity, based on screening of sera obtained longitudinally from young children and from healthy adults (Ling et al., Clin Exp Immunol 2004, 138, 290-8). International Patent Application Publication No. WO 03/082183 of one of the inventors of the present application, disclose a defined group of cell wall and cell membrane *S. pneumoniae* proteins for use as components of vaccines for preventing infection by *S. pneumoniae*. One of the disclosed proteins is Pneumococcal surface immunogenic protein B (PsipB), corresponding to GenBank accession number NP_358083.

U.S. Patent No. 6,699,703 discloses *S. pneumoniae* polypeptides and methods for producing such polypeptides by recombinant techniques, compositions comprising same and methods of use in the preparation of a vaccine.

U.S. Patent Nos. 6,887,480 and 7,132,107 disclose vaccines and immunogenic compositions comprising the polypeptides designated Sp128 and Sp130 of *S. pneumoniae*, and methods of immunizing an animal using same.

U.S. Patent No. 7,078,042 discloses vaccines comprising Pneumococcal surface protein C (PsipC), epitopic regions and different clades thereof, and use thereof for eliciting an immunological response against *S. pneumoniae*.

U.S. Patent No. 6,764,686 discloses modified pneumolysin polypeptides and vaccines against *S. pneumoniae* comprising such polypeptides.

U.S. Patent No. 5,965,141 discloses a truncated pneumococcal surface protein A (PsipA) fragment and a vaccine comprising same.

None of the aforementioned publication discloses modified forms of PsipB having specific amino acid substitutions or use thereof in vaccine compositions. In its native conformation, PsipB occurs as a dimer. PsipB dimers having limited solubility may be obtained in purified form *in vitro*, but their isolation and maintenance requires either use of pH in the highly basic range, or inclusion of transitional metal salts in neutral range

buffers. Neither high pH nor transitional metal salts are suitable for use in human vaccine compositions.

There remains an unmet need for an improved *S. pneumoniae* polypeptide-based vaccine which can induce long-lasting immunological responses in all age groups, including young children and elderly people, and which has broad specificity against a wide range of different *S. pneumoniae* serotypes.

SUMMARY OF THE INVENTION

The present invention provides modified PsipB polypeptides and fragments thereof and immunogenic compositions and vaccines comprising them. The modified PsipB polypeptides comprise one or more amino acid substitutions, such that at least one of the two or three cysteine residues normally present in the wild type protein is replaced by a different amino acid residue. Advantageously, the modified PsipB polypeptides of the invention exhibit enhanced stability and enhanced immunogenicity as compared to the wild type PsipB polypeptide.

Surprisingly, the modified PsipB polypeptides and fragments of the present invention retain their solubility and dimer conformation at physiological pH ranges and for extended period of time, without requiring transitional metal salts for their stability. These improved properties render the subject polypeptides useful for human vaccine preparation.

The present invention is based in part on the unexpected discovery that a modified PsipB polypeptide and a fragment thereof having a single cysteine to alanine substitution at position 66 of SEQ NO:1 (designated herein PsipB C66A) has enhanced stability in maintaining its conformation as a dimer at pH in the physiologic range. Moreover, when used as a vaccine, PsipB C66A and a fragment thereof having same substitution, provide greater immunoprotective effect against *S. pneumoniae* challenge, as compared to wild type PsipB.

Without wishing to be bound by any particular theory or mechanism of action, the enhanced *in vitro* stability of the modified PsipB polypeptides disclosed herein may be attributed to elimination of free cysteine residues which normally tend to crosslink among different PsipB molecules, leading to PsipB oligomerization. In addition, the enhanced immunoprotective effect of the modified PsipB polypeptides may be attributed to exposure of antigenic epitopes which are masked in the oligomerized forms of wild type PsipB. Moreover, the modified PsipB polypeptides and fragments of the present invention are

advantageous for creating composite or multimeric vaccines comprising a plurality of *S. pneumoniae* protein antigens which can provide specific immunoprotection against a wide range of different *S. pneumoniae* serotypes.

In a first aspect, the invention provides a modified PsipB polypeptide or a fragment thereof comprising at least amino acids 48-112 of SEQ ID NO:1 or of a sequence having at least 95% identity to SEQ ID NO:1 and at least one amino acid substitution of a cysteine residue naturally present in a wild type PsipB polypeptide.

According to some embodiments, the wild type PsipB polypeptide is according to any one of SEQ ID NO:1-12.

In a particular embodiment, the modified PsipB polypeptide comprises at least two cysteine residues naturally present in a wild type PsipB polypeptide while the third cysteine is substituted for an amino acid residue selected from the group consisting of alanine, serine, threonine, glycine, valine, leucine and tyrosine.

In a particular embodiment, the wild type PsipB polypeptide comprises an amino acid sequence that has at least about 95% identity with the region of position 48 to position 112 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide comprises an amino acid sequence that has at least about 97% identity with the region of position 48 to position 112 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide comprises an amino acid sequence that has at least about 98% identity with the region of position 48 to position 112 of SEQ ID NO:1.

In a particular embodiment, the wild type PsipB polypeptide comprises a cysteine residue at a position corresponding to a position of SEQ ID NO:1 selected from the group consisting of position 66, position 72, position 84 and a combination of residues thereof. In a particular embodiment, the wild type PsipB polypeptide has two cysteine residues at the positions corresponding to position 66, position 72 or position 84 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide has a cysteine residue at each of the positions corresponding to position 66 and position 84 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide has a cysteine residue at each of the positions corresponding to position 66 and position 84 of SEQ ID NO:1 and further has a tyrosine residue at the position corresponding to position 72 of SEQ ID NO:1. Each possibility represents a separate embodiment of the invention.

In a particular embodiment, the wild type PsipB polypeptide has an amino acid sequence that has at least about 90% identity with SEQ ID NO:1, such as 95%, 96%, 97%, 97% or 98% identity. In a particular embodiment, the wild type PsipB polypeptide has an amino acid sequence that has at least about 90% identity with SEQ ID NO:1, and said wild type PsipB polypeptide comprises a cysteine residue at a position corresponding to a position of SEQ ID NO:1 selected from the group consisting of position 66, position 72, position 84 and a combination of residues thereof. In a currently preferred embodiment, the wild type PsipB polypeptide has two cysteine residues at positions corresponding to position 66, position 72 or position 84 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide has an amino acid sequence selected from the group consisting of: SEQ ID NO:1 [NP_358083]; SEQ ID NO:2 [ZP_01829414]; SEQ ID NO:3 [NP_345081]; SEQ ID NO:4 [YP_002742080]; SEQ ID NO:5 [ZP_01817263]; SEQ ID NO:6 [ZP_01835575]; SEQ ID NO:7 [ZP_01821338]; SEQ ID NO:8 [ZP_01827178]; SEQ ID NO:9 [ZP_01833557]; SEQ ID NO:10 [YP_001835244]; SEQ ID NO:11 [YP_002037228]; and SEQ ID NO:12 [YP_002735619]. Each possibility represents a separate embodiment of the invention.

In a particular embodiment, the amino acid substitution is of a residue corresponding to a residue of the wild type PsipB of SEQ ID NO:1 selected from the group consisting of: the cysteine residue at position 66 (C66); the cysteine residue at position 72 (C72); the cysteine residue at position 84 (C84), and a combination of residues thereof. In a particular embodiment, the amino acid substitution comprises substitution of a cysteine residue by an amino acid residue selected from the group consisting of alanine, serine, threonine, glycine, valine, leucine, tyrosine and a combination of residues thereof. In a currently preferred embodiment, the amino acid substitution comprises substitution of a cysteine residue by an alanine residue. Each possibility represents a separate embodiment of the invention.

In a particular embodiment, at least one amino acid substitution is selected from the group consisting of: substitution of the cysteine residue at position 66 by an alanine residue (C66A); substitution of the cysteine residue at position 72 by an alanine residue (C72A); substitution of the cysteine residue at position 84 by an alanine residue (C84A), and a combination of residues thereof. In a particular embodiment, the modified PsipB polypeptide comprises C66A. In a particular embodiment, the modified PsipB polypeptide comprises C66A as the sole amino acid substitution of a cysteine residue. In a particular

embodiment, the modified PsipB polypeptide comprises all of C66A, C72A and C84A. In a particular embodiment, the modified PsipB polypeptide is devoid of cysteine residues. Each possibility represents a separate embodiment of the invention.

In particular embodiments, the modified PsipB polypeptide is selected from the group consisting of SEQ ID NO:13 (PsipB C66A); SEQ ID NO:14 (PsipB C72A); SEQ ID NO:15 (PsipB C84A), and SEQ ID NO:16 (PsipB C66A, C72A, C84A). Each possibility represents a separate embodiment of the invention.

In particular embodiments, the modified PsipB polypeptide is selected from the group consisting of SEQ ID NOS:21-62.

In a particular embodiment, the modified PsipB polypeptide corresponds to an immunogenic fragment of 50 to 120 amino acids. In a particular embodiment, the modified PsipB polypeptide consists of 65 to 120 amino acids. In a particular embodiment, the immunogenic fragment comprises at least 65 amino acids of residues 48-112 of SEQ ID NO:1, of a wild type PsipB polypeptide, and comprises an amino acid substitution at a position corresponding to a position of the wild type PsipB polypeptide of SEQ ID NO:1 selected from the group consisting of: the cysteine residue at position 66 (C66); the cysteine residue at position 72 (C72); the cysteine residue at position 84 (C84), and a combination of residues thereof. In a particular embodiment, the amino acid substitution is selected from the group consisting of: C66A; C72A; C84A, and a combination of residues thereof. In a particular embodiment, the immunogenic fragment is selected from the group consisting of SEQ ID NOS:64-67 and 73-79. Each possibility represents a separate embodiment of the invention.

In a particular embodiment, the modified PsipB polypeptide or fragment thereof further comprises at least one amino acid substitution of an amino acid residue other than cysteine that is naturally present in a wild type PsipB polypeptide. In particular embodiments, the modified PsipB polypeptide is the product of a chemical or recombinant synthesis. In a particular embodiment, a fusion protein is provided comprising the modified PsipB polypeptide.

According to one embodiment, the fusion protein comprises detoxified pneumolysin or a fragment thereof. According to another embodiment, the fusion protein comprises heat shock protein 60 (hsp60) or a fragment thereof.

In another aspect, the invention provides an isolated polynucleotide sequence encoding a modified PsipB polypeptide or fragment thereof, wherein the modified PsipB polypeptide or fragment encoded by the polynucleotide has an amino acid residue other than cysteine at a position corresponding to a position of the wild type PsipB polypeptide of SEQ ID NO:1 selected from the group consisting of position 66, position 72, position 84, and a combination of residues thereof. Each possibility represents a separate embodiment of the invention.

In a particular embodiment, the polynucleotide sequence encodes a modified PsipB polypeptide or fragment thereof comprising an amino acid substitution selected from the group consisting of C66A, C72A, C84A and a combination of residues thereof. Each possibility represents a separate embodiment of the invention.

In particular embodiments, the polynucleotide sequence encodes a modified PsipB polypeptide selected from the group consisting of SEQ ID NO:13 [PsipB C66A]; SEQ ID NO:14 [PsipB C72A]; SEQ ID NO:15 [PsipB C84A], and SEQ ID NO:16 [PsipB C66AC72AC84A]. In particular embodiments, the polynucleotide sequence comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19 and SEQ ID NO:20.

In yet other embodiments, the polynucleotide sequence encodes a modified PsipB fragment selected from the group consisting of SEQ ID NOs:73-79. In a specific embodiment, the polynucleotide sequence encoding the modified PsipB fragment is according to SEQ ID NO: 72.

According to another aspect, an expression vector comprising the polynucleotide sequence encoding a modified PsipB polypeptide, operatively linked to one or more transcriptional control sequences is provided.

According to yet another aspect, a host cell transformed with the expression vector is provided.

According to a further aspect, the present invention provides a pharmaceutical composition comprising an effective amount of the expression vector, wherein the composition further comprises a pharmaceutically acceptable carrier, diluent or adjuvant. In a particular embodiment, the pharmaceutical composition further comprises at least one polynucleotide sequence encoding a *S. pneumoniae* immunogenic polypeptide other than PsipB or a modified PsipB.

In another aspect, the invention provides a vaccine composition comprising an effective amount of a modified PsipB polypeptide or a modified PsipB fragment, wherein the modified PsipB polypeptide has an amino acid residue other than cysteine at a position corresponding to a position of SEQ ID NO:1 selected from the group consisting of position 66, position 72, position 84, and a combination of residues thereof, and wherein the composition further comprises a pharmaceutically acceptable carrier, diluent, delivery system and/or adjuvant. In a particular embodiment, the modified PsipB polypeptide or fragment is selected from the group consisting of SEQ ID NOs: 21-62, 64-67 and 73-79. In yet another particular embodiment, the modified PsipB polypeptide is selected from the group consisting of SEQ ID NO:13 [PsipB C66A]; SEQ ID NO:14 [PsipB C72A]; SEQ ID NO:15 [PsipB C84A], and SEQ ID NO:16 [PsipB C66AC72AC84A]. Each possibility represents a separate embodiment of the invention.

In particular embodiments, the vaccine is formulated for administration by a route selected from the group consisting of: intramuscular, intranasal, oral, intraperitoneal, subcutaneous, topical, intradermal and transdermal delivery. In particular embodiments, the vaccine is formulated for intramuscular administration. In other particular embodiments, the vaccine is formulated for oral administration. In yet other embodiments, the vaccine is formulated for intranasal administration.

In a particular embodiment, the vaccine formulation comprises an adjuvant and/or delivery system. In a particular embodiment, the adjuvant and the modified PsipB polypeptide are present in the vaccine as an admixture. In a particular embodiment, the adjuvant and the modified PsipB polypeptide are present in the vaccine as a conjugate. In a particular embodiment, the conjugate is a fusion protein. In a particular embodiment, the adjuvant/delivery system and the modified PsipB polypeptide together form a fusion protein. In particular embodiment the modified PsipB polypeptide or PsipB is conjugated to one or more *S. pneumoniae* capsular polysaccharides. In a particular embodiment, the vaccine is substantially devoid of an adjuvant.

In a particular embodiment, the vaccine comprises a plurality of modified PsipB polypeptides or fragments thereof. The modified PsipB polypeptides or fragments may be identical or non-identical.

In a particular embodiment, the vaccine further comprises at least one heterologous *S. pneumoniae* derived polypeptide or fragment thereof, in addition to the modified PsipB polypeptide.

In a particular embodiment, the vaccine further comprises a plurality of *S. pneumoniae* derived polypeptides or polypeptide fragments comprising at least one modified PsipB polypeptide or fragment thereof. Such a vaccine is also referred to herein as a "multimer". In a particular embodiment, the multimer comprises a plurality of modified PsipB polypeptides or fragments. In a particular embodiment, the modified PsipB polypeptides comprising the plurality are adjacent or non-adjacent. In a particular embodiment, the plurality of the modified PsipB polypeptides comprises a repeating unit within the multimer. In a particular embodiment, the multimer comprises a conjugate.

In a particular embodiment, the vaccine comprises a fusion protein comprising a modified PsipB polypeptide or fragment thereof.

In a particular embodiment, the modified PsipB polypeptide of the vaccine corresponds to an immunogenic fragment of a wild type PsipB polypeptide, wherein the immunogenic fragment further comprises an amino acid substitution selected from the group consisting of C66A, C72A, C84A, and a combination of residues thereof, wherein the position numbers refer to the positions of the wild type PsipB polypeptide of SEQ ID NO:1. In particular embodiment, the vaccine comprises a modified PsipB immunogenic fragment selected from the group consisting of SEQ ID NOs: 64-67 and 73-79.

In another aspect, the invention provides a method of inducing an immune response against *S. pneumoniae* in a subject, the method comprising administering to a subject in need thereof an effective amount of a modified PsipB polypeptide or fragment according to the invention, or a polynucleotide encoding the modified PsipB polypeptide or fragment.

In another aspect, the invention provides a method of conferring protection against *S. pneumoniae* infection in a subject, the method comprising administering to a subject in need thereof an effective amount of a modified PsipB polypeptide or fragment according to the invention, or a polynucleotide encoding the modified PsipB polypeptide or fragment.

In yet another aspect, the invention provides use of a modified PsipB polypeptide or fragment according to the invention, or a polynucleotide encoding the modified PsipB polypeptide or fragment, for preparation of a medicament for inducing an immune

response in a subject against *S. pneumoniae* or for conferring protection against *S. pneumoniae* infection.

A modified PsipB polypeptide or fragment according to the invention, or a polynucleotide encoding the modified PsipB polypeptide or fragment are used in conferring protection against *S. pneumoniae* infection in a subject and in inducing an immune response in a subject against *S. pneumoniae*.

In another aspect, the invention provides use of a modified PsipB polypeptide or fragment according to the invention, or a polynucleotide encoding the modified PsipB polypeptide, for preparation of a medicament

In particular embodiments, the medicament is a vaccine.

In another aspect, the invention provides a method of enhancing the stability at physiological pH of a PsipB polypeptide, the method comprising providing a modified PsipB polypeptide or fragment thereof having at least one amino acid substitution of a cysteine residue naturally present in a wild type PsipB polypeptide. In a particular embodiment, the amino acid substitution is of a residue corresponding to a residue of the wild type PsipB polypeptide of SEQ ID NO:1 selected from the group consisting of: the cysteine residue at position 66 (C66); the cysteine residue at position 72 (C72); the cysteine residue at position 84 (C84), and a combination of residues thereof. In a particular embodiment, the amino acid substitution comprises substitution of a cysteine residue by an amino acid residue selected from the group consisting of alanine, serine, threonine, glycine, valine, leucine tyrosine and a combination of residues thereof.

It is to be understood explicitly that the scope of the present invention encompasses homologs, analogs, variants and derivatives, including shorter and longer polypeptides and polynucleotides, and analogs comprising amino acid or nucleic acid derivatives, as are known in the art, with the stipulation that these variants preserve the immunogenic properties of the modified PsipB polypeptides disclosed herein.

Other objects, features and advantages of the present invention will become clear from the following description and drawings.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a multiple sequence alignment of wild type PsipB amino acid sequences, bolded and underlined numbers on the left of the first alignment panel correspond to SEQ ID number 1.

Figure 2 shows expression of recombinant wild type PsipB.

Fig. 2A shows mRNA expression of the gene corresponding to GenBank accession No. NP_358083 S. *pneumonia* WU2 cells. Total RNA prepared from growing *S. pneumoniae* strain WU2 culture was used for cDNA library preparation. Primers for PsipB were used to identify the corresponding cDNA. Lane 1 – MW markers, lane 2 – NP_358083 gene transcription, lane 3 – control without cDNA, with PsipB primers.

Fig. 2B shows dimerization of recombinant Hat-tagged wild type PsipB protein. The recombinant protein was purified with Ni-NTA beads and separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using sample buffer with (lane 2) and without β-mercaptoethanol (lane 3).

Fig. 2C shows immunoblotting of the recombinant wild type HAT-tagged PsipB protein. The recombinant product was separated using SDS-PAGE with β-mercaptoethanol, transferred to nitrocellulose membrane and probed with rabbit antiserum against PsipB. The antiserum reacted with two bands of apparent molecular weight 22 kDa and 45 kDa.

Fig. 2D shows expression of wild type PsipB product in wild type *S. pneumoniae* serotype 3 strain WU2 and its Δ*PsipB*null mutant. Cytoplasmic and cell wall fractions were prepared and were analyzed by SDS-PAGE without β-mercaptoethanol and immunoblotting rabbit antiserum against PsipB.

Figure 3 shows analytical gel filtration chromatography profiles obtained with the purified untagged wild type PsipB polypeptide (Figs. 3A and 3C) and the modified polypeptide PsipB C66A (Figs. 3B and 3D). Lyophilized samples (0.5 mg) were dissolved in 1 ml DDW and applied to a Superdex® 75 column pre-equilibrated with 25 mM Tris-HCl buffer pH 8, containing 300 mM NaCl, either within 5 minutes after solubilization (Figs. 3A and 3B), or the next day following freezing and thawing (Figs. 3C and 3D). The column was run at room temperature at a rate of 0.8 ml/min.

Figure 4 shows analytical gel filtration chromatography profiles obtained with the modified polypeptide PsipB C66A. Samples lyophilized after dialysis against sodium

bicarbonate (0.5 mg) were dissolved in 2 ml of DDW and the pH was adjusted to 8.0 with dilute HCl. Samples were maintained at room temperature (23 °C) and gel filtration analysis was performed at the following time points following solubilization: 5 min (Fig 4A); 1 hour (Fig 4B); 3 hours (Fig 4C); and 24 hours (Fig 4D).

Figure 5 shows analytical SDS-PAGE of the modified polypeptide PsipB C66A using samples prepared as described for Figure 4. Samples containing 3 μg (lanes 2, 3, 6 and 8) or 6 μg (lanes 1, 4, 7 and 9) of PsipB C66A and β-mercaptoethanol were applied to the gel at the following time points following solubilization: 5 min (lanes 1 and 2); 1 hour (lanes 3 and 4); 3 hours (lanes 6 and 7); and 24 hours (lanes 8 and 9). Molecular weight markers are shown in lane 5.

Figure 6 shows analysis of recombinant wild type PsipB.

Fig. 6A shows preparative gel filtration of recombinant wild type PsipB following solubilization and refolding of inclusion bodies (IBs). IBs from 500 ml of fermentation culture were suspended in DDW and refolded in 33 ml of 4.5 M urea, 40 mM Tris and 1 mM cysteine pH 11.3. After 1.5 hr. at 4 °C, 100 ml of 0.67 M arginine was added and the solution was stirred overnight at 4 °C. The next day, the refold mixture was concentrated to 30 ml and applied in two consecutive 15 ml portions onto a Superdex® 200 column pre-equilibrated with TN/Urea/Arg pH 10.4. The column was run at 4 °C at a rate of 2 ml/min.

Fig. 6B shows the analytical gel filtration profile of recombinant wild type PsipB obtained from fractions 8-15 from Fig 7B. The pooled fractions were concentrated, and desalted on a Sephadex® G-25 column equilibrated with $NaHCO_3$ (1.2 mg/ml) pH 11.5. The obtained material was divided into aliquots, lyophilized and stored at -20 °C. The lyophilized PsipB was dissolved in 0.2% $NaHCO_3$ pH 11.5, and applied to analytical Superdex® 75 column equilibrated with TN buffer pH 10.4.

Fig. 6C shows an immunoblot of recombinant wild type PsipB. Lyophilized samples prepared as described for Fig. 6B were subjected to SDS-PAGE under reducing conditions followed by transblotting and immunodetection using rabbit polyclonal antiserum against PsipB (1:20,000 dilution).

Figure 7 shows analytical gel filtration of recombinant wild type PsipB following lyophilization and freezing for either 24 h (Fig. 7A) or 6 days (Fig. 7B). Lyophilized samples were solubilized in 0.2% $NaHCO_3$ adjusted to pH11.5, frozen for the desired time

period, thawed and applied to a Superdex® 75 column equilibrated with TN buffer pH 10.4.

Figure 8 shows SDS-PAGE analysis of recombinant wild type PsipB ("Original") and a recombinant modified PsipB (C66A). Lyophilized and reconstituted PsipB (original), prepared as described for Fig. 6C, and PsipB (C66A) were analyzed under reducing (right panel; "+ME") and non-reducing (left panel; "-ME") conditions.

Figure 9A shows preparative gel filtration of recombinant modified PsipB C66A following solubilization and refolding of inclusion bodies (IBs). IBs from 500 ml of fermentation culture were suspended in DDW and refolded in 33 ml of 4.5 M urea, 40 mM Tris and 1 mM cysteine pH 11.3. After 1.5 hr. at 4 °C, 100 ml of 0.67 M arginine was added and the solution was stirred overnight at 4°C. The next day, the refold mixture was concentrated to 30 ml and applied in two consecutive 15 ml portions onto a Superdex® 200 column pre-equilibrated with TN/Urea/Arg pH 10.0. The column was run at 4 °C at a rate of 2 ml/min. One hour after the application, 25 tubes containing samples (5 ml each) were collected. Protein concentration was estimated by absorbance at 280 nm.

Figure 9B shows SDS-PAGE (15%) of fractions eluted from the preparative column (shown in Fig 9A) run in presence of β-mercaptoethanol. Lanes 1 to 9 correspond to aliquots from fractions 14 to 22.

Figure 10 shows analytical gel filtration carried out on either Superdex 75 (Figs. 10A-C) or Superdex® 200 (Fig. 10D) of recombinant modified PsipB C66A following lyophilization and reconstitution. Fractions 15-20 from the experiment of Figure 9 were pooled, and applied to a Sephadex® G-25 column equilibrated with $NaHCO_3$ (1.2 mg/ml) pH 10. The obtained material was divided into aliquots, lyophilized and stored at -20 °C. The lyophilized PsipB C66A samples were reconstituted in TN buffer, pH 10.2 (Fig. 10A); 0.2% $NaHCO_3$ pH 11.0 (Fig. 10B), or 0.2% $NaHCO_3$ pH 11.5 (Fig. 10C and Fig. 10D), and applied to columns pre-equilibrated with TN buffer pH 10.2. The columns were run at 23 °C at a rate of .8 ml/min.

Figure 11 shows SDS-PAGE (15%) analysis of lyophilized PsipB C66A under reducing and non-reducing conditions.

Figure 12 shows reverse phase HPLC of recombinant modified PsipB C66A, using a lyophilized sample.

Figure 13 shows an immunoblot of recombinant modified PsipB C66A. Lyophilized samples prepared as described for Fig. 10 were subjected to SDS-PAGE under reducing conditions followed by transblotting and immunodetection using rabbit polyclonal antiserum against wild type PsipB (1:20,000 dilution).

Figure 14 shows reduction in bacterial colonization after immunization with the modified PsipB polypeptide PsipB C66A ("PsipBa"). BALB/c mice (7 weeks old) were immunized subcutaneously with three doses of either wild type PsipB or PsipB C66A formulated with adjuvant (5μg/mouse; CFA/IFA/IFA adjuvant) or with adjuvant-alone, with two weeks between immunizations. Two weeks after the last immunization the mice were challenged intranasally with *S. pneumoniae* serotype 3 strain WU2. The nasopharynx and lungs were excised, homogenized and plated onto blood agar plates (overnight). The extent of colonization in the nasopharynx (Fig. 14A; Naso WU2) and lungs (Fig. 14B; Lung WU2) at 48 hours is presented as bacterial CFU per 1 ml homogenate.

Figure 15. Immunoblot of different partially purified fractions of PsipBas stained with polyclonal anti PsipBa.

Figure16. SDS-PAGE (12 %) of lyophilized PsipBas dissolved in UPW in presence of ME and applied at 2, 6 and 20 μg per lane.

Figure 17 - Gel-filtration analysis of the purified lyophilized PsipBas on analytical Superdex 75 column pre-equilibrated with TN buffer, pH 10.5. The main peak with retention time of 15.22 min corresponds to dimer. To estimate the molecular mass, the column was calibrated with BSA (66 kDa), rat CNTF (22 kDa) and human leptin (16 kD).

Figure 18. Survival rates following IN challenge with *S. pneumoniae* serotype 3 strain WU2 of BALB/c mice immunized subcutaneously (SC) 3 times with 20 μg of PsipBas protein in adjuvant or with adjuvant alone.

Figure 19. Survival of mice following IP challenge with 1000 CFU of *S. pneumoniae* strain WU2 pre-treated with mouse anti-PsipBas sera *ex-vivo*. Following 1h incubation at RT, mixtures of antisera and 1000 CFU of WU2 bacteria were inoculated intraperitoneally (IP) to 7-week-old BALB/c mice, 10 mice per experimental group. Survival was monitored daily for seven consecutive.

DETAILED DESCRIPTION OF THE INVENTION

PsipB has previously been identified as a candidate for inclusion in a vaccine for protection against *S. pneumoniae* infection (see for example, Ling et al., Clin Exp Immunol

2004; 138:290-298). However, attempts to purify PsipB, either from *S. pneumoniae* extracts or from recombinant expression systems, generally yield an insoluble product at pH values in the physiological range, and a soluble product is obtained only when the protein is maintained in an environment of high pH, e.g. above about pH 10, or in an environment of neutral pH comprising heavy metal salts. Either of such formulations would be unsuitable for a human vaccine, thus not viable for product development. Isolated preparations of dimeric wild type PsipB have been obtained upon carrying out all processing and storage operations above pH 10 environment.

The present invention provides modified PsipB polypeptides and fragments which exhibit enhanced stability at physiological pH *in vitro* as compared to the wild type PsipB. Moreover, the modified PsipB polypeptides and fragments induce enhanced protective effect against *S. pneumoniae*, as compared to wild type PsipB. These characteristics render the modified PsipB polypeptides and fragments and the polynucleotides encoding them, as disclosed herein, viable and superior candidates for inclusion in immunogenic compositions, in particular vaccines, including multivalent vaccines.

Some of the polypeptide and polynucleotide sequences of the present invention are disclosed in the Sequence Listing. Sequences 1-12 correspond to wild type PsipB amino acid sequences. Sequences 13-16 correspond to modified PsipB polypeptides. Sequences 17-20 correspond to polynucleotides encoding the polypeptides of sequences 13-16. Sequences 21-24 correspond to mutant polypeptides based on SEQ ID NO 2. Sequences 25-28 correspond to mutant polypeptides based on SEQ ID NO 3. Sequences 29-32 correspond to mutant polypeptides based on SEQ ID NO 4. Sequences 33-36 correspond to mutant polypeptides based on SEQ ID NO 5. Sequences 37-40 correspond to mutant polypeptides based on SEQ ID NO 6. Sequences 41-44 correspond to mutant polypeptides based on SEQ ID NO 7. Sequences 45-48 correspond to mutant polypeptides based on SEQ ID NO 8. Sequences 49-51 correspond to mutant polypeptides based on SEQ ID NO 9. Sequences 52-54 correspond to mutant polypeptides based on SEQ ID NO 10. Sequences 55-58 correspond to mutant polypeptides based on SEQ ID NO 11. Sequences 59-62 correspond to mutant polypeptides based on SEQ ID NO 12. Sequence 63 corresponds to highly conserved fragment and sequences 64-67 to its mutants. Sequences 68-71 correspond to primers. Sequence 72 corresponds to polynucleotide encoding the fragment of sequence 73 and sequences 73-79 correspond to PsipBa mutant fragments.

Definitions

The terms "wild type Pneumococcus surface immunogenic protein B" and "wild type PsipB" are used herein interchangeably to refer to a naturally occurring protein comprising a region having at least about 95% identity with the region of position 48 to position 112 of GenBank accession number NP_358083, shown herein as SEQ ID NO:1, and wherein said region of said protein comprises cysteine residues at each of the positions corresponding to position 66 and position 84 of SEQ ID NO:1. Accordingly, a wild type PsipB may comprise a region having about 95%, 96%, 97%, 98%, 99% identity with the region of position 48 to position 112 of SEQ ID NO:1. Further, the complete amino acid sequence of a wild type PsipB may display a high degree of identity or similarity with SEQ ID NO:1, such as for example, about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity with SEQ ID NO:1. For example, the wild type PsipB proteins of SEQ ID NO:2 and SEQ ID NO:3 respectively have 99% and 97% identity with SEQ ID NO:1.

The terms "modified PsipB", "modified form of PsipB", "mutant PsipB", "variant PsipB", "derivative of PsipB" and the like interchangeably refer to a polypeptide which differs from a wild type PsipB amino acid sequence due to one or more amino acid substitutions, amino acid deletions, amino acid modifications and addition of heterologous amino acid sequences. It is to be explicitly understood that in preferred embodiments, the modified PsipB polypeptide or fragment of the invention induces an immunogenic response that is at least equivalent to that induced by a wild type PsipB, and in some more preferred embodiments, the induced immunogenic response is greater than induced by the wild type PsipB. In yet other embodiments, the modified PsipB polypeptide or fragment of the invention is more stable or possesses other advantageous properties over the wild type PsipB.

The term "residue at a position corresponding to a position of SEQ ID NO:1" refers to the amino acid residue present at a numerically represented position (from N-terminus to C-terminus) on a first stated PsipB protein sequence which is the counterpart of an amino acid present at the stated position of the PsipB protein sequence of SEQ ID NO:1, when those protein sequences are aligned with each other so as to optimize homology (i.e. similarity or identity). For example, the amino acid cysteine at position 66 (C66) of SEQ NO:2 corresponds to C66 of SEQ NO:1; C22 of SEQ NO:11 corresponds to C66 of SEQ NO:1; C19 of SEQ NO:12 corresponds to C66 of SEQ NO:1, and so forth.

Similarly, the term "a residue corresponding to a residue of the wild type PsipB polypeptide of SEQ ID NO:1" refers to the amino acid residue on a first stated PsipB protein sequence which is the counterpart of an amino acid of the PsipB protein sequence of SEQ ID NO:1, when those protein sequences are aligned with each other so as to optimize homology (i.e. similarity or identity).

The terms "*Streptococcus pneumoniae*" and "*S. pneumoniae*" as used herein encompass any strain, subtype or serotype of that organism.

The term "wild type" as used herein in reference to a polypeptide, refers to the amino acid sequence of the polypeptide as it occurs in nature. The term "in nature" encompasses for example, any strain, subtype or serotype of *S. pneumoniae*, including those maintained in strain collections and libraries. Similarly, the term "wild type" as used herein in reference to a nucleic acid sequence encoding the polypeptide, refers to the nucleic acid sequence as it occurs in nature.

The terms "derived from *S. pneumoniae*" and "*S. pneumoniae* derived" in reference to nucleic acid or polypeptide sequences, are used interchangeably herein to refer to the source from which the sequence was originally isolated or identified. Such sequences may or may not be present in other bacterial species, and may or may not be present in all *S. pneumoniae* strains.

The term "native conformation" as used herein in reference to a polypeptide, refers to the three dimensional conformation and/or subunit structure of a protein molecule as it occurs in its correctly folded state. For example, a wild type protein in its native conformation may be in the form of a dimer.

The term "mutant" as used herein in reference to an amino acid, DNA or RNA sequence means that such a sequence differs from, but has sequence identity with the wild type or disclosed sequence. The degree of sequence identity between the wild type or disclosed sequence and the mutant sequence is preferably greater than about 50%, and in many cases is about 60%, 70%, 80%, 90%, 95, 98% or more.

The terms "amino acid substitution" and "amino acid replacement" are used herein interchangeably to indicate a mutation at a given position in the amino acid sequence of a polypeptide, whereby a different amino acid residue occurs in place of the amino acid residue occurring in the original, parent or wild type sequence. Such a mutation does not alter the overall length of the polypeptide, in contrast to other types of mutations such as

deletions and additions.

The amino acids referred to herein encompass the natural coded amino acids, their derivatives, and D isomers, represented by either one-letter or three-letter codes according to conventions well known in the art. When there is no indication, the L isomer is intended, and the D isomers are indicated by "D" before the residue abbreviation. For use in the invention, amino acids are those which are available commercially or are available by routine synthetic methods. Certain residues may require special methods for incorporation into the peptide, and sequential, divergent and convergent synthetic approaches to the peptide sequence may be used.

The terms "protein" and "polypeptide" are used interchangeably herein to refer to amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain modified amino acids other than the 20 gene-encoded amino acids. The polypeptides may be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Modifications can occur anywhere in the polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given polypeptide. Also a given polypeptide may have many types of modifications. The term "polypeptide" further includes fusion proteins, including, but not limited to, fusion proteins with a heterologous amino acid sequence, fusions with heterologous and homologous leader sequences, with or without N-terminal methionine residues; immunologically tagged proteins; and the like.

Modifications of polypeptides and amino acids include acetylation; acylation; ADP-ribosylation; amidation; covalent attachment of non-peptide molecules such as flavin, a heme moiety, a nucleotide or nucleotide derivative, a lipid or lipid derivative, or a phosphytidylinositol; cross-linking cyclization; disulfide bond formation; demethylation; formation of covalent cross-links; formation of cysteine; formation of pyroglutamate; formylation; gamma-carboxylation; glycosylation; GPI anchor formation; hydroxylation; iodination; methylation; myristolyation; oxidation; pegylation; proteolytic processing; phosphorylation; prenylation; racemization; selenoylation; sulfation; and transfer-RNA mediated addition of amino acids to protein such as arginylation (see for example, Creighton, T. E., Proteins-Structure and Molecular Properties 2nd Ed., W. H. Freeman and

Company, New York (1993); Posttranslational Covalent Modification of Proteins, B. C. Johnson, Ed., Academic Press, New York, pp. 1-12 (1983)).

As used herein, a "fusion protein" or "chimeric protein" refers to a protein or polypeptide which comprises at least a portion of a first protein or polypeptide fused to least a portion of a second heterologous protein or polypeptide. For example, a fusion protein may comprise a modified PsipB polypeptide genetically fused to a his-tag.

As used herein, "heterologous" refers to two biological components that are not found together in nature. The components may be proteins or fragments thereof, host cells, genes or control sequences such as promoters. Although the heterologous components are not found together in nature, they can function together, such as when a promoter heterologous to a gene is operably linked to the gene.

The terms "polynucleotide", "nucleic acid sequence" and "nucleic acid" are used interchangeably herein to refer to polymeric forms of nucleotides of any length, either ribonucleotides or deoxynucleotides, including but are not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. Further included are mRNA or cDNA that comprise intronic sequences (see, e.g., Niwa et al. (1999) Cell 99(7):691-702). The backbone of the polynucleotide can comprise sugars and phosphate groups (as typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidites and thus can be an oligodeoxynucleoside phosphoramidate or a mixed phosphoramidate-phosphodiester oligomer (see e.g., Peyrottes et al. (1996) Nucl. Acids Res. 24:1841-1848; Chaturvedi et al. (1996) Nucl. Acids Res. 24:2318-2323). A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars, and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component, capping, substitution of one or more of naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support.

The terms "coding sequence of" and "coding region of", in reference to a particular polypeptide or protein, are used interchangeably herein to refer to a nucleic acid sequence which is transcribed and translated into the particular polypeptide or protein when placed under the control of appropriate regulatory sequences.

The term "polynucleotide sequence encoding a polypeptide" encompasses a polynucleotide which includes only coding sequence for the polypeptide, as well as a polynucleotide which includes additional coding and/or non-coding sequence. Examples of additional coding sequences include leader or secretory sequences. Examples of non-coding sequences or regulatory sequences such as promoters, transcription enhancers, etc., are well known in the art.

The term "identity", as used herein and as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences.

The term "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, described for example in, Computational Molecular Biology, Lesk, A.M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D.W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., SIAM J. Applied Math 1988, 48:1073).

Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, the GCG program package (Devereux, J., et al., Nucleic Acids Research 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F. et al., J. Molec. Biol. 215: 403-410 (1990). The BLAST X program is publicly available from NCBI and other sources (BLAST Manual, Altschul, S., et al., NCBI NLM NIH Bethesda, MD 20894; Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). As an illustration, by a polynucleotide having a

nucleotide sequence having at least, for example, 95% "identity" to a reference nucleotide sequence it is intended that the nucleotide sequence of the tested polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence. Analogously, a polypeptide having an amino acid sequence having for example, 95% identity to a reference amino acid sequence means that the test amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

The phrase "substantially identical" in the context of two nucleic acids or polypeptides, refers to two or more sequences that have at least 50%, 60%, 70%, 80%, and in some aspects 90-95% nucleotide or amino acid residue identity, when compared and aligned for maximum correspondence, as measured using one of the known sequence comparison algorithms or by visual inspection. Typically, the substantial identity exists over a region of at least about 100 residues, and most commonly the sequences are substantially identical over at least about 150-200 residues. In some embodiments, the sequences are substantially identical over the entire length of the coding regions.

A "substantially identical" amino acid sequence is a sequence that differs from a

reference sequence by one or more conservative or non-conservative amino acid substitutions, deletions, or insertions, and provided that the polypeptide essentially retains its functional and/or immunogenic and/or antibody binding properties. A conservative amino acid substitution, for example, substitutes one amino acid for another of the same class (e.g., substitution of one hydrophobic amino acid, such as isoleucine, valine, leucine, or methionine, for another, or substitution of one polar amino acid for another, such as substitution of arginine for lysine, glutamic acid for aspartic acid or glutamine for asparagine).

The term "complementary" as used herein refers to the broad concept of subunit sequence complementarity between two nucleic acids, e.g., two DNA molecules or a DNA/RNA hybrid. When a nucleotide position in both of the molecules is occupied by nucleotides normally capable of base pairing with each other, then the nucleic acids are considered to be complementary to each other at this position. Thus, two nucleic acids are complementary to each other when a substantial number (at least 50%) of corresponding positions in each of the molecules are occupied by nucleotides which normally base pair with each other (e.g., A:T and G:C nucleotide pairs).

"Hybridization" refers to the process by which a nucleic acid strand joins with a complementary strand through base pairing. Hybridization reactions can be sensitive and selective so that a particular sequence of interest can be identified even in samples in which it is present at low concentrations. Suitably stringent conditions (i.e. "high", "medium" or "low") can be defined by, for example, the concentrations of salt or formamide in the prehybridization and hybridization solutions, or by the hybridization temperature, and are well known in the art. In particular, stringency can be increased by reducing the concentration of salt, increasing the concentration of formamide, or raising the hybridization temperature.

The term "oligonucleotide" refers to either a single stranded polydeoxynucleotide or two complementary polydeoxynucleotide strands which may be chemically synthesized. Synthetic oligonucleotides generally lack 5' phosphate and thus will not ligate to another oligonucleotide without adding a phosphate with an ATP in the presence of a kinase.

The term "primer" as used herein, refers to an oligonucleotide which is capable of acting as a point of initiation of nucleic acid synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid

strand is induced, i.e. in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH. Primers may be obtained from a biological source, as in a purified restriction digest of genomic DNA, or produced synthetically. The primers are preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare amplification products. Preferably, the primers are oligodeoxyribonucleotides but must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and use of the method. The primers typically contain 10 or more nucleotides.

Synthetic oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods (Narang, S. A. et al. (1979) Meth. Enzymol. 68:90; Brown E. L., et al. (1979) Meth. Enzymol. 68:109) or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucauge et al. (1981) Tetrahedron Let. 22:1859-1962. One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066 which is incorporated herein by reference.

The term "digestion" in reference to a nucleic acid, in particular a DNA, refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes used herein are commercially available and their reaction conditions, cofactors and other requirements may be readily ascertained by the ordinarily skilled artisan. After digestion, gel electrophoresis may be performed to isolate the desired fragment, the latter of which is also referred to as a "restriction fragment".

As used herein, the term "isolated" means that the material is removed from its original environment. The original environment may be a natural environment if the material is naturally occurring, for example in a bacterial cell wall, or the original environment may be an artificial environment, if the material is artificial or engineered. For example, a naturally occurring polynucleotide or polypeptide present in a living organism, when separated from some or all of the coexisting materials in the natural system, is isolated. Similarly, a recombinantly engineered polynucleotide or the

corresponding expressed polypeptide, are referred to as isolated, when separated from a vector or expression system respectively containing the recombinant polynucleotide or expressed polypeptide.

As used herein, the term "purified" does not require absolute purity; rather, it is intended as a relative definition. The purified nucleic acid sequences of the invention have been purified from other sequences, such as the remainder of genomic DNA or from other sequences in a library or other environment by at least one order of magnitude, typically two or three orders, and more typically four or five orders of magnitude.

As used herein, the term "recombinant", in reference to a nucleic acid, means that the nucleic acid is adjacent to a "backbone" nucleic acid to which it is not adjacent in its natural environment. Backbone molecules according to the invention include nucleic acids such as expression vectors, self-replicating nucleic acids, viruses, integrating nucleic acids, and other vectors or nucleic acids used to maintain or manipulate a nucleic acid insert of interest.

As used herein, the term "recombinant", in reference to polypeptides or proteins, means polypeptides or proteins produced by recombinant DNA techniques, i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide or protein.

As used herein, "host cell" refers to a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous polynucleotide sequence, either in the form of a recombinant vector or other transfer DNA, and includes the progeny of the original cell which has been transfected or transformed.

As used herein, the term "control sequence" refers to a nucleic acid having a base sequence which is recognized by the host organism to effect the expression of encoded sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include a promoter, ribosomal binding site, terminators, and in some cases operators; in eukaryotes, generally such control sequences include promoters, terminators and in some instances, enhancers. The term control sequence is intended to include at a minimum, all components whose presence is necessary for expression, and may also include additional components whose presence is advantageous, for example, leader sequences.

As used herein, the term "operably linked" refers to sequences joined or ligated to

function in their intended manner. For example, a control sequence is operably linked to coding sequence by ligation in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequence and host cell. For example, a promoter sequence is "operably linked to" a coding sequence when RNA polymerase which initiates transcription at the promoter will transcribe the coding sequence into mRNA.

As used herein, the term "synthetic" in reference to polypeptides or protein sequences, means those that are those prepared by chemical synthesis.

The term "fragment" as used herein refers to a portion of the full-length polypeptide sequence of a protein. Fragments may be generated by synthetic methods known in the art (e.g. solid phase or liquid phase synthesis) or by recombinant techniques using polynucleotides that encode the desired fragment, or alternately fragments may be generated by enzymatic or chemical cleavage of the full-length protein, either that recombinantly or synthetically produced, or that isolated from a native source.

Physiological pH refers to the pH of the blood, typically within the range of 7.4-7.

The term “antibody” as used herein is used in the broadest sense and specifically encompasses monoclonal antibodies, humanized antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), single chain antibodies and antibody fragments (e.g., $F(ab')_2$, Fab', Fab, Fv) so long as they bind specifically to a target antigen or epitope of interest.

The term “epitope” as used herein refers to that portion of an antigen that is specifically recognized by a particular antibody and makes contact with the antigen-binding region of that antibody. When a protein or fragment of a protein is immunogenic in a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as “epitopes” or “antigenic determinants”. An antigenic determinant may compete with the intact antigen which elicited the immune response, for binding to an antibody.

The terms "specifically interacts" and "specifically binds" as used herein in reference to an antibody, interchangeably refer to high avidity and/or high affinity binding of the antibody to a specific polypeptide or epitope thereof, e.g., an epitope of PsipB. Antibody binding to its epitope is stronger than binding of the same antibody to any other epitope,

particularly those which may be present in molecules in association with, or in the same sample, as the specific polypeptide of interest. Antibodies which bind specifically to a polypeptide of interest may be capable of binding other polypeptides at a weak, yet detectable, level (e.g., 10% or less of the level of binding shown to the polypeptide of interest). Such weak binding, or background binding, is readily discernible from the specific antibody binding to the compound or polypeptide of interest, e.g., by use of appropriate controls.

The terms "immunogenic activity" and "immunogenicity" in reference to a PsipB polypeptide, are used herein interchangeably to refer to the ability of that polypeptide of raising an immune response which recognizes the PsipB polypeptide. The immune response may be a humoral and/or a cellular immune response.

The term "immunogenic composition" as used herein refers to a composition comprising a moiety, such as an *S. pneumoniae* polypeptide, analog or fragment thereof that is capable of eliciting a humoral and/or a cellular immune response in a host animal.

The term "vaccine" as used herein refers to a pharmaceutical composition comprising an attenuated or inactivated infectious agent or component thereof (for example an isolated protein or carbohydrate component) which is intended for immunizing a subject against that infectious agent, and thus has activity in preventing infection or treating infection by that infectious agent.

As used herein, an "immunogenic fragment of a wild type PsipB polypeptide" refers to a fragment of the PsipB polypeptide that is shorter than the full-length polypeptide and has at least about 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, 100%, or more of the ability of the full-length polypeptide to induce a humoral and/or cellular immune response in a host animal following administration to that host. It is especially preferred that the immune response be production of antibodies specific for PsipB. Fragments of interest can be made by recombinant, synthetic, or proteolytic digestive methods. Such fragments can then be isolated and tested for their ability to induce production of antibody specific for PsipB, using methods known in the art.

The term "effective amount" as used herein refers to an amount capable of producing the desired response, such as induction of a protective immune response.

The singular forms "a", "and", and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polypeptide" includes a

plurality of such polypeptides and reference to "the reagent" includes reference to one or more reagents and equivalents thereof known to those skilled in the art, and so forth.

Wild type PsipB polypeptides

The present invention provides modified PsipB polypeptides and polynucleotides encoding same, which are derived from or based on wild type PsipB polypeptide and polynucleotide sequences.

PsipB is a cell wall protein of *S. pneumoniae*, the antigenicity of which in humans increases with age PsipB, was isolated from *S. pneumoniae* strain WU2 and has an amino acid sequence 100% identical to that of the hypothetical protein having GenBank accession number NP_358083 (SEQ ID NO:1), identified by genomic sequencing of *S. pneumoniae* strain R6 (Hoskins et al., J. Bacteriol. 2001, 183(19):5709-5717). A wild type PsipB polypeptide having SEQ ID NO:1 was also identified by genomic sequencing of *S. pneumoniae* strain D39 (Lanie et al., J. Bacteriol. 2007, 189(1):38-51).

The recombinant cloning and expression of the gene corresponding to SEQ ID NO:1 (NP_358083) is described herein in Example 1. This wild type PsipB sequence was used as the basis for constructing modified PsipB polypeptides according to the invention, as described herein in Example 2.

Figure 1 shows a multiple sequence alignment of amino acid sequences of wild type PsipB polypeptides. Analysis using the SMART protein domain database indicates that all of these proteins contain a protein domain typically associated with flavoprotein oxygenases classified in the DIM6/NTAB family (flavin reductases).

As evident from Figure 1, these polypeptides share a high degree of homology, particularly in the region corresponding to residues 48-112 of SEQ ID NO:1, which is 96 to 100% identical among the sequences in Figure 1. Many PsipB polypeptides are highly homologous along their entire sequences; for example SEQ ID NOS:1-4 display 97 to 99% identity among each other.

Further, most wild type PsipB polypeptides include a conserved cluster of three cysteine residues. That is, a cysteine residue occurs at each of the positions corresponding to residues 66, 72 and 84 of SEQ ID NO:1, in each of SEQ ID NOS: 1 to 8, 11 and 12. Some wild type PsipB polypeptides, for example, SEQ ID NOS: 9 and 10, have a tyrosine residue at the second position in the cluster, corresponding to residue 72 of SEQ ID NO:1.

Any wild type PsipB, such as the sequences shown in Figure 1, may be used for producing the modified PsipB polypeptide of the invention, for example, SEQ ID NO:2, identified by genomic sequencing of *S. pneumoniae* strain 14453, as disclosed in U.S. Patent No. 6,699,703; SEQ ID NO:3, identified by genomic sequencing of *S. pneumoniae* strain 4, as disclosed in International Patent Application Publication No. WO 2002/077021 and corresponding to the hypothetical protein having GenBank accession number NP_345081; and SEQ ID NO:4, corresponding to the hypothetical protein having GenBank accession number YP_002742080, identified by genomic sequencing of *S. pneumoniae* Taiwan19F-14. Other wild type PsipB polypeptide sequences may be used as the basis for producing the modified PsipB polypeptide of the invention, for example SEQ ID NO:5 (ZP_01817263); SEQ ID NO:6 (ZP_01835575); SEQ ID NO:7 (ZP_01821338); SEQ ID NO:8 (ZP_01827178); SEQ ID NO:9 (ZP_01833557); SEQ ID NO:10 (YP_001835244); SEQ ID NO:11 (YP_002037228); and SEQ ID NO:12 (YP_002735619).

In a particular embodiment, the wild type PsipB polypeptide comprises an amino acid sequence that has at least about 90% identity with the region of position 48 to position 112 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide comprises an amino acid sequence that has at least about 95% identity with the region of position 48 to position 112 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide comprises an amino acid sequence that has at least about 97% identity with the region of position 48 to position 112 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide comprises an amino acid sequence that has at least about 98% identity with the region of position 48 to position 112 of SEQ ID NO:1.

In a particular embodiment, the wild type PsipB polypeptide comprises a cysteine residue at a position corresponding to a position of SEQ ID NO:1 selected from the group consisting of position 66, position 72, position 84 and a combination of residues thereof. In a particular embodiment, the wild type PsipB polypeptide has cysteine residues at each of the positions corresponding to position 66, position 72 and position 84 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide has a cysteine residue at each of the positions corresponding to position 66 and position 84 of SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide has a cysteine residue at each of the positions corresponding to position 66 and position 84 of SEQ ID NO:1 and further has a tyrosine residue at the position corresponding to position 72 of SEQ ID NO:1.

In a particular embodiment, the wild type PsipB polypeptide has an amino acid sequence that has at least about 90% identity with SEQ ID NO:1. In a particular embodiment, the wild type PsipB polypeptide has an amino acid sequence that has at least about 90% identity with SEQ ID NO:1, and said wild type PsipB polypeptide comprises a cysteine residue at a position corresponding to a position of SEQ ID NO:1 selected from the group consisting of position 66, position 72, position 84 and a combination of residues thereof. In a currently preferred embodiment, the wild type PsipB polypeptide has cysteine residues at each of the positions corresponding to position 66, position 72 and position 84 of SEQ ID NO:1.

As used herein, "at least 90% identity" includes 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% and 99% identity and intervals thereof. Further, there is no particular limitation on the strain, subtype or serotype which is the source of the wild type PsipB polypeptide.

Modified PsipB polypeptides

The modified PsipB polypeptides of the invention comprise at least one amino acid substitution of a cysteine residue naturally present in a wild type PsipB polypeptide. In a particularly preferred embodiment, the PsipB sequence to be modified is the sequence shown as SEQ ID NO:1 or a homologous protein comprising a sequence having at least about 90% identity with the region position 48 to position 112 of SEQ ID NO:1.

As shown in Figure 1, wild type PsipB polypeptides generally have a cysteine residue at the positions corresponding to positions 66, 72, and 84 of SEQ ID NO:1, denoted herein respectively as C66, C72 and C84. Accordingly, the amino acid substitutions incurred in the modified PsipB polypeptides may be at a position corresponding to any of C66, C72, C84, or a combination of residues thereof of SEQ ID NO:1. Thus, the modified PsipB polypeptides include those which are single, double and triple cysteine mutants, meaning that respectively one, two and three of the cysteine residues is/are mutated to a different residue. The amino acids C66, C72 and C84 are also referred to herein as the "first cysteine residue", the "second cysteine residue" and the "third cysteine residue", respectively.

In certain embodiments, the amino acid substitution may be a conservative or non-conservative substitution. In certain embodiments, the amino acid substitution comprises substitution of a cysteine residue by an amino acid residue selected from the group

consisting of alanine, serine, threonine, glycine, valine, leucine tyrosine and a combination of residues thereof.

In accordance with certain embodiments, double and triple cysteine mutants may comprise different kinds of amino acid substitutions. This means that in the same modified polypeptide, a cysteine at one position is substituted with one kind of amino acid, while a cysteine at a different position is substituted with a different kind of amino acid. For example, a modified PsipB polypeptide may be produced by substitution of the cysteine residue at position 66 by an alanine residue and substitution of the cysteine residue at position 72 by a serine residue. Many other variations may be envisioned and produced by one of skill in the art.

Suitable amino acid substitutions encompassed by the invention include: substitution of the cysteine residue at position 66 by an alanine residue (C66A); substitution of the cysteine residue at position 72 by an alanine residue (C72A); substitution of the cysteine residue at position 84 by an alanine residue (C84A), and any combination of residues thereof. Modified PsipB polypeptides which have a single mutation selected from C66A, C72A and C84A are referred to herein as PsipB C66A, PsipB C72A and PsipB C84A, respectively. A modified PsipB polypeptide which is a triple mutant, and thus includes all of the substitutions C66A, C72A and C84A, is referred to herein as PsipB C66AC72AC84A.

In a particular embodiment, the modified PsipB polypeptide comprises C66A. In some cases, C66A is the sole amino acid substitution of a cysteine residue in the modified PsipB polypeptide. Single mutants comprising C66A (or a mutation corresponding thereto) are disclosed herein and include SEQ ID NO:13; SEQ ID NO:21; SEQ ID NO:25; SEQ ID NO:29; SEQ ID NO:33; SEQ ID NO:37; SEQ ID NO:41; SEQ ID NO:45; SEQ ID NO:49; SEQ ID NO:52; SEQ ID NO:55; SEQ ID NO:59 and SEQ ID NO:64.

In a particular embodiment, the modified PsipB polypeptide comprises C66 and C84A i.e. "double mutants". Such double mutants are disclosed herein and include SEQ ID NO: 51 and SEQ ID NO:54.

In other cases, it may be preferable that the modified PsipB polypeptide comprises all of C66A, C72A and C84A i.e. "triple mutants". Such triple mutants are disclosed herein and include SEQ ID NO: 16; SEQ ID NO: 24; SEQ ID NO: 28; SEQ ID NO: 32; SEQ ID NO: 36; SEQ ID NO: 40; SEQ ID NO: 44; SEQ ID NO: 48; SEQ ID NO: 58; SEQ ID NO:

62; and SEQ ID NO: 67.

In a particular embodiment, the modified PsipB polypeptide is devoid of cysteine residues. In particular embodiments, the modified PsipB polypeptide has an amino acid sequence selected from the group consisting of SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15 and SEQ ID NO:16. Construction and expression of exemplary modified polypeptides is described herein in Example 2.

The invention further encompasses modified PsipB polypeptides which have at least about 80% identity to any of SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15 and SEQ ID NO:16. Such polypeptides may have about 85%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity to any of SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15 and SEQ ID NO:16.

The invention also provides a modified PsipB polypeptide which corresponds to an immunogenic fragment of a wild type PsipB polypeptide and which has been mutated in one or more positions corresponding to the cysteine residues C66, C72 or C84 of the wild type PsipB polypeptide of SEQ ID NO:1, as described above. Such a fragment is also referred to herein as a "modified PsipB immunogenic fragment".

An immunogenic fragment is a contiguous portion of a PsipB polypeptide which has at least the same or substantially the same immunogenic activity as the full-length PsipB polypeptide, for example the immunogenic activity of a PsipB amino acid sequence selected from SEQ ID NOS:1-4.

In a particular embodiment, the modified PsipB immunogenic fragment may be selected from SEQ ID NOs:64-67 and SEQ ID NOs: 73-79.

Use of a modified PsipB immunogenic fragment may be preferably provided, for example, as a part of a fusion protein with other immunogenic *S. pneumoniae* proteins. For such a fusion protein, it may be preferable and advantageous to eliminate, where possible, non-epitope regions of the protein, so as to avoid steric hindrance with the other proteins and /or epitopes of the fusion protein.

Identification of one or more immunogenic fragments of a wild type PsipB polypeptide may be readily carried out by one of skill in the art. An immunogenic fragment may be devoid of a specific region of the wild type PsipB that is not part of an immunogenic epitope, for example, the N-terminal leader sequence, and/or a C-terminal

anchor domain.

In a particular embodiment, the immunogenic fragment comprises a B-cell epitope.

In particular embodiments, the immunogenic fragment comprises one or more structural or functional features, such as alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding

A modified PsipB immunogenic fragment may be in the range of about 50 to about 120 amino acids. According to some embodiments the modified PsipB immunogenic fragment is in the range of 65-120 amino acids. In a particular embodiment, the immunogenic fragment comprises an amino acid substitution selected from the group consisting of: C66A; C72A; C84A, and a combination of residues thereof, using the position numbering of SEQ ID NO:1. In a particular embodiment, the immunogenic fragment comprises all of C66A, C72A and C84A. It is to be specifically understood that the immunogenic fragment may have amino acid deletions at either or both of the amino-terminal portion and the carboxy-terminal portion relative to the wild type PsipB polypeptide. Accordingly, the numbering used herein refers to the amino acid residues of the original wild type PsipB polypeptide, and may not necessarily reflect the position numbering of the modified PsipB immunogenic fragment of the invention.

The modified PsipB polypeptide may further comprise one or more mutations at non-cyteine residues present in a wild type PsipB polypeptide. For example, one or more Asp and/or Glu residues present in a wild type PsipB polypeptide may be replaced by an Asn and/or Gln residue, respectively. Also of potential interest is the replacement of one or more Lys residues present in a wild type PsipB polypeptide by an Arg. The modified PsipB polypeptides or immunogenic fragments thereof possess a degree of immunogenicity that is at least equivalent to or greater than, the degree of immunogenicity of the corresponding wild type PsipB polypeptide. As shown herein in Example 6, a modified PsipB polypeptide of SEQ ID NO:13 exhibits enhanced immunogenicity and protective effect as compared to the wild type PsipB polypeptide of SEQ ID NO:1.

The invention further encompasses a fusion protein comprising a modified PsipB polypeptide or immunogenic fragment thereof, and a heterologous peptide or polypeptide sequence. The heterologous sequence may be, for example, a different *S. pneumoniae*

immunogenic polypeptide or fragment thereof, or a sequence which enables purification of the modified PsipB polypeptide e.g. poly-his, or one which facilitates formation of a conjugate or multimer.

Production of modified PsipB polypeptides

The modified PsipB polypeptides disclosed herein may be produced by either recombinant or chemical synthetic methods. In currently preferred embodiments, the modified PsipB polypeptide is a product of recombinant expression.

The modified PsipB polypeptides of the invention are preferably synthesized by expressing a polynucleotide molecule encoding the modified polypeptide in a host microorganism transformed with the nucleic acid molecule. Such a polynucleotide may be produced by mutation of a first polynucleotide encoding a wild type PsipB polypeptide, so as to provide a second polynucleotide which encodes a modified PsipB polypeptides having replacements of one or more cysteine residues which are normally present in the wild type PsipB.

A DNA sequence encoding a wild type PsipB polypeptide may be isolated from any strain, subtype or serotype of *S. pneumoniae* or other microorganism producing the recombinant PsipB, using various methods well known in the art (see for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989)). Any of genomic DNA, a genomic DNA library and/or cDNA library may be prepared using chromosomal DNA or messenger RNA from the microorganism that produces the PsipB to be modified. When the DNA sequence of the wild type PsipB is known, homologous, labeled oligonucleotide probes may be synthesized and used to identify DNA fragments or clones containing the PsipB gene present in the genomic DNA or library using well known hybridization techniques. DNA fragments and/or clones carrying DNA identical to that of the probe can then be distinguished using stringent hybridization conditions. Sequencing of the individual fragments identified by hybridization may be performed with sequencing primers designed from the original polypeptide or polynucleotide sequence, and it is further possible to extend the polynucleotide sequence in both directions to determine a full length gene sequence. The relevant DNA fragment(s) may be isolated and further manipulated for cloning in an appropriate vector, using appropriate techniques, well known in the art.

Appropriate probes generally comprise at least 15 nucleotide residues or base pairs,

preferably, at least 30 nucleotide residues or base pairs and may have at least 50 nucleotide residues or base pairs. Particularly preferred are probes having between about 20-30 nucleotide residues or base pairs. Appropriate probes may be constructed for example, to correspond to DNA segments encoding a region of a known wild type PsipB, such as those shown in SEQ ID NOS:1-12. DNA sequences used to prepare the probes may be determined on the basis of reference to a database including the sequence of the known wild type PsipB, such as the TIGR database maintained by the Institute for Genomic Research (http://www.tigr.org/).

Alternately, a DNA sequence encoding a wild type PsipB polypeptide of known sequence may be isolated from genomic DNA of an appropriate *S pneumoniae* strain or other microorganism by polymerase chain reaction (PCR) using specific primers, constructed on the basis of the nucleotide sequence of a known wild type PsipB sequence, such as for example those shown in SEQ ID NOS:1-12. Suitable techniques are well known in the art, described for example in U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159 and 4,965,188.

Upon isolation and cloning of a polynucleotide encoding a wild type PsipB polypeptide, the desired mutation(s) may be introduced by modification at one or more base pairs, using methods known in the art, such as for example, site-specific mutagenesis (see for example, Kunkel Proc. Natl. Acad. Sci. USA 1985, 82:488-492; Weiner et al., Gene 1994, 151:119-123; Ishii et al., Methods Enzymol. 1998, 293:53-71); cassette mutagenesis (see for example, Kegler-Ebo et al., Nucleic Acids Res. 1994 May 11; 22(9):1593–1599); recursive ensemble mutagenesis (see for example, Delagrave et al., Protein Engineering 1993, 6(3):327-331), and gene site saturation mutagenesis (see for example, U.S. Pat. Application Publication No. 2009/0130718).

Methods are also well known for introducing multiple mutations into a polynucleotide (see for example, Michaelian et al., Nucleic Acids Res. 1992, 20:376; Dwivedi et al., Anal. Biochem. 1994, 221:425-428; Bhat Methods Mol. Biol. 1996, 57:269-277; Meetei et al., Anal. Biochem. 1998, 264:288-291; Kim et al., Biotechniques 2000, 28:196-198; and International patent Application Publication Nos. WO 03/002761A1 and WO 99/25871).

An alternative method to PCR is the use of synthetic gene. The method allows production of an artificial gene which comprises an optimized sequence of nucleotide to be

express in desired species (for example *E. coli*). Redesigning a gene offers a means to improve gene expression in many cases. Rewriting the open reading frame is possible because of the redundancy of the genetic code. Thus it is possible to change up to about a third of the nucleotides in an open reading frame and still produce the same protein. For a typical protein sequence of 300 amino acids there are over 10^{150} codon combinations that will encode an identical protein. Using optimization methods such as replacing rarely used codons with more common codons can result in dramatic effects on levels of expression of protein encoded by the target gene. Further optimizations such as removing RNA secondary structures can also be included. Computer programs are available to perform these and other simultaneous optimizations. Because of the large number of nucleotide changes made to the original DNA sequence, the only practical way to create the newly designed genes is to use gene synthesis.

An alternative approach is cloning the gene into pET30+ vector omitting the His-tag sequence by the use of NdeI restriction enzyme to produce the first metionine.

Alternatively, a polynucleotide encoding a modified PsipB or immunogenic fragment thereof may be prepared synthetically, for example using the phosphoroamidite method (see, Beaucage et al., Curr Protoc Nucleic Acid Chem. 2001 May; Chapter 3:Unit 3.3; Caruthers et al., Methods Enzymol.1987, 154:287-313).

The polynucleotide thus produced may then be subjected to further manipulation including one or more of purification, annealing, ligation, amplification, restriction endonuclease digestion and cloning in appropriate vectors. The polynucleotide may be ligated either initially into a cloning vector, or directly into an expression vector that is appropriate for its expression in a particular host cell type.

Polypeptides of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences, such as a thrombin cleavage site.

Upon transformation of a suitable host cell, and propagation under conditions appropriate for protein expression, the modified PsipB polypeptide may be identified and analyzed for its various properties including immunogenicity, using methods known in the art. Transformed hosts expressing modified PsipB may be identified by analyzing the proteins expressed by the host using SDS-PAGE and comparing the gel to an SDS-PAGE

gel obtained from the host which was transformed with the same vector but not containing a nucleic acid sequence encoding PsipB or modified PsipB.

Modified PsipB protein can also be identified by other known methods such as immunoblot blot analysis using anti-PsipB antibodies, dot blotting of total cell extracts, limited proteolysis, mass spectrometry analysis, and combinations thereof.

Modified PsipB protein which has been identified in cell extracts may be isolated and purified by conventional methods, including ammonium sulfate or ethanol precipitation, acid extraction, salt fractionation, ion exchange chromatography, hydrophobic interaction chromatography, gel permeation chromatography, affinity chromatography, and combinations thereof.

Modified PsipB protein in the form of inclusion bodies may be isolated following several inclusion body washes using for example, combinations of buffers and detergent additives. The protein may be further purified under denaturing conditions by dissolving the washed inclusion bodies in urea or guanidine HCl followed by gel filtration chromatography. Protein refolding may be carried out by procedures known in the art, and may be followed by additional purification steps such as gel filtration chromatography, ion exchange chromatography and combinations thereof.

Conditions for carrying out the aforementioned procedures as well as other useful methods are readily determined by those of ordinary skill in the art (see for example, Current Protocols in Protein Science, 1995 John Wiley & Sons).

In particular embodiments, the polypeptides of the invention can be produced and/or used without their start codon (methionine or valine) to favor production and purification of recombinant polypeptides. It is known that cloning genes without sequences encoding leader peptides will restrict the polypeptides to the cytoplasm of *E. coli* and will facilitate their recovery (see for example, Glick, B. R. and Pasternak, J. J. (1998) In "Molecular biotechnology: Principles and applications of recombinant DNA", 2nd edition, ASM Press, Washington D.C., p. 109-143).

An analytical purification generally utilizes three properties to separate proteins. First, proteins may be purified according to their isoelectric points by running them through a pH graded gel or an ion exchange column. Second, proteins can be separated according to their size or molecular weight via size exclusion chromatography or by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis. Proteins are

often purified by using 2D-PAGE and are then analysed by peptide mass fingerprinting to establish the protein identity. Thirdly, proteins may be separated by polarity/hydrophobicity via high pressure liquid chromatography or reversed-phase chromatography. The purified protein is followed by its molecular mass or other methods known in the art.

In order to evaluate the process of multistep purification, the amount of the specific protein has to be compared to the amount of total protein. The latter can be determined by the Bradford total protein assay or by absorbance of light at 280 nm, however some reagents used during the purification process may interfere with the quantification. For example, imidazole (commonly used for purification of polyhistidine-tagged recombinant proteins) is an amino acid analogue and at low concentrations will interfere with the bicinchoninic acid (BCA) assay for total protein quantification. Impurities in low-grade imidazole will also absorb at 280 nm, resulting in an inaccurate reading of protein concentration from UV absorbance.

Another method to be considered is Surface Plasmon Resonance (SPR). SPR can detect binding of label free molecules on the surface of a chip. If the desired protein is an antibody, binding can be translated to directly to the activity of the protein. One can express the active concentration of the protein as the percent of the total protein. SPR can be a powerful method for quickly determining protein activity and overall yield.

While the modified PsipB polypeptides of the invention are typically made by recombinant means, as described above, they may also be produced by synthetic means using well known techniques, in particular solid phase synthesis (see for example, Merrifield, R. B., J. Am. Chem. Soc., 85:2149-2154, 1963; Stewart, J. M. and Young, J. D., Solid Phase Peptide Synthesis, 2nd Ed., Pierce Chemical Co., Rockford, Ill., pp. 11-12). Synthetic peptides may be produced using commercially available laboratory peptide design and synthesis kits (see for example, Geysen et al, Proc. Natl. Acad. Sci., USA 1984, 81:3998). In addition, a number of available FMOC peptide synthesis systems are available. Assembly of a polypeptide or fragment can be carried out on a solid support using for example, an Applied Biosystems, Inc. Model 431A automated peptide synthesizer. The polypeptides may be made by either direct synthesis or by synthesis of a series of fragments that can be coupled using other known techniques.

Polynucleotides

The invention further provides isolated polynucleotide sequences encoding the disclosed modified PsipB polypeptides. In currently preferred embodiments, the modified PsipB polypeptide encoded by the polynucleotide has an amino acid residue other than cysteine at a position corresponding to a position of the wild type PsipB polypeptide of SEQ ID NO:1 selected from the group consisting of positions 66, 72, 84 , and a combination of residues thereof. In a particular embodiment, the amino acid residue other than cysteine is selected from the group consisting of alanine, serine, threonine, glycine, valine, tyrosine and leucine.

In a particular embodiment, the polynucleotide sequence encodes a modified PsipB polypeptide comprising an amino acid substitution selected from the group consisting of C66A, C72A, C84A and a combination of residues thereof.

As is readily apparent to those of skill in the art, the codon used in the polynucleotide for encoding a particular amino acid which is to substitute for cysteine, should be selected in accordance with the known and favored codon usage of the host cell selected for expressing the polynucleotide.

In particular embodiments, the polynucleotide sequence encodes a modified PsipB polypeptide selected from: SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15, and SEQ ID NO:16. In particular embodiments, the polynucleotide sequence encodes a modified PsipB polypeptide selected from: SEQ ID NOS:21-62, or an immunogenic fragment of a modified PsipB polypeptide selected from: SEQ ID NOS:64-67 and 73-79. In particular embodiments, the polynucleotide sequence comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19 and SEQ ID NO:20. In particular embodiments, the polynucleotide sequence has a nucleic acid sequence selected from the group consisting of SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19 and SEQ ID NO:20. Further encompassed by the invention are polynucleotides that are at least 70% identical over their entire length to a polynucleotide encoding a modified PsipB polypeptide, and polynucleotides that are complementary to such polynucleotides. In particular embodiments, the modified PsipB polypeptide has an amino acid sequence selected from: SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15, and SEQ ID NO:16. . In particular embodiments, the modified PsipB polypeptide has an amino acid sequence selected from: SEQ ID NOS:21-67. More preferably, such polynucleotides are at least 80%

identical, or at least 90% identical over their entire length to a polynucleotide encoding a modified PsipB polypeptide. Even more preferably, such polynucleotides are at least 95% identical over their entire length to a polynucleotide encoding a modified PsipB polypeptide. Most preferably, such polynucleotides are at least 97% identical, such as 98% identical or 99% identical, over their entire length to a polynucleotide encoding a modified PsipB polypeptide.

Further encompassed by the invention are polynucleotides that are at least 70% identical over their entire length to a polynucleotide selected from the group consisting of SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19 and SEQ ID NO:20, and polynucleotides that are complementary to such polynucleotides. More preferably, such polynucleotides are at least 80% identical, or at least 90% identical over their entire length to a polynucleotide selected from the group consisting of SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19 and SEQ ID NO:20. Even more preferably, such polynucleotides are at least 95% identical over their entire length to a polynucleotide selected from the group consisting of SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19 and SEQ ID NO:20. Most preferably, such polynucleotides are at least 97% identical, such as 98% identical or 99% identical, over their entire length to a polynucleotide selected from the group consisting of SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19 and SEQ ID NO:20.

The invention further encompasses polynucleotides that contain non-coding sequences, including for example, non-coding 5' and 3' sequences, such as transcribed, non-translated sequences, termination signals, ribosome binding sites, sequences that stabilize mRNA, introns and polyadenylation signals. Further included are polynucleotides that comprise coding sequences for additional amino acids heterologous to the modified PsipB polypeptide, in particular a marker sequence, such as a poly-his tag, that facilitates purification of the polypeptide in the form of a fusion protein.

The invention further encompasses polynucleotides that hybridize to the polynucleotide sequences provided herein. In this regard, the invention especially encompasses polynucleotides that hybridize under stringent conditions to the polynucleotides described herein. As herein used, the terms "stringent conditions" and "stringent hybridization conditions" mean hybridization occurring only if there is at least 95% and preferably at least 97% identity between the sequences. A specific example of stringent hybridization conditions is overnight incubation at 42° C in a solution comprising:

50% formamide, 5xSSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5.times.Denhardt's solution, 10% dextran sulfate, and 20 micrograms/ml of denatured, sheared salmon sperm DNA, followed by washing the hybridization support in 0.1xSSC at about 65° C. Hybridization and wash conditions are well known (see for example, Sambrook, et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989)).

Vectors, expression systems and host cells

The polynucleotide encoding the polypeptide of the invention may be incorporated into a wide variety of expression vectors, which may be transformed into in a wide variety of host cells. The host cell may be prokaryotic or eukaryotic.

Introduction of a polynucleotide into the host cell can be effected by well known methods, such as calcium phosphate transfection, DEAE-dextran mediated transfection, transvection, microinjection, cationic lipid-mediated transfection, electroporation, conjugation, transduction, scrape loading, ballistic introduction and infection.

Representative examples of appropriate hosts include bacterial cells, such as cells of streptococci, staphylococci, enterococci, *E. coli*, streptomyces, cyanobacteria, *Bacillus subtilis, Neisseria meningitidis, Haemophilus influenzae and Moraxella catarrhalis*; fungal cells, such as cells of a yeast, *Kluveromyces, Saccharomyces, Pichia*, a basidiomycete, *Candida albicans* and *Aspergillus*; insect cells such as cells of Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS, HeLa, C127, 3T3, BHK, 293, CV-1 and Bowes melanoma cells; and plant cells, such as cells of a gymnosperm or angiosperm.

Appropriate expression vectors that can be used to produce the polypeptides of the invention include, among others, chromosomal-, episomal- and virus-derived vectors, for example, vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses, picornaviruses, retroviruses, and alphaviruses and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. The expression system constructs may contain control regions that regulate as well as engender expression. Generally, any system or vector suitable to maintain, propagate or express polynucleotides and/or to express a polypeptide in a host may be used for expression. The

appropriate DNA sequence may be inserted into the expression system by any of a variety of well-known and routine techniques, such as, for example, those

Vectors for expressing proteins in *E. coli* are particularly useful for expressing prokaryotic proteins, such as those from *S. pneumoniae*. Such vectors include, but are not limited to, pK233, pT7, pET and lambda pSKF. Examples of vectors that express fusion proteins include PATH vectors (Dieckmann and Tzagoloff (1985) J. Biol. Chem. 260:1513-1520) which contain DNA sequences that encode anthranilate synthesis (TrpE) followed by a polylinker at the carboxy terminus. Two non-limiting examples of fusion constructs are Thiofusion and His-Tag which can be isolated and purified by conventional methods. Other expression vector systems are based on beta-galactosidase (pEX); maltose binding protein (pMAL); and glutathione S-transferase (pGST).

Selection of a host cell transformed with the desired vector may be accomplished using standard selection protocols involving growth in a selection medium which is toxic to non-transformed cells. For example, *E. coli* is grown in a medium containing an antibiotic selection agent; cells transformed with the expression vector which further provides an antibiotic resistance gene, will grow in the selection medium.

Vaccines and pharmaceutical compositions

The invention further provides vaccines and pharmaceutical compositions comprising a modified PsipB polypeptide and a pharmaceutically acceptable carrier, diluent or adjuvant. In currently preferred embodiments, the modified PsipB polypeptide has an amino acid residue other than cysteine at a corresponding to a position selected from the group consisting of positions 66, 72, 84 and a combination of residues thereof of the wild type PsipB polypeptide of SEQ ID NO:1. In particular embodiments, the modified PsipB polypeptide is selected from the group consisting of SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15, and SEQ ID NO:16. In particular embodiments, the modified PsipB polypeptide has an amino acid sequence selected from: SEQ ID NOS:21-62. The modified PsipB polypeptide of the vaccine may be an immunogenic fragment of a wild type PsipB polypeptide, wherein the immunogenic fragment further comprises an amino acid substitution selected from the group consisting of C66A, C72A, C84A, and a combination of residues thereof. In particular embodiments, the immunogenic fragment of a wild type PsipB polypeptide is selected from the group consisting of SEQ ID NOS:64-67

and 73-79. As used herein, the stated position numbers refer to the positions of the wild type PsipB polypeptide of SEQ ID NO:1.

The vaccine may comprise a plurality or mixture of modified PsipB polypeptides. In particular embodiments, the modified PsipB polypeptides comprising the plurality are identical or non-identical. In particular embodiments, the modified PsipB polypeptides comprising the plurality are adjacent or non-adjacent. In particular embodiments, the modified PsipB polypeptides comprising the plurality form a repeating unit.

In a particular embodiment, the vaccine comprises a fusion protein.

The vaccine may further comprise one ore more additional *S. pneumoniae* derived polypeptides, fragments, or variants thereof in addition to the modified PsipB polypeptide. Such additional *S. pneumoniae* derived polypeptides, fragments, or variants thereof are also termed herein "heterologous *S. pneumoniae* polypeptides". A vaccine comprising a modified PsipB polypeptide and at least one heterologous *S. pneumoniae* polypeptide is also referred to herein as a "multimer". In another aspect the present invention provides a synthetic or recombinant polypeptide (herein denoted "multimer") comprising a plurality of *S. pneumoniae* derived polypeptide fragments. The multimer may contain a plurality of repeats not necessarily adjacent, of a specific fragment, a plurality of different fragments, a plurality of repeats of a plurality of fragments, or a combination of any of those options.

Polypeptide candidates for inclusion in a multimer vaccine are disclosed for example in WO 02/077021; WO 03/082183; U.S. Patent Application Publication No. 2009/0252756; U.S. Patent Nos. 5,965,141; 6,764,686; 6,699,703; 6,887,480; 7,078,042 and 7,132,107.

In a particular embodiment, the multimer comprises a plurality of modified PsipB polypeptides, as described herein. In a particular embodiment, the multimer comprises a fusion protein. In a particular embodiment, the multimer comprises a conjugate with a carrier protein. Suitable carrier proteins include, without limitation, tetanus toxin, diphtheria toxin, hepatitis B virus surface antigen, poliomyelitis virus VP1 antigen or any other viral or bacterial toxin or antigen which is capable of stimulating the development of a stronger immune response upon administration of the vaccine to a subject.

Multimers and conjugates may be formed using chemical techniques, for example employing cross-linkers such as avidin/biotin, gluteraldehyde or dimethylsuperimidate. Methods of peptide-carrier conjugation are known in the art, as described for example in

Van Regenmortel et al., (Synthetic Polypeptides as antigens. in Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 19 (ed.) Burdou, R. H. & Van Knippenberg P. H. (1988), Elsevier N. Y.).

Vaccines according to the invention may either be prophylactic (i.e. to prevent infection) or therapeutic (i.e. to treat disease after infection), but will typically be prophylactic.

The compositions and vaccines comprise an effective amount of antigen, which means that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention of disease. This amount varies depending upon the health and physical condition of the individual to be treated, age, the taxonomic group of individual to be treated (e.g. non-human primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials.

The vaccines and compositions of the invention will typically comprise one or more pharmaceutically acceptable carriers, which include any carrier that is not harmful to the individual receiving the composition. Suitable carriers include macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, trehalose and lipid aggregates (such as oil droplets or liposomes). The vaccines may also contain diluents, such as water, saline, glycerol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present. A thorough discussion of pharmaceutically acceptable excipients is available for example, in Handbook of Pharmaceutical Excipients, 6th Edition (2009) Rowe R.C., Sheskey P.J., Quinn M.E. (Eds.) Pharmaceutical Press. The vaccine may be substantially devoid of an adjuvant, or alternately may include an adjuvant. Pharmaceutically acceptable adjuvants include, but are not limited to water-in-oil emulsion formulations, oil-in-water emulsions, lipid emulsions, and liposomes. According to specific embodiments the adjuvant may be selected from: Montanide®; alum; muramyl dipeptide; Gelvac®; MF59™; chitin microparticles; chitosan; cholera toxin subunit B; labile toxin; AS21A; AS02V; Intralipid®; Lipofundin; monophosphoryl lipid A (MPL) or 3-O-deacylated MPL; RIBI ®(monophosphoryl lipid A with mycobacterial cell wall

components); saponin adjuvants, such as QS21 or Stimulon™; ISCOMs (immune stimulating complexes); oligonucleotides comprising CpG; DNA vaccines such as pVAC; Complete Freund's Adjuvant (CFA); Incomplete Freund's Adjuvant (IFA); immune enhancers such as cytokines such as interleukins (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 (WO99/44636), etc.), interferons (e.g. gamma interferon), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF); a polyoxyethylene ether or a polyoxyethylene ester; a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol; or other substances that act as immunostimulating agents to enhance the efficacy of the composition.

The vaccine composition may be formulated by: encapsulating an antigen or an antigen/adjuvant complex in liposomes to form liposome-encapsulated antigen and mixing the liposome-encapsulated antigen with a carrier comprising a continuous phase of a hydrophobic substance. If an antigen/adjuvant complex is not used in the first step, a suitable adjuvant may be added to the liposome-encapsulated antigen, to the mixture of liposome-encapsulated antigen and carrier, or to the carrier before the carrier is mixed with the liposome-encapsulated antigen. The order of the process may depend on the type of adjuvant used. Typically, when an adjuvant like alum is used, the adjuvant and the antigen are mixed first to form an antigen/adjuvant complex followed by encapsulation of the antigen/adjuvant complex with liposomes. The resulting liposome-encapsulated antigen is then mixed with the carrier. The term "liposome-encapsulated antigen" may refer to encapsulation of the antigen alone or to the encapsulation of the antigen/adjuvant complex depending on the context. This promotes intimate contact between the adjuvant and the antigen and may, at least in part, account for the immune response when alum is used as the adjuvant. When another adjuvant is used, the antigen may be first encapsulated in liposomes and the resulting liposome-encapsulated antigen is then mixed into the adjuvant in a hydrophobic substance.

In formulating a vaccine composition that is substantially free of water, antigen or antigen/adjuvant complex is encapsulated with liposomes and mixed with a hydrophobic substance. In formulating a vaccine in an emulsion of water-in-a hydrophobic substance, the antigen or antigen/adjuvant complex is encapsulated with liposomes in an aqueous medium followed by the mixing of the aqueous medium with a hydrophobic substance. In the case of the emulsion, to maintain the hydrophobic substance in the continuous phase,

the aqueous medium containing the liposomes may be added in aliquots with mixing to the hydrophobic substance.

In all methods of formulation, the liposome-encapsulated antigen may be freeze-dried before being mixed with the hydrophobic substance or with the aqueous medium as the case may be. In some instances, an antigen/adjuvant complex may be encapsulated by liposomes followed by freeze-drying. In other instances, the antigen may be encapsulated by liposomes followed by the addition of adjuvant then freeze-drying to form a freeze-dried liposome-encapsulated antigen with external adjuvant. In yet another instance, the antigen may be encapsulated by liposomes followed by freeze-drying before the addition of adjuvant. Freeze-drying may promote better interaction between the adjuvant and the antigen resulting in a more efficacious vaccine, as well as maintenance of stability.

Formulation of the liposome-encapsulated antigen into a hydrophobic substance may also involve the use of an emulsifier to promote more even distribution of the liposomes in the hydrophobic substance. Typical emulsifiers are well-known in the art and include mannide oleate (Arlacel™ A), lecithin, Tween™ 80, Spans™ 20, 80, 83 and 85. The emulsifier is used in an amount effective to promote even distribution of the liposomes. Typically, the volume ratio (v/v) of hydrophobic substance to emulsifier is in the range of about 5:1 to about 15:1.

Microparticles and nanoparticles employ small biodegradable spheres which act as depots for vaccine delivery. The major advantage that polymer microspheres possess over other depot-effecting adjuvants is that they are extremely safe and have been approved by the Food and Drug Administration in the US for use in human medicine as suitable sutures and for use as a biodegradable drug delivery system (Langer R. Science. 1990; 249(4976):1527-33). The rates of copolymer hydrolysis are very well characterized, which in turn allows for the manufacture of microparticles with sustained antigen release over prolonged periods of time (O'Hagen, et al., Vaccine. 1993;11(9):965-9).

CCS/C® is a synthetic polycationic sphingolipid derived from D-erythro ceramide to which spermine is covalently attached, thereby forming Ceramide Carbamoyl Spermine (CCS). CCS mixed with cholesterol (CCS/C) self-assembles into liposomes known as VaxiSome. Based on its structure and components (ceramide, CO_2 and spermine), CCS is predicted to be biocompatible and biodegradable. In vitro and in vivo studies suggest that the CCS/C formulation up-regulates levels of CD40 and B7 co-stimulatory molecules,

which are essential in antigen presentation and T-helper cell activation. As a result, VaxiSome is a potent liposomal adjuvant/delivery system for stimulating enhanced immune responses via the Th1 and Th2 pathways.

Parenteral administration of microparticles elicits long-lasting immunity, especially if they incorporate prolonged release characteristics. The rate of release can be modulated by the mixture of polymers and their relative molecular weights, which will hydrolyze over varying periods of time. Without wishing to be bound to theory, the formulation of different sized particles (1 μm to 500 μm) may also contribute to long-lasting immunological responses since large particles must be broken down into smaller particles before being available for macrophage uptake. In this manner a single- injection vaccine could be developed by integrating various particle sizes, thereby prolonging antigen presentation.

In some applications an adjuvant or excipient may be included in the vaccine formulation. Alum for example, is a preferred adjuvant for human use. The choice of the adjuvant will be determined in part by the mode of administration of the vaccine. One preferred mode of administration is intramuscular administration. Another preferred mode of administration is intranasal administration. Non-limiting examples of intranasal adjuvants include chitosan powder, PLA and PLG microspheres, QS-21, AS02V, calcium phosphate nanoparticles (CAP); mCTA/LTB (mutant cholera toxin E112K with pentameric B subunit of heat labile enterotoxin), and detoxified *E. coli* derived heat-labile toxin.

The adjuvant used may also be, theoretically, any of the adjuvants known for peptide- or protein-based vaccines. For example: inorganic adjuvants in gel form (aluminium hydroxide/aluminium phosphate, Warren et al., 1986; calcium phosphate, Relyvelt, 1986); bacterial adjuvants such as monophosphoryl lipid A (Ribi, 1984; Baker et al., 1988) and muramyl peptides (Ellouz et al., 1974; Allison and Byars, 1991; Waters et al., 1986); particulate adjuvants such as the so-called ISCOMS ("immunostimulatory complexes", Mowat and Donachie, 1991; Takahashi et al., 1990; Thapar et al., 1991), liposomes (Mbawuike et al. 1990; Abraham, 1992; Phillips and Emili, 1992; Gregoriadis, 1990) and biodegradable microspheres (Marx et al., 1993); adjuvants based on oil emulsions and emulsifiers such as Montanide™ (Incomplete Freund's adjuvant, Stuart-Harris, 1969; Warren et al., 1986), SAF (Allison and Byars, 1991), saponins (such as QS-

21; Newman et al., 1992), squalene/squalane (Allison and Byars, 1991); synthetic adjuvants such as non-ionic block copolymers (Hunter et al., 1991), muramyl peptide analogs (Azuma, 1992), synthetic lipid A (Warren et al., 1986; Azuma, 1992), synthetic polynucleotides (Harrington et al., 1978) and polycationic adjuvants (WO 97/30721).

Adjuvants for use with immunogens of the present invention include aluminum or calcium salts (for example hydroxide or phosphate salts). A particularly preferred adjuvant for use herein is an aluminum hydroxide gel such as Alhydrogel™. Calcium phosphate nanoparticles (CAP) is another potential adjuvant. The immunogen of interest can be either coated to the outside of particles, or encapsulated on the inside (He et al., 2000, Clin. Diagn. Lab. Immunol., 7,899-903).

Another adjuvant for use with an immunogen of the present invention is an emulsion. A contemplated emulsion can be an oil-in-water emulsion or a water-in-oil emulsion. In addition to the immunogenic chimer protein particles, such emulsions comprise an oil phase of squalene, squalane, peanut oil or the like, as are well known, and a dispersing agent. Non-ionic dispersing agents are preferred, and such materials include mono- and di-C_{12}-C_{24}-fatty acid esters of sorbitan and mannide such as sorbitan mono-stearate, sorbitan mono-oleate and mannide mono-oleate.

Such emulsions are for example water-in-oil emulsions that comprise squalene, glycerol and a surfactant such as mannide mono-oleate (Arlacel™ A), emulsified with the chimer protein particles in an aqueous phase. Alternative components of the oil-phase include alpha-tocopherol, mixed-chain di- and tri-glycerides, and sorbitan esters. Well-known examples of such emulsions include Montanide™ ISA-720, and Montanide™ ISA 703 (Seppic, Castres, France. Other oil-in-water emulsion adjuvants include those disclosed in WO 95/17210 and EP 0 399 843.

The use of small molecule adjuvants is also contemplated herein. One type of small molecule adjuvant useful herein is a 7-substituted-8-oxo- or 8-sulfo-guanosine derivative described in U.S. Pat. No. 4,539,205, U.S. Pat. No. 4,643,992, U.S. Pat. No. 5,011,828 and U.S. Pat. No. 5,093,318. 7-allyl-8-oxoguanosine(loxoribine) has been shown to be particularly effective in inducing an antigen-(immunogen-) specific response.

A useful adjuvant includes monophosphoryl lipid A (MPL®), 3-deacyl monophosphoryl lipid A (3D-MPL®), a well-known adjuvant manufactured by Corixa Corp. of Seattle, formerly Ribi Immunochem, Hamilton, Mont. The adjuvant contains three

components extracted from bacteria: monophosphoryl lipid (MPL) A, trehalose dimycolate (TDM), and cell wall skeleton (CWS) (MPL+TDM+CWS) in a 2% squalene/Tween™ 80 emulsion. This adjuvant can be prepared by the methods taught in GB 2122204B.

Other compounds are structurally related to MPL® adjuvant called aminoalkyl glucosamide phosphates (AGPs) such as those available from Corixa Corp under the designation RC-529™ adjuvant {2-[(R)-3-tetra-decanoyloxytetradecanoylamino]-ethyl-2-deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyloxytetra-decanoyl]-2-[(R)-3-tetra-decanoyloxytet-radecanoyl-amino]-p-D-glucopyranoside triethylammonium salt}. An RC-529 adjuvant is available in a squalene emulsion sold as RC-529SE and in an aqueous formulation as RC-529AF available from Corixa Corp. (see, U.S. Pat. No. 6,355,257 and U.S. Pat. No. 6,303,347; U.S. Pat. No. 6,113,918; and U.S. Publication No. 03-0092643).

Further contemplated adjuvants include synthetic oligonucleotide adjuvants containing the CpG nucleotide motif one or more times (plus flanking sequences). The adjuvant designated QS21, available from Aquila Biopharmaceuticals Inc., is an immunologically active saponin fractions having adjuvant activity derived from the bark of the South American tree Quillaja Saponaria Molina (e.g., Quil ™ A), and the method of its production is disclosed in U.S. Pat. No. 5,057,540. Derivatives of Quil™ A, for example QS21 (an HPLC purified fraction derivative of Quil ™) and other fractions such as QA17 are also disclosed. Semi-synthetic and synthetic derivatives of Quillaja Saponaria Molina saponins are also useful, such as those described in U.S. Pat. No. 5,977,081 and U.S. Pat. No. 6,080,725. The adjuvant denominated MF59 is described in U.S. Pat. No. 5,709,879 and U.S. Pat. No. 6,086,901.

Muramyl dipeptide adjuvants are also contemplated and include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thur-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine [CGP 11637, referred to as nor-MDP], and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmityol-s-n-glycero-3-hydroxyphosphoryloxy) ethylamine [(CGP) 1983A, referred to as MTP-PE]. The so-called muramyl dipeptide analogues are described in U.S. Pat. No. 4,767,842.

Other adjuvant mixtures include combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil-in-water emulsions comprising 3D-MPL and QS21 (WO 95/17210, PCT/EP98/05714), 3D-MPL formulated with other carriers (EP 0 689 454 B1), QS21 formulated in cholesterol-containing liposomes (WO 96/33739), or immunostimulatory

oligonucleotides (WO 96/02555). Adjuvant SBAS2 (now ASO2) from GlaxoSmithKline containing QS21 and MPL in an oil-in-water emulsion is also useful. Alternative adjuvants include those described in WO 99/52549 and non-particulate suspensions of polyoxyethylene ether (UK Patent Application No. 9807805.8).

The use of an adjuvant that contains one or more agonists for toll-like receptor-4 (TLR-4) such as an MPL® adjuvant or a structurally related compound such as an RC-529® adjuvant or a Lipid A mimetic, alone or along with an agonist for TLR-9 such as a non-methylated oligodeoxynucleotide-containing the CpG motif is also optional.

A heat-shock protein, fragment or peptide is also an optional adjuvant, as a carrier protein or peptide, in a mixture, or as part of a fusion polypeptide expressed or synthesized together with at least one polypeptide according to the invention. For example, U.S. Patent Nos. 5,736,146 and 5,869,058 provide peptides derived from humans and *E. coli* heat-shock protein 60 (hsp60) as carriers for vaccination against viral and bacterial pathogens. Defined peptides present uniquely effective characteristics in conjugate vaccines due to the following reasons:

i. HSP60 epitopes provide natural T-cell help; Humans are born with a high frequency of T cells responsive to HSP60, so no induction is needed and youngsters respond.

ii. Defined HSP60-peptide conjugates function as built-in adjuvants activating innate TLR-4 receptors on APC; the HSP60-conjugate vaccine administered in aqueous solution serves as its own adjuvant.

iii. Defined HSP60-peptide conjugates do not induce the production of competing antibodies and therefore do not suppress vaccination responses, even with multiple administrations.

iv. Boosting to the HSP60-epitope occurs naturally, since HSP60 is up-regulated at the site of any immune response (infection or tumor); the vaccination effect does not decline for prolonged periods. Immune memory is robust and effective.

Detoxified pneumolysin, known as a carrier protein and as an adjuvant (for example Michon et al., Vaccine, 18, 1732-1741, 1998), or fragment or analog thereof, can be also used in conjunction or conjugation of the polypeptides of the present invention.

Another type of adjuvant mixture comprises a stable water-in-oil emulsion further containing aminoalkyl glucosamine phosphates such as described in U.S. Pat. No. 6,113,918. Of the aminoalkyl glucosamine phosphates the molecule known as RC-529 {(2-[(R)-3-tetradecanoyloxytetradecanoylamino]ethyl-2-deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyloxy-tetradecanoyl]-2-[(R)-3—tetradecanoyloxytetra-decanoylamino]-p-D-glucopyranoside triethylammonium salt.)} is most preferred. A preferred water-in-oil emulsion is described in WO 99/56776.

Adjuvants are utilized in an adjuvant amount, which can vary with the adjuvant, host animal and immunogen. Typical amounts can vary from about 1 μg to about 10 mg per immunization. Those skilled in the art know that appropriate concentrations or amounts can be readily determined.

Vaccine compositions comprising an adjuvant based on oil in water emulsion is also included within the scope of the present invention. The water-in-oil emulsion may comprise a metabolisable oil and a saponin, such as for example as described in US 7,323,182.

According to several embodiments, the vaccine compositions of the present invention may contain one or more adjuvants, characterized in that it is present as a solution or emulsion which is substantially free from inorganic salt ions, wherein said solution or emulsion contains one or more water soluble or water-emulsifiable substances which are capable of making the vaccine isotonic or hypotonic. The water soluble or water-emulsifiable substances may be, for example, selected from the group consisting of: maltose; fructose; galactose; saccharose; sugar alcohol; lipid; and combinations thereof.

The polypeptides, multimers, and fusion proteins of the present invention may comprise according to several specific embodiments a proteosome adjuvant. The proteosome adjuvant comprises a purified preparation of outer membrane proteins of meningococci and similar preparations from other bacteria. These proteins are highly hydrophobic, reflecting their role as transmembrane proteins and porins. Due to their hydrophobic protein-protein interactions, when appropriately isolated, the proteins form multi-molecular structures consisting of about 60-100 nm diameter whole or fragmented membrane vesicles. This liposome-like physical state allows the proteosome adjuvant to act as a protein carrier and also to act as an adjuvant.

The use of proteosome adjuvant has been described in the prior art and is reviewed by Lowell GH in "New Generation Vaccines", Second Edition, Marcel Dekker Inc, New York, Basel, Hong Kong (1997) pages 193-206. Proteosome adjuvant vesicles are described as comparable in size to certain viruses which are hydrophobic and safe for human use. The review describes formulation of compositions comprising non-covalent complexes between various antigens and proteosome adjuvant vesicles which are formed when solubilizing detergent is selectably removed using exhaustive dialysis technology.

Vaccine compositions comprising different immunogenic polypeptides can be produced by mixing or linking a number of different polypeptides according to the invention with or without an adjuvant. In addition, an immunogenic polypeptide according to the present invention may be included in a vaccine composition comprising any other *S. pneumoniae* protein or protein fragment, including mutated proteins such as detoxified pneumolysin, or they can be linked to or produced in conjunction with any such *S. pneumoniae* protein or protein fragment.

Vaccine compositions according to the present invention may include, for example, influenza polypeptides or peptide epitopes, conjugated with or coupled to at least one immunogenic *S. pneumoniae* polypeptide according to the invention.

The vaccines and compositions will generally be sterile and/or pyrogen-free, and may comprise a detergent in order to minimize adsorption of antigens to containers.

The vaccines and compositions may or may not comprise a microbial preservative. Preservatives include for example, mercurial preservatives (e.g. thimerosal), 2-phenoxy-ethanol, methyl parabens, propyl parabens, benzyl alcohol and mixtures thereof.

The vaccines and compositions may be formulated for administration by a route selected from the group consisting of: intramuscular, intranasal, oral, intraperitoneal, subcutaneous, topical, intradermal and transdermal delivery. In particular embodiments, the vaccine is formulated for intramuscular administration. In other particular embodiments, the vaccine is formulated for oral administration. In yet other embodiments, the vaccine is formulated for intranasal administration.

Typically, the compositions are prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect. Direct delivery of the compositions will generally

be parenteral, e.g., by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions can also be administered into a lesion. Other modes of administration include oral and pulmonary administration, suppositories, and transdermal or transcutaneous applications. Dosage treatment may be a single dose schedule or a multiple dose schedule (e.g. including booster doses).

Also provided are pharmaceutical compositions comprising at least one polynucleotide sequence encoding a modified PsipB polypeptide, and optionally further comprising at least one polynucleotide sequence encoding an *S. pneumoniae* immunogenic polypeptide other than PsipB or a modified PsipB. The use of a polynucleotide of the invention in genetic immunization will generally employ a suitable delivery method, such as direct injection of plasmid DNA into muscles (Wolff et al., Hum Mol Genet (1992) 1:363; Manthorpe et al., Hum. Gene Ther. (1983) 4: 419); delivery of DNA complexed with specific protein carriers (Wu et al., J Biol. Chem. (1989) 264: 16985); coprecipitation of DNA with calcium phosphate (Benvenisty & Reshef, PNAS USA, (1986) 83: 9551); encapsulation of DNA in various forms of liposomes (Kaneda et al., Science (1989) 243: 375); particle bombardment (Tang et al., Nature (1992) 356:152, Eisenbraun et al., DNA Cell Biol (1993) 12: 791), and *in vivo* infection using cloned retroviral vectors (Seeger et al., PNAS USA (1984) 81: 5849).

Methods of protecting against *S. pneumoniae* infection

The invention further provides methods of inducing an immune response against *S. pneumoniae* in a subject, and of conferring protection against *S. pneumoniae* infection in a subject. The methods comprise administering to a subject in need thereof an effective amount of a modified PsipB polypeptide, or a polynucleotide encoding same.

The invention further provides use of a modified PsipB polypeptide, or of a polynucleotide encoding same, for preparation of a medicament for inducing an immune response in a subject against *S. pneumoniae*.

The invention further provides use of a modified PsipB or of a polynucleotide encoding same, for preparation of a medicament for conferring protection against *S. pneumoniae* infection.

The subject methods are carried using the modified PsipB polypeptides of the invention which are antigenic, immunogenic, and capable of eliciting an immune response

in a subject to which they are administered.

In accordance with the invention, "protection" means to reduce bacterial load or increase in the survival rate or period of a test animal compared to a control animal, following administration to the test animal a modified PsipB polypeptide or polynucleotide, and subsequent challenge (or infect) with a strain, subtype or serotype of *S. pneumoniae* wherein the challenge dose is sufficient to cause disease or death in a non-protected animal. Statistical analyses, for example, using the Log rank test to compare survival curves, and the Fisher exact test to compare survival rates, may be used to calculate P values and determine whether an observed difference between two groups is statistically significant.

The vaccine preparations of the present invention may be used to protect or treat a human subject susceptible to *S. pneumoniae* infection, by means of administering said vaccine via systemic or mucosal route. A human subject may be an infant (under 12 months), a toddler (12-24 months), a child (2-12years), an adolescent (12-20 years), or an adult. These administrations may include injection via the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or via mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Intranasal administration of vaccines for the treatment of pneumonia or otitis media may be preferred as a means of preventing nasopharyngeal carriage of pneumococci, thus attenuating infection at its earliest stage. Although the vaccine of the invention may be administered as a single dose, components thereof may also be co-administered together at the same time or at different times (for instance if saccharides are present in a vaccine these could be administered separately at the same time or 1-2 weeks after the administration of a bacterial protein vaccine for optimal coordination of the immune responses with respect to each other). In addition to a single route of administration, 2 different routes of administration may be used. For example, bacterial proteins may be administered intramuscularly or intranasally. In addition, the vaccines of the invention may be administered intramuscularly for priming doses and intranasally for booster doses.

As with all immunogenic compositions or vaccines, an effective amount of modified PsipB polypeptide, or polynucleotide encoding same, must be determined empirically. Factors to be considered include the immunogenicity, whether or not the immunogen will be complexed with or covalently attached to an adjuvant or carrier protein or other carrier,

route of administrations and the number of dosages to be administered. Typically, a single dose for a protein antigen is about 5 to 100 μg.

"*S. pneumoniae* infection" as used herein refers to a state in which disease-causing *S. pneumoniae* have invaded, colonized, spread, adhered, disseminated or multiplied in body cells or tissues. This term encompass the term "inoculation", namely the state in which the bacteria colonized the nasopharynx but no symptoms of infection are yet manifest.

The following examples are presented in order to more fully illustrate certain embodiments of the invention. They should in no way, however, be construed as limiting the broad scope of the invention. One skilled in the art can readily devise many variations and modifications of the principles disclosed herein without departing from the scope of the invention.

EXAMPLES

Example 1. Recombinant cloning and expression of wild type PsipB.

Methods

The gene encoding wild type PsipB corresponding to Genbank accession number NP_358083 was amplified from *S. pneumoniae* strain WU2 genomic DNA by PCR with the following primers: forward: 5'-TCG GAT CCG TGA TTG GAG TCG TTT GCG -3' (SEQ ID NO: 68); reverse: 5'-TCG AGC TCT CAG TTC TTG CCA TCC TTCT -3' (SEQ ID NO: 69).

The forward and reverse primers contain *BamH*I and *Sac*I recognition sequences, respectively, and all primers contain 5'-TC spacers. The primers flank the entire open reading frame. The amplified and *BamH*I-*Sac*I (Takara Bio Inc, Shiga, Japan)-digested DNA-fragments were cloned into the pHAT expression vector (the HAT epitope is a 19-amino-acid sequence with six non-consecutive His residues [BD Biosciences Clontech, Palo Alto, CA, USA]) and transformed in DH5a E. coli cells. The pHAT-PsipB vector was purified from DH5α cells using the Qiagen High Speed Plasmid Maxi Kit (Qiagen GMBH, Hilden, Germany) and transformed in *E. coli* host expression strain BL21(DE3)pLysS (Stratagene, La Jolla, CA, USA). The identity of the insert was confirmed by sequencing. An overnight culture of the bacteria was diluted 1:50 and grown till OD600 = 0.6. The expression of the recombinant HAT-tagged PsipB (rPsipB) was induced by the addition of 1 mM IPTG to the cells for 5 h at 37 °C. The cells were

harvested by centrifugation and lysed by sonication in Lysis Buffer (0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 8.0). The fraction with inclusion bodies was isolated and lysed in Lysis Buffer plus 0.1% SDS or 8 M urea. The HAT-tagged recombinant proteins were purified using Ni-NTA agarose beads (Qiagen GMBH) by binding for 1 h at room temperature. The beads were washed with wash buffer (0.1% SDS or 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl pH 6.3), and the recombinant proteins were recovered from the beads using elution buffer (0.1% SDS or 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-HCl, 100 mM Imidazole). After purification, the PsipB preparation was dialyzed against PBS pH 8.0 or PBS pH 7.3 with 2mM FeSO4 buffer for 48 h with three exchanges of the buffer. Isolation of protein was confirmed by immunoblotting analysis using anti-HAT antibodies (BD Biosciences Clontech), and in-gel digestion of protein to derive peptides for MALDI-TOF mass spectrometry sequencing.

For MALDI TOF analysis, the suspected rPsipB band was cut out from the gel and then subjected to acetonitrile treatment, alkylation and reduction. In-gel digestions were performed using 12.5 ng/mL trypsin. Following overnight incubation, 100mM ammonium carbonate and acetonitrile were added, followed by 5% formic acid. The peptides were subjected to MALDI TOF analysis using the Bruker Reflex-IV mass spectrometer (Bruker-Daltonik, Bremen, Germany). Alignment to existing databases was performed using both the Mascot software package (Matrix Science Ltd., UK, http://www.matrixscience.com) and Program "Profound" provided by the Rockefeller University. The supernatant containing the peptides was subjected to MALDI-TOF mass spectrometry sequencing using Bruker Reflex-IV mass spectrometer (Bruker-Daltonik, Bremen, Germany).

For protein gel electrophoresis, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Hoefer mini VE vertical electrophoresis system (Amersham Biosciences, San Francisco, CA, USA). Protein samples were suspended in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and boiled prior to electrophoresis.

To produce antibodies against rPsipB, rabbits were immunized with Ni-NTA-purified rPsipB formulated with Complete Freund's Adjuvant (CFA) four times biweekly. The rabbits were bled on the 3rd day after three and four immunizations, and total serum was isolated after coagulation of the blood from the immunized rabbits. Animal experimental

protocols were approved by the Institutional Animal Care and Use Ethics Committee of Ben-Gurion University of the Negev, Beer Sheva, Israel.

Isolation of *S. pneumoniae* cell wall proteins was carried out by the method of Siegel et al. (Siegel et al., 1981. Infect Immun. 31:808-14). Briefly, bacterial cells were harvested by centrifugation at 4700 g for 15 min, washed with phosphate buffered saline (PBS) and incubated with mutanolysin for 1 h at 37 °C. The supernatant containing the soluble proteins released from bacteria was collected after centrifugation and stored at −70 °C.

For immunoblotting analysis, protein mixtures were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Carlsbad, CA, USA), as described by the manufacturer. Immunoblot analysis of PsipB protein expression was done with rabbit anti-sera obtained against purified rPsipB.

RT-PCR was done with MasterAmp High Fidelity RT-PCR kit, Epicentre (Madison, WI) in accordance with the manufacturer's recommendations. The total RNA from *S. pneumoniae* strain WU2 was used as a template.

The nucleotide and amino acid sequences of PsipB were analyzed using the TIGR4 and R6 sequences available in the TIGR databases. Blast (NCBI) and SMART programs were used to detect homology and functional domains in PsipB protein sequence.

Data were expressed as the mean ± standard deviation of mean. Differences between groups in the experiments were analyzed by using two-tailed Student's t-test. $P < 0.001$ was considered to be statistically significant. Each experimental point represents the mean of the data ± standard deviation obtained from three independent experiments.

Results

The identical loci spr0489 for strain R6 or sp0565 for strain TIGER4 of *S. pneumoniae* were considered to be open reading frames for the hypothetical protein NP_358083 (Hoskins et al., J Bacteriol 183:5709-17). Using primers constructed according to the R6 sequence in RT-PCR analysis, an mRNA product was detected in *S. pneumoniae* serotype 3 strain WU2 (Fig. 2A).

The protein with the accession number NP_358083 was cloned and expressed to obtain HAT-tagged recombinant protein. The recombinant protein was found in an insoluble form in the inclusion bodies fraction. The recombinant protein was purified with Ni-NTA beads under denaturating conditions. To obtain anti-serum, rabbits were

immunized with the purified recombinant protein. This protein was designated Pneumococcal surface immunogenic protein B (PsipB) on the basis of its localization in external capsule of *S. pneumoniae* and its immunogenic properties.

The theoretical molecular weight of PsipB is 17.2 kDa. Separation of the recombinant HAT-tagged PsipB on SDS-PAGE with sample buffer, containing β-mercaptoethanol, revealed a major band of 22 kDa and a minor band of 45 kDa (Fig. 2B, lane 2). Omission of β-mercaptoethanol in the sample buffer yielded the 45 kDa band as the major band and the 22 kDa band as the minor band (Fig. 2B, lane 3). Both of the bands reacted with anti-PsipB anti-serum (Fig. 2C). The identity of the upper and the lower bands as PsipB was confirmed by MALDI-TOF analysis of the samples excised from the bands stained with Coomassie blue. Nine peptides derived from in-gel digestion of the lower band and 5 peptides derived from in-gel digestion of the upper band significantly match the database sequence of PsipB from R6 *S. pneumoniae* strain ($p<0.05$). The presence of two bands of recombinant PsipB is likely due to dimerization of PsipB molecules, since the apparent molecular weight of the upper band is approximately double that of the lower one.

Rabbit anti-PsipB antiserum recognized bands of 17kDa and 34kDa in immunoblotting of cytoplasmic fraction and the 34kDa band in the cell wall fraction prepared from *Streptococcus pneumonia* serotype 3 strain WU2. Neither could be detected in the cytoplasm or in the cell wall of a null-mutated bacteria lacking *PsipB* (Fig.2D). The molecular weight of recombinant PsipB dimer resembles the molecular weight of the bands that reacted with anti-PsipB serum in the bacterial cytoplasm and cell wall lysates.

To further characterize PsipB, its sequence was analyzed using SMART protein domain database. A flavin binding domain was identified, corresponding to the region between amino acid residues 1 to 79. For the SCOP protein classification this domain was found to be related to the ferredoxin reductase-like FAD-binding domain. Notably, there is a cluster of three cysteine residues cluster in the middle of the sequence – Cys66Cys72Cys84.

The recombinant PsipB was found to be insoluble under physiological conditions. To obtain a soluble protein, different refolding and dialysis conditions were tested. PsipB turned fully soluble after dialysis against PBS with 2mM FeSO4. Partial solubility could be obtained following dialysis against PBS with 2mM $CoCl_2$ or $CuSO_4$.

Example 2. Recombinant cloning and expression of four different modified PsipB polypeptides.

DNA encoding the modified PsipB polypeptides, designated PsipB C66A, in which the cysteine residue at position 66 of wild type PsipB was substituted by an alanine residue; PsipB C72A, in which the cysteine residue at position 72 of wild type PsipB was substituted by an alanine residue; PsipB C84A, in which the cysteine residue at position 84 of wild type PsipB was substituted by an alanine residue; and PsipB C66AC72AC84A, in which the cysteine residues at each of positions 66, 72 and 84 of wild type PsipB were substituted by alanine residues, was produced. Plasmid clones of each of the aforementioned modified forms of PsipB (four clones of each mutant) were used to transform *E. coli* XL-1 cells. Mini expression of each clone was carried out by induction with 0.4 mM IPTG for 4 hours. Partially purified IBs were prepared and analyzed for expression by SDS-PAGE (15%) and Coomassie blue staining.

Example 3. Comparison of solubilization properties between wild type PsipB and PsipB C66A mutant.

Samples of each of wild type PsipB and PsipB C66A were lyophilized with $NaHCO_3/Na_2CO3$ adjusted to pH 11.5 at a protein:salt ratio of ~ 1:1 in 0.5 mg portions and stored for several months at -20 °C.

Experiment No. 1. Reconstitution at pH 9 and stability after 24 h of freezing. Lyophilized samples of wild type PsipB and PsipB C66A were easily solubilized in 1.0 ml of DDW, resulting in apparent pH of 9.0 to 9.5. Aliquots of 200 µl were applied onto an analytical column of Superdex® 75 pre-equilibrated with 25 mM Tris-HCl buffer pH 8, containing 300 mM NaCl, either within 5 minutes after solublization (Fig. 3A and Fig. 3B) or frozen and tested next day (Fig. 3C and Fig. 3D). The column was run at room temperature at a rate of 0.8 ml/min. The results are presented in Fig 3, which shows that wild type PsipB (Fig. 3A and Fig. 3C) was almost immediately oligomerized, whereas PsipB C66A (Fig. 3B and Fig. 3D) retained its original profile, with the main peak eluting at a retention time corresponding to the dimeric protein.

Experiment No. 2. Reconstitution at pH 11.5 and stability after 24 h of freezing. Lyophilized samples of wild type PsipB and PsipB C66A were easily solubilized with 1.0 ml of $NaHCO_3/Na_2CO3$ (2.5 mg/ml) adjusted to pH 11.5 and analyzed by analytical gel filtration chromatography. Aliquots of 200 µl were applied onto an analytical Superdex®

75 column pre-equilibrated with 25 mM Tris-HCl buffer, pH 8 containing 300 mM NaCl within 5 minutes after solublization. The column was run at room temperature at a rate of 0.8 ml/min. The results indicate that the elution profile of both wild type PsipB and PsipB C66A were unchanged after reconstitution.

Experiment No. 3. Solubilization at pH 8 and stability after 1 to 24 h at room temperature. Due to the immediate oligomerization of wild type PsipB after solubilization at pH 8-9, this experiment was conducted with PsipB C66A only. PsipB C66A (0.5 mg) was dissolved in 2 ml of DDW and the pH was adjusted to 8.0 with dilute HCl. The sample was held at room temperature (23 °C), and aliquots were taken for gel filtration analysis at zero time (5 min after solubilization) and after 1, 3 and 24 hours. Aliquots of the different time points were applied onto an analytical Superdex 75 column pre-equilibrated with 25 mM Tris-HCl buffer, pH 8 containing 300 mM NaCl within 5 minutes after solublization. The column was run at room temperature at a rate of 0.8 ml/min. In parallel, aliquots from the same time points were removed and frozen for subsequent analysis by SDS-PAGE.

As shown in Fig. 4, the profile of PsipB C66A after 5 min is characterized by a main peak with retention time of 13.9 (Fig. 4A). After one hour (Fig. 4B), 3 hours (Fig. 4C) and 24 hours (Fig. 4D), this peak was gradually reduced and peaks of retention times 20 and 22 min appeared. To test whether this change resulted from degradation of PsipB C66A, aliquots taken at each time point were analyzed by SDS-PAGE (Fig 5). The results show clearly that no proteolytic degradation occurred at zero time, and degradation was negligible at the later time points. Therefore the changes in the gel filtration profile likely resulted from changes in protein conformation which led to retention of the protein.

Example 4. Refolding and purification of wild type PsipB.

Four clones transformed with the polynucleotide encoding wild type PsipB were analyzed for expression and clone 3 was chosen for large scale expression experiments.

For large scale fermentation, 2.5 l (5 x 500 ml) of clone 3 of PsipB was prepared. Frozen precipitates corresponding to 2.5 L bacterial culture were suspended in 600 ml of 10 mM Tris-HCl buffer, pH 8 containing 10 mM EDTA, all prepared in pyrogen-free (<0.05 EU/ml) ultrapure water (UPW), and stirred or mixed for 30 min at 4 °C. Then 120 mg of lysozyme and 6 mg of DNAse were added, and the suspension was stirred for an additional 2 h. Subsequently, the suspensions were sonicated for 6-7 min at 50%-cycle program (the sonicator works in pulses that last 50% of the time) in 100 ml portions, and

centrifuged for 20 min at 13,000 rpm. The pellet was then suspended in UPW, and the sonication procedure was repeated. The IB pellet was suspended in 1% Triton X-100, sonicated and centrifuged as described above. Subsequently the pellet was suspended in UPW and sonicated, which was repeated 5-6 times. The resulting IBs were suspended in 50 ml DDW, divided into 10 x 5ml aliquots and frozen. Ten ml of IB (corresponding to 500 ml of fermentation culture) were refolded in 33 ml of 4.5 M urea, 40 mM Tris base and 1 mM cysteine adjusted to pH 11.3. After 1.5 hr. at 4 °C, 100 ml of 0.67 M arginine was added and the solution was stirred overnight at 4 °C. The next day, the 133 ml refold mixture was concentrated to 30 ml and two 15 ml samples were successively applied onto preparative Superdex® 200 columns pre-equilibrated with TN/Urea/Arg at pH 10.4 in cold at 2 ml/min (Fig. 6A). Fractions 8-15 from Fig. 6A, (along with corresponding tubes from the second separation) were pooled yielding ~ 65 ml with OD_{280} = 0.451, namely >50 mg. Aliquots from those fractions were also analyzed by gel filtration on an analytical Superdex® 200 column equilibrated with TN/Urea/Arg, pH 10.0. All showed a single peak corresponding to a mixture of a dimer of ~ 34-35 kDa and a trimer or tetramer.

The pooled eluate of PsipB (~50 ml) from the two preparative Superdex® 200 columns was rapidly concentrated to ~ 20 ml and desalted on Sephadex® G-25 column in 5 x 4 ml aliquots. The column was equilibrated with $NaHCO_3$ (1.2 mg/ml) adjusted to pH 11.5. This procedure effectively preserved the dimeric structure of PsipB and prevented aggregation. The pooled desalted eluate was divided into vials (0.5 mg/vial) and lyophilized. Total 58 tubes (29 mg) were obtained and stored at -20 °C. The lyophilized PsipB could be easily dissolved in $NaHCO_3$ adjusted to pH 11.5. The gel-filtration profile of the dissolved PsipB is presented in Fig. 6B.

The molecular mass of the purified protein was determined on an analytical Superdex® 75 column, using a calibration curve obtained with protein molecular weight markers of 132 kDa (BSA dimer), 66 kDa (BSA monomer), 32 kDa and 16 kDa. The column was pre-equilibrated with TN buffer pH, 10.4 and developed at 0.8 ml/min at room temperature. The wild type PsipB was found to be a mixture of two peaks: a peak with RT of 12.22 min corresponding to molecular mass of 70 kDa and a peak of 13.18 corresponding to molecular mass of 38.2 kDa, indicating that the pure PsipB forms a mixture of tetramers and dimers under the above described experimental conditions. The theoretical molecular mass of the PsipB dimer is 34,460 Da and that of a PsipB tetramer is 68,929 Da. SDS-PAGE confirmed that under denaturating and non-reducing conditions

PsipB resolves into a mixture of dimers (the major band) and monomers (the minor band), but under denaturating and reducing conditions it resolves into a monomer (Fig. 8 left panel). In contrast, the PsipB C66A mutant is almost totally a monomer, even under non-reducing conditions (Fig. 8 right panel).

Two peaks were also observed using RP-chromatography, likely corresponding to the tetramer and dimer forms. To verify the protein identity, the lyophilized PsipB was subjected to SDS-PAGE under reducing conditions followed by blotting and immunodetection using rabbit anti-PsipB polyclonal antiserum at 1:20,000 dilution The protein was also recognized as a >95% pure single band by immunoblot analysis (Fig. 7C). The small band corresponding to molecular mass of ~ 35 kDa represents <5% of the total material and is a dimeric form of PsipB.

The lyophilized PsipB was solubilized 0.2% $NaHCO_3$ adjusted to pH 11.5. After freezing for 24 h or 6 days and thawing, gel filtration analysis was run. After 24 h (Fig. 7A), the profile was almost identical to that prior to freezing as shown in Fig 5C. However, after 6 days the protein underwent partial degradation, indicated by the peak at retention time 17.52 min (Fig. 7B).

Example 5. Refolding and purification of modified PsipB C66A.

Clones encoding each of the four PsipB mutants (PsipB C66A; PsipB C72A; PsipB C84A and PsipB C66AC72AC84A; four clones per mutant) in plasmid pTOPO were obtained from Entelechon GmbH. The plasmids were transfected into *E. coli* XL-1 cells. Respective DNAs were prepared and stored along with the corresponding glycerol cultures. Mini expression of the clones was carried out by induction with 0.4 mM IPTG for 4 hours. Partially purified IB (2 x sonications) were prepared and analyzed for expression by SDS-PAGE. All clones expressed PsipB polypeptides very well, as indicated by Coomassie blue staining of the sonicates separated by SDS-PAGE.

Protocols for refolding and purification of PsipB C66A were established, as follows.

Experiment A. Ten ml of IB (corresponding to 500 ml of fermentation culture) were refolded in 33 ml of 4.5 M urea, 40 mM Tris base and 1 mM cysteine adjusted to pH 11.3. After 1.5 hr at 4 °C, 100 ml of 0.67 M arginine was added and the solution was stirred overnight at 4 °C. The next day, the 133 ml refold mixture was concentrated to 30

ml and two 15 ml samples were successively applied onto a preparative Superdex® 200 column pre-equilibrated with TN/Urea/Arg at pH 10.0 in cold at 2 ml/min (Fig 9).

Aliquots from every tube were analyzed by SDS-PAGE and next day the tubes 15-20 from Fig 9, (along with corresponding tubes from the second separation) were pooled yielding >120 mg protein. Aliquots from those tubes were also analyzed by gel filtration on an analytical Superdex® 200 column equilibrated with TN/Urea/Arg, pH 10.0. All showed a single peak corresponding to molecular mass of ~ 34-35 kDa.

Fifteen ml was dialyzed against $NaHCO_3$ (1 mg/ml) adjusted to pH 10.0 and lyophilized. The lyophilized material was tested by analytical gel filtration and showed aggregation. The remainder of the pooled fraction was rapidly (within 10 min) transferred to $NaHCO_3$ (1.2 mg/ml) by separating successively 8 ml aliquots on a Sephadex® G-25 column (volume 100 ml) pre-equilibrated with the same buffer. This procedure preserved the dimeric structure and prevented aggregation. The pooled eluate of PsipB (~ 50 ml with OD_{280} = 0.603) was divided into 0.5 mg aliquots in vials and lyophilized. A total of 48 mg (96 tubes) was obtained and stored at -20 °C.

Experiment B. This experiment was aimed to optimize the reconstitution conditions after lyophilization and to determine the purity of the PsipB C66A by several methods. PsipB C66A (lyophilized in vials) was reconstituted by addition of 1 ml of either TN buffer pH 10.2 or 0.2% $NaHCO_3$ adjusted to either pH 11.0 or pH 11.5. The vials were gently mixed and the material was rapidly solubilized. Aliquots were then applied to a analytical Superdex® 75 or 200 column pre-equilibrated with TN buffer pH, 10.2 and run at 0.8 ml/min at room temperature. The results, shown in Figure 10, indicate that as a progressively higher pH is used for solubilization oligomer formation is prevented. As shown, the pH of the solubilizing solution has an effect on the gel filtration profile. At pH 10.2, over 24 % of the polypeptide corresponds to oligomeric forms (Fig 10A), whereas at pH 11.0 18 % of the polypeptide corresponds to oligomeric forms (Fig 10B), and at pH 11.5 less than 2 % of the polypeptide corresponds to oligomeric forms (Figs. 10C and 10D). The molecular mass of the purified protein was determined on an analytical Superdex® 75 or 200 column, using a calibration curve obtained with protein molecular weight markers of 132 kDa (BSA dimer), 66 kDa (BSA), 40 kDa (complex of chicken LBD/leptin) and 16 kDa (human leptin). The column was pre-equilibrated with TN buffer pH 10.2 and developed at 0.8 ml/min at room temperature. The molecular mass of the

PsipB C66A was found to be 33.9 kDa indicating that the pure PsipB C66A forms dimers under the above described experimental conditions. The theoretical molecular mass of the dimer is 34,460 Da. SDS-PAGE analysis confirmed that under denaturating and reducing (or non-reducing) conditions PsipB C66A resolved as a monomer (Fig 11). A single main peak was also observed using RP-chromatography (Fig. 12). A slight shoulder observed on the left of the peak was likely related to dimer/monomer conversion under the hydrophobic conditions of RP-chromatography.

To verify the protein identity, the lyophilized PsipB C66A was subjected to SDS-PAGE followed by transblotting and immunodetection using rabbit anti-PsipB polyclonal antiserum at 1:20,000 dilution The protein was also recognized as an >95% pure single band by immunoblot analysis (Fig. 13). The small band corresponding to molecular mass of ~ 33 kDa represents less than 5% of the total material and is a dimeric form of PsipB.

The purified lyophilized dimeric PsipB C66A was tested for its endotoxin content, and 0.5 mg of protein was found to contain less than 13.2 EU/ml or 1.32 ng/ml or less than 26.4 EU per mg.

Example 6. Immunoprotection induced by a modified PsipB in an animal model.

In a mouse model for upper respiratory *S. pneumoniae* colonization, BALB/c mice (7 weeks old) were immunized subcutaneously with three doses of either wild type PsipB or PsipB C66A (5μg/mouse; CFA/IFA/IFA adjuvant), with two-week intervals between immunizations. Mice in the control group were administered adjuvant alone. Two weeks after the final immunizing dose, the mice were anesthetized with isoflurane, and challenged intranasally with a sublethal dose of *S. pneumoniae* serotype 3 strain WU2 (in 25 μl PBS). The nasopharynx and lungs were excised, homogenized and plated onto blood agar plates (overnight) for bacterial enumeration. The extent of colonization in the nasopharynx and lungs at 48 hours was determined as bacterial CFU per 1 ml homogenate.

As shown in Fig. 14A, vaccination of mice with PsipB and PsipB C66A reduced by 70% and 86.5%, respectively the extent of bacterial colonization in nasopharynx respective to PBS adjuvant). Vaccination of mice with PsipB did not result in reduction of lung colonization, while vaccination with PsipB C66A provided 100% protection against lung colonization (Fig. 14B). Accordingly, a modified PsipB polypeptide according to the invention is more effective than the wild type polypeptide in conferring immunoprotection against *S. pneumoniae* infection and colonization.

Example 7. Testing the efficacy of modified PsipB polypeptides and multimers.

Immunogenic polypeptides are produced and used individually, as multimers, or in different combinations as part of fusion polypeptides with or without a carrier or adjuvant sequence and are tested, with or without an external adjuvant for their vaccine potential in several *in vitro*, *ex vivo* and *in vivo* models. Cross protection against capsularly and genetically unrelated bacterial strains is also tested. In certain cases, antibodies produced against selected peptides and polypeptides are used. The following models are used to test the efficacy:

In vitro model in which the ability of polypeptides, chimeric polypeptides and antisera against them to interfere with bacterial adhesion to cultured upper respiratory tract epithelial cells and to endothelial cells is evaluated.

To evaluate the stage at which the immune system prevents disease two *in vivo* tests are used: in vaccinated mice the extent of nasopharyngeal, lung blood and spleen colonization in determined; and *S. pneumoniae* tagged with luciferase is monitored using the bioluminescence imaging using an IVIS imaging system;

Ex vivo immunization with antiserum against the polypeptides. Several 100s CFU of *S. pneumoniae* serotype 3 strain WU2 are ex vivo neutralized with rabbit antiserum against the polypeptides diluted serums for 1 hr and used to challenge 7 week old BALB/c female mice. Negative control mice are challenged with *S. pneumoniae* serotype 3 strain WU2 after neutralization with pre-immune diluted serums obtained from the same rabbit. Positive control mice are challenged with *S. pneumoniae* serotype 3 strain WU2, after neutralization with rabbit anti Non-lectins serum. Survival is monitored for seven days.

Mouse model for systemic infection. -For systemic *S. pneumoniae* lethal challenge mice immunized with a polypeptide formulated with adjuvant and with adjuvant alone, as control, are inoculated intraperitoneally (i.p.) or intravenous (i.v.), with a lethal dose of *S. pneumoniae* serotype 3 strain WU2. The inoculum size is determined to be the lowest causing 100% mortality in the control mice within 96-120 hours. Survival is monitored daily.

Mouse models for upper respiratory infections. – For respiratory *S. pneumoniae* lethal challenge mice immunized with polypeptide in adjuvant, and with adjuvant alone as control, are anaesthetized with isoflurane, and inoculated intranasally with a lethal dose of *S. pneumoniae* serotype 3 strain WU2 (in 25 µl PBS). The inoculum size is determined to

be the lowest causing 100% mortality in the control mice within 96-120 hours. Survival is monitored daily.

Ex-vivo inhibition of nasopharyngeal and lung colonization. To determine whether peptides and polypeptides derived from age-dependent *S. pneumoniae* proteins are capable of inducing antibodies that inhibit colonization, mice are inoculated intranasally with *S. pneumoniae* serotype 3 WU2 prior and after mixture ex vivo with antibodies to the polypeptide. Alternatively, the polypeptide is mixed with S. *pneumoniae* serotype 3, strain WU2 bacteria, and the mixture is inoculated intranasally with $5x10^5$ to $5x10^7$ *S. pneumoniae*. At 3, 6, 24 and 48 hours following inoculation mice are sacrificed and the nasopharynx and lungs excised homogenized and plated onto blood agar plates for colony number enumeration.

Otitis media models. Otitis media models in chinchilla and the rat (developed according to Chiavolini et al., 2008, Clinical Microbiology Reviews, 21:666-685; Giebink, G. S. 1999, Microb. Drug Resist., 5:57-72; Hermansson et al., 1988, Am. J. Otolaryngol. 9:97-101; and Ryan et al., 2006, Brain Res. 1091:3-8), are utilized to test the effectiveness of PsipB mutants according to the invention. The ability of GtS (333-486) to protect those animals from developing otitis media following intranasal challenge is studied.

Example 8. Production and purification of a PsipB 1-112 (PsipBas) fragment (p712as).

Preparation of new plasmid by PCR – The original plasmid encoding PsipBa (C66A of Seq ID NO 1, Example 2) was prepared from the glycerol culture of the bacteria expressing this protein using Qiagene mini prep kit. Then the DNA encoding PsipBa (residues 1-112) was shortened using PCR reaction (3 min - 95°C; [30 sec - 95°C; 30 sec - 55°C; 35 sec - 70°C] x 30 cycles; 10 min - 70°C; overnight 4°C) and employing the 5' primer of AAAAACATATGGTGATTGGAGTC (SEQ ID NO:70, the NdeI site is underlined) and the 3' primer TTTTTGCTAAGCTTATTAGGCAAACAGTTG (SEQ ID NO:71) encoding the double stop codon TAATAA, and followed by HindIII and Bpu1102I restriction sites. Subsequently the expressed protein has the following C-terminal sequence QLFA**. The PCR product was electrophorized on 1.5% agarose gel showing a single band of ~ 350 bp. This band was eluted from the gel and ligated to pGEM-T vector. XL-1 bacteria were transformed with the ligation product and propagated on agar in presence of ampicilin. Six colonies were picked up and grown in 5 ml of LB overnight.

Mini-preps from all six colonies were digested with NdeI and HindIII and all gave the expected ~ 350 bp fragment. Two clones were sequenced and the expected sequence was verified. The respective molecular mass calculated by DNAman program is 12861.6 Da, the specific extinction (1 mg/ml) at 280 nm is 0.22 and the expected isoelectric point is 4.76. The nucleotide (from pGEM-T clone 2, SEQ ID NO:72) and the full translated sequence of p712as (shown in bold, ORF starts at MVIG and ends at QLFA) containing 113 amino acids (SEQ ID NO:73), are presented herein:

```
1          NTGGCCGNTCCNGNCTCCNGCCGCCTGGCGGCCGCGGGAATTCGATTAAAAACATATGGT
1            W  P  X  X  X  P  A  A  W  R  P  R  E  F  D  *  K  H  M  V
61         GATTGGAGTCGTTGCGAGAGAGAATGCCGCAGAGCAGATCAAACAGTATCAAAAATTTAC
21           I  G  V  V  A  R  E  N  A  A  E  Q  I  K  Q  Y  Q  K  F  T
121        TGTGAATATTTCTGATGAAACTTCTATGCTTGCGATGGAGCAGGCTGGTTTTATCAGTCA
41           V  N  I  S  D  E  T  S  M  L  A  M  E  Q  A  G  F  I  S  H
181        TCAGGAGAAATTGGAACGTTTGGGAGTGCATTATGAAATTTCTGAACGAACTCAGATTCC
61           Q  E  K  L  E  R  L  G  V  H  Y  E  I  S  E  R  T  Q  I  P
241        TATTTTAGACGCCGCACCACTTGTTTTAGATTGTCGGGTAGATAGGATTGTTGAGGAAGA
81           I  L  D  A  A  P  L  V  L  D  C  R  V  D  R  I  V  E  E  D
301        CGGTATTTGCCACATCTTTGCCAAGATTCTTGAGCGACTTGTTGCCCCAGAACTCCTGGA
101          G  I  C  H  I  F  A  K  I  L  E  R  L  V  A  P  E  L  L  D
361        TGAAAAGGGACATTTTAAAAATCAACTGTTTGCCTAATAAGCTTAGCAAAAAAATCACTA
121          E  K  G  H  F  K  N  Q  L  F  A  *  *  A  *  Q  K  N  H  *
```

To prepare the expression plasmid the fragment encoding the PsipBas was cut off from the pGEM-T clone 2 with NdeI and HindIII and ligated into a linearized plasmid from which the encoding fragment was cut off with the same enzymes. The ligation product was first transfected into XL-1 cells, grown under kanamycin restriction and the DNA was prepared as described above. Then BL-21 Codon and pLys bacteria were transformed. The existence of the proper plasmid was verified by its isolation and restriction enzymes (NdeI and HindIII and NdeI and Bpu1 102i) analysis which has shown a ~ 350 bp insert as expected.

Expression – Four clones in BL-21 pLys and Codon cells were grown in 30 ml TB medium at 37°C until the OD595 reached 0.9, and then expression was induced with 0.4 mM IPTG. After 4 hours the cells were spun and frozen. Next day, each pellet was suspended in 20 ml cold 10mM EDTA 10mM Tris-HCl buffer pH 8.0, shaken for 30 min, sonicated and centrifuged. The supernatant was kept on ice, the pellet was sonicated and centrifuged again and suspended in 1 ml of ultrapure water (UPW). The soluble (cytosolic)

and insoluble inclusion bodies (IBs) fractions were evaluated for mini-expression using 12% SDS-PAGE. The expression of PsipBas was detected in the insoluble fraction of Codon and pLys cells. No specific expression was found in the soluble fraction of either Codon or pLys cells.

In order to elucidate whether the accompanying 14 kDa protein is related to PsipBas polypeptide, different fractions containing the mixture of 12 kDa and 14 kDa bands were tested by immunoblot with a rabbit anti PsipBa antibody at 1:20,000 and goat anti-rabbit Ab at 1:10,000 (Fig. 15). PsipBa served as a positive control (left lane). As shown the specific Ab recognized only the 12 kDa band.

Large-scale fermentation and preparation of inclusion bodies - Clone no 1 from pLys cells was chosen for large-scale preparation. Fermentation of 2.5 L (5 x 500 ml flasks) was carried out at 37°C in TB medium. After 3 hours when the cells reached OD595 = 0.9, IPTG was added to 0.4 mM, the fermentation was continued for 4 hr, and then the cells were centrifuged and stored in -20 °C. The cell pellets were suspended in 400 ml UPW containing 10mM EDTA, 10mM Tris-HCl buffer pH 8.0, DNAse (10 μg/ml), and the IBs were prepared. The final IB pellet was suspended in 100ml of UPW and frozen in aliquots. Aliquot of purified IBs were checked by SDS-PAGE to verify PsipBas expression. Approximate calculation shows that 1ml of IBs suspension contains between 2.5 to 5 mg of PsipBas.

Purification protocol - 20ml of IBs suspension were thawed and refolded in total volume of 66 ml. The refold was performed in at 1mM of cysteine at pH 11. After 1.0 h of shaking the sample were diluted with 3 vol of 0.667 M Arg and the shaking was continued for 1.5 h. The solution (266ml) was left on stirrer overnight. Next morning the solution was concentrated to 40ml and two portions of 20ml each were applied consecutively onto preparative Superdex column pre-equilibrated with 25mM Tris buffer pH 10 containing 0.3 M arginine and 1 M urea. The fractions containing PsipBas along with the contaminating 14 kDa protein were identified by SDS-PAGE, pooled and next day applied onto Q-Sepharose (8 ml) column pre-equilibrated with Tris base, pH 10. The fraction eluted with 150mM and 200mM NaCl were each pooled and checked for purity by SDS-PAGE.

Eluates of 150mM and 200mM pools along with the similar fractions obtained from the previous experiment were pooled, concentrated to 35 ml, desalted on a G-25 Sephadex column (2.6 x 100cm) equilibrated with 1.2 mg/ml of $NaHCO_3$, and immediately

lyophilized in 0.25 mg/vial, each vial containing also ~ 0.6 mg $NaHCO_3$. The protein concentration was calculated from the A280, assuming that absorbance of 0.22 = 1 mg/ml as calculated by the DNAman program. The G-25 Sephadex eluate was adjusted to 500μg/ml, filter sterilized and lyophilized in 250 μg per vial. The lyophilized PsipBas could be easily dissolved in UPW. SDS-PAGE presented in Fig. 16 shows its purity level of ~89% as estimated by gel scanning. In a gel filtration analysis, lyophilized PsipBas appears mainly as dimer along with some oligomer (Fig.17).

Endotoxin - The purified PsipBas (0.25 mg/ml) was tested for its endotoxin content. It contained <10 EU/ml or <40 EU/mg or 4 ng/mg.

Example 9. Lethal challenge and ex- vivo animal study with PsipBsa)

Lethal challenge study was performed in order to evaluate the protective capacity of subcutaneously (SC) immunization with cell-wall-derived recombinant protein against intranasal (IN) challenge with a lethal dose of S. pneumoniae bacteria. Ten 7-week-old BALB/c mice were immunized SC 3 times with 20 μg of PsipBas protein formulated with CFA adjuvant (on Day 0) and IFA adjuvant (on Days 7 and 21). An additional group of 10 mice was treated with CFA/IFA adjuvant alone as a negative control. One week after the last immunization, the mice were challenged IN with 4.7 X 10^7 of *S. pneumoniae*. Survival was monitored daily for seven consecutive days. Survival was monitored daily for seven consecutive days. As shown in figure 18, immunizations with PsipBas protein resulted in 20% survival, while negative control mice did not survived (0% survival).

Ex-vivo neutralization test was performed on 1000 CFU of WU2 bacteria incubated for 1 hour at room Temperature (RT) with 1:20 diluted pooled mouse anti-PsipBas sera. Mouse post-immune sera (but pre-challenge) were collected and pooled from the 2 individual mice that were immunized with PsipBas and survived later on IN challenge with S. pneumoniae serotype 3 strain WU2 (see above). Negative control sera were collected and pooled from 3 out of 10 random mice, following injection with CFA/IFA adjuvant alone (but pre-challenge) and that died following IN challenge with *S. pneumoniae* serotype 3 strain WU2. In order to test the neutralization activity of the antisera following incubation, mixture of antisera and bacteria were inoculated intraperitoneally (IP) to nine 7-week-old BALB/c mice. Survival was monitored daily for seven consecutive days. The final results (figure 19) showed 33% survival of mice treated with anti-PsipBas sera, while 10 negative control mice did not survived (0% survival).

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention.

CLAIMS

1. A modified PsipB polypeptide or fragment comprising at least amino acid residues 48-112 of SEQ ID NO:1 or of a sequence having at least 95% homology to SEQ ID NO:1 and comprising an amino acid substitution of at least one cysteine residue naturally present in a wild type PsipB polypeptide.

2. The modified PsipB polypeptide or fragment of claim 1, wherein the amino acid substitution is at a residue corresponding to a residue of the wild type PsipB polypeptide of SEQ ID NO:1 selected from the group consisting of: the cysteine residue at position 66 (C66); the cysteine residue at position 72 (C72); the cysteine residue at position 84 (C84), and a combination of residues thereof.

3. The modified PsipB polypeptide or fragment of claim 1 or 2, wherein the amino acid substitution is of a cysteine residue by an amino acid residue selected from the group consisting of alanine, serine, threonine, glycine, valine, leucine, tyrosine and a combination of residues thereof.

4. The modified PsipB polypeptide or fragment of claim 2, wherein the amino acid substitution is selected from the group consisting of: substitution of the cysteine residue at position 66 by an alanine residue (C66A); substitution of the cysteine residue at position 72 by an alanine residue (C72A); substitution of the cysteine residue at position 84 by an alanine residue (C84A), and a combination of residues thereof.

5. The modified PsipB polypeptide or fragment of claim 4, comprising 1-3 substitutions selected from the group consisting of: C66A, C72A and C84A.

6. The modified PsipB polypeptide or fragment of claim 1, wherein the modified PsipB polypeptide or fragment is devoid of cysteine residues.

7. The modified PsipB polypeptide or fragment of claim 1, wherein the modified PsipB polypeptide is selected from the group consisting of SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; SEQ ID NO:21; SEQ ID NO:22; SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:29; SEQ ID NO:30; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; SEQ ID NO:40; SEQ ID NO:41; SEQ ID NO:42; SEQ ID NO:43; SEQ ID NO:44; SEQ ID NO:45; SEQ

ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; SEQ ID NO:50; SEQ ID NO:51; SEQ ID NO:52; SEQ ID NO:53; SEQ ID NO:54; SEQ ID NO:55; SEQ ID NO:56; SEQ ID NO:57; SEQ ID NO:58; SEQ ID NO:59; SEQ ID NO:60; SEQ ID NO:61, and SEQ ID NO:62.

8. The modified PsipB polypeptide or fragment of claim 1, wherein the modified PsipB polypeptide is selected from the group consisting of SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15; and SEQ ID NO:16.

9. The modified PsipB fragment of claim 1 consisting of 65-120 amino acids.

10. The modified PsipB fragment of claim 9 comprising residues 1-112 of SEQ ID NO:1.

11. The modified PsipB fragment of claim 10, selected from the group consisting of SEQ ID NO:64; SEQ ID NO:65; SEQ ID NO:66; SEQ ID NO:67, SEQ ID NO:73; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID NO:77; SEQ ID NO:78; SEQ ID NO:79.

12. The modified PsipB polypeptide or fragment of any of claims 1-11, in the form of a fusion protein.

13. The modified PsipB polypeptide or fragment of any of claims 1-11 conjugated or fused to a carrier protein or conjugated to one or more *S. pneumoniae* capsular polysaccharides.

14. An isolated polynucleotide sequence encoding a modified PsipB polypeptide or fragment, wherein the modified PsipB polypeptide or fragment encoded by the polynucleotide has an amino acid residue other than cysteine at a position corresponding to a position of the wild type PsipB polypeptide of SEQ ID NO:1 selected from the group consisting of position 66, position 72, position 84, and a combination of residues thereof.

15. The isolated polynucleotide of claim 14, wherein the modified PsipB polypeptide or fragment comprises at least one amino acid substitution selected from the group consisting of C66A, C72A, C84A and a combination of residues thereof.

16. The isolated polynucleotide of claim 14, wherein the modified PsipB polypeptide or fragment encoded by the polynucleotide is selected from the group consisting of SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; SEQ ID

NO:21; SEQ ID NO:22; SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:29; SEQ ID NO:30; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; SEQ ID NO:40; SEQ ID NO:41; SEQ ID NO:42; SEQ ID NO:43; SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; SEQ ID NO:50; SEQ ID NO:51; SEQ ID NO:52; SEQ ID NO:53; SEQ ID NO:54; SEQ ID NO:55; SEQ ID NO:56; SEQ ID NO:57; SEQ ID NO:58; SEQ ID NO:59; SEQ ID NO:60; SEQ ID NO:61; SEQ ID NO:62; SEQ ID NO:64; SEQ ID NO:65; SEQ ID NO:66; SEQ ID NO:67, SEQ ID NO:73; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID NO:77; SEQ ID NO:78; and SEQ ID NO:79.

17. The isolated polynucleotide of claim 14, comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19, SEQ ID NO:20 and SEQ ID NO:72.

18. An expression vector comprising the polynucleotide sequence of any one of claims 14-17, wherein the polynucleotide sequence is operatively linked to one or more control sequences.

19. A host cell transformed with the expression vector of claim 18.

20. A pharmaceutical composition comprising an effective amount of the expression vector of claim 19, and at least one pharmaceutically acceptable carrier, diluent or adjuvant.

21. A vaccine composition comprising an effective amount of a modified PsipB polypeptide or fragment according to claim 1, further comprising at least one pharmaceutically acceptable carrier, diluent, adjuvant or delivery system.

22. The vaccine composition of claim 21, wherein the modified PsipB polypeptide or fragment is selected from the group consisting of SEQ ID NO:13; SEQ ID NO:14; SEQ ID NO:15; SEQ ID NO:16; SEQ ID NO:21; SEQ ID NO:22; SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:29; SEQ ID NO:30; SEQ ID NO:31; SEQ ID NO:32; SEQ ID NO:33; SEQ ID NO:34; SEQ ID NO:35; SEQ ID NO:36; SEQ ID NO:37; SEQ ID NO:38; SEQ ID NO:39; SEQ ID NO:40; SEQ ID NO:41;

SEQ ID NO:42; SEQ ID NO:43; SEQ ID NO:44; SEQ ID NO:45; SEQ ID NO:46; SEQ ID NO:47; SEQ ID NO:48; SEQ ID NO:49; SEQ ID NO:50; SEQ ID NO:51; SEQ ID NO:52; SEQ ID NO:53; SEQ ID NO:54; SEQ ID NO:55; SEQ ID NO:56; SEQ ID NO:57; SEQ ID NO:58; SEQ ID NO:59; SEQ ID NO:60; SEQ ID NO:61; SEQ ID NO:62; SEQ ID NO:64; SEQ ID NO:65; SEQ ID NO:66; SEQ ID NO:67; SEQ ID NO:73; SEQ ID NO:74; SEQ ID NO:75; SEQ ID NO:76; SEQ ID NO:77; SEQ ID NO:78; and SEQ ID NO:79.

23. The vaccine composition of claim 21 or 22, comprising a plurality of modified PsipB polypeptides.

24. The vaccine of any one of claims 21-23, further comprising at least one heterologous *S. pneumoniae* derived polypeptide or fragment thereof, in addition to the modified PsipB polypeptide.

25. The vaccine composition of any of claims 23-27, comprising a modified PsipB polypeptide immunogenic fragment.

26. The vaccine composition of any one of claims 21-25, formulated for administration by a route selected from the group consisting of: intramuscular, intranasal, oral, intraperitoneal, subcutaneous, topical, intradermal and transdermal delivery.

27. A method of inducing an immune response against *S. pneumoniae* in a subject, the method comprising administering to a subject in need thereof an effective amount of a modified PsipB polypeptide or fragment according to any one of claims 1-13, thereby inducing an immune response against *S. pneumoniae* in the subject.

28. A method of inducing an immune response against *S. pneumoniae* in a subject, the method comprising administering to a subject in need thereof an effective amount of a pharmaceutical composition of claim 20 under conditions which allow expression of the polynucleotide encoding the modified PsipB polypeptide, thereby inducing an immune response against *S. pneumoniae* in the subject.

29. A method of conferring protection against *S. pneumoniae* infection in a subject, the method comprising administering to a subject in need thereof an effective amount of a modified PsipB polypeptide or fragment according to any one of claims 1-13, thereby conferring protection against *S. pneumoniae* infection in the

subject.

30. A method of conferring protection against *S. pneumoniae* infection in a subject, the method comprising administering to a subject in need thereof an effective amount of a pharmaceutical composition of claim 20 under conditions which allow expression of the polynucleotide encoding the modified PsipB polypeptide, thereby conferring protection against *S. pneumoniae* infection in the subject.

31. Use of a modified PsipB polypeptide or fragment according to any one of claims 1-13 for the preparation of a medicament for inducing an immune response in a subject against *S. pneumoniae* or for conferring protection against *S. pneumoniae* infection.

32. A modified PsipB polypeptide or fragment according to any one of claims 1-13 for use in inducing an immune response in a subject against *S. pneumoniae* or for use in conferring protection against *S. pneumoniae* infection.

33. A method of enhancing the stability at physiological pH formulation of a PsipB polypeptide, the method comprising providing a modified PsipB polypeptide having at least one amino acid substitution of a cysteine residue naturally present in a wild type PsipB polypeptide, wherein the amino acid substitution is of a residue corresponding to a residue of the wild type PsipB polypeptide of SEQ ID NO:1 selected from the group consisting of: the cysteine residue at position 66 (C66); the cysteine residue at position 72 (C72); the cysteine residue at position 84 (C84), and a combination thereof.

Figure 1


1-NP_358083 1----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQIPILDACP 67
2-NP_345081 1----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQTPILDACP 67
3-ZP_01817263 1----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQIPILDACP 67
4-ZP_01835575 1----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQIPILDACP 67
5-YP_0027420801----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQTPILDACP 67
6-ZP_01829414 1----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQISILDACP 67
7-ZP_01821338 1----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQTPILDACP 67
8-ZP_01827178 1----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQTPILDACP 67
9-ZP_01833557 1----------MIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQTPILDACP 67
10YP_0018352441MTCSSSYSLGDWVVIGVVARENAAEQIKQYQKFTVNISDETSMLAMEQAGFISHQEKLERLGVHYEISERTQTPILDACP 80
11YP_0020372281----------MERLGVHYEISERTQIPILDACP 23
12YP_0027356191----------MGVHYEISERTQTPILDTCP 20
NP_358083 68LVLDCRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFAPTYFMGDGYQRVYRYLDKRVDMKGSFIKKARKKDG 147
NP_345081 68LVLDCRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFAPTYFMGDGYQRVYRYLDKRVDMKGSFIKKARKKDG 147
ZP_01817263 68LVLDCRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFAPTYFMGDGYQRVYRYLDKRVDIKGSFIKKARKKDG 147
ZP_01835575 68LVLDCRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFAPTYFMGDGYQRVYRYLDKRVDMKGGFIKKARKKDG 147
YP_0027420806 8LVLDCRVDRIVEEDGICHIFAKILERLVVPELLDEKGHFKNQLFAPTYFMGDGYQRVYRYLDKRVDMKGSFIKKARKKDG 147
ZP_01829414 68LVLDCRVDRIVEEDGICHIFAKILERLVAPEFLDEKGHFKNQLFAPTYFMGDGYQRVYRYLDKRVDMKGSFIKKARKKDG 147
ZP_01821338 68LVLDCRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFAPTYFMGDVYQRVYRYLDKRVDMKGSFIKKSEEEGW 147
ZP_01827178 68LVLDCRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFA----------QPISWEMDIS-AFIAIWTSV-- 131
ZP_01833557 68LVLDMRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFA----------QPISWEMDIS-AFIAIWTSV-- 131
YP_0018352448 1LVLDMRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFA----------QPISWEMDIS-AFIAIWTSV-- 144
YP_0020372282 4LVLDCRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFAPTYFMGDGYQRVYRYLDKRVDMKGSFIKKARKKDG 103
YP_0027356192 1LVLDCRVDRIVEEDGICHIFAKILERLVAPELLDEKGHFKNQLFAPTYFMGDGYQRVYRYLDKRVDMKGSFIKKARKKDG 100
NP_358083 148KN----------149
NP_345081 148KN----------149
ZP_01817263 148KN----------149
ZP_01835575 148KN----------149
YP_002742080148KN----------149
ZP_01829414 148KN----------149
ZP_01821338 148QELSCQIKIETERLVLRVRTVADAEDIFDYASLPEVAYPAGFPPVKTLEDEIYYLEHILPERNQKENLPAGYGIVVKGTD227
ZP_01827178 ----------
ZP_01833557 ----------
YP_001835244 ----------
YP_002037228 104KN ---------- 105
YP_002735619 101KN ---------- 102

Figure 2A

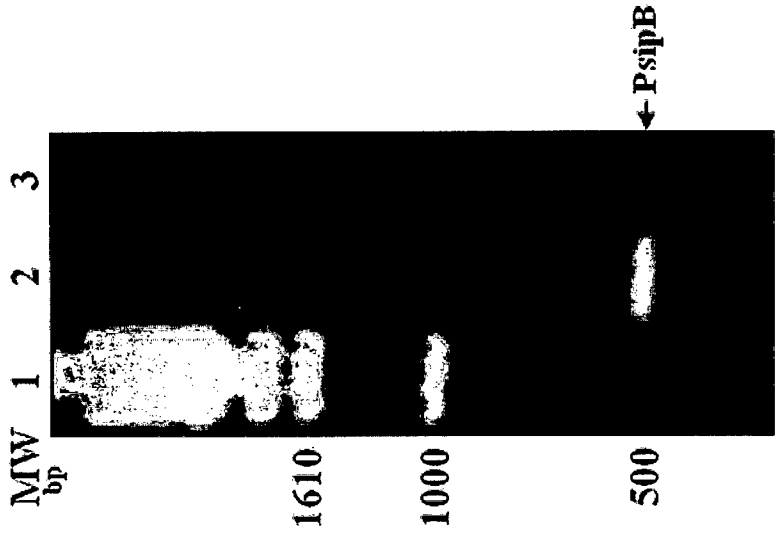


Figure 2B

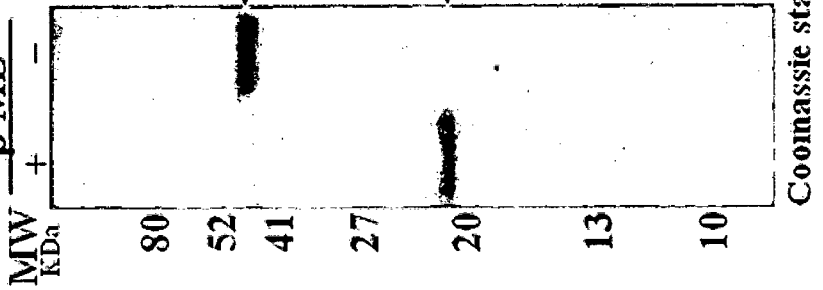


Figure 2C

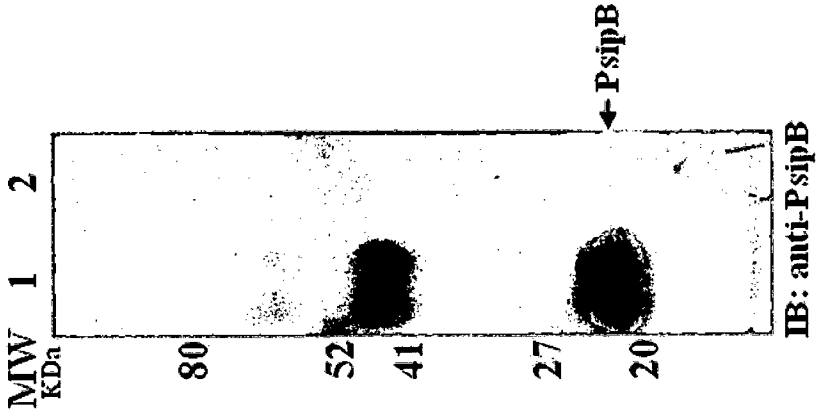


Figure 2D

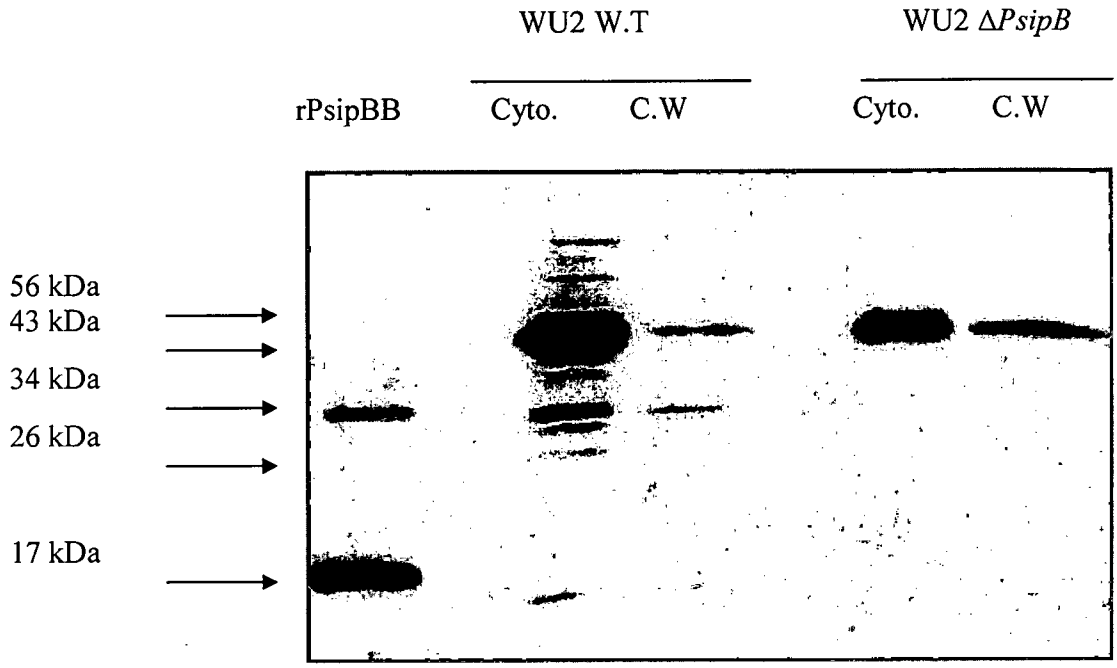

WU2 W.T
WU2 ΔPsipB
rPsipBB
Cyto.
C.W
Cyto.
C.W
56 kDa
43 kDa
34 kDa
26 kDa
17 kDa

Figure 3A

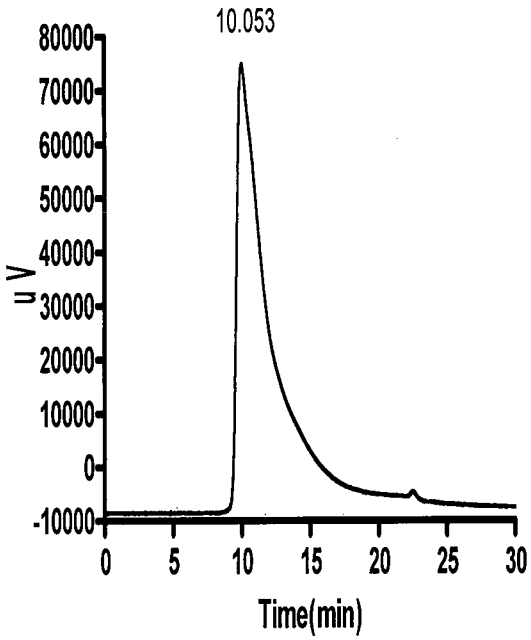
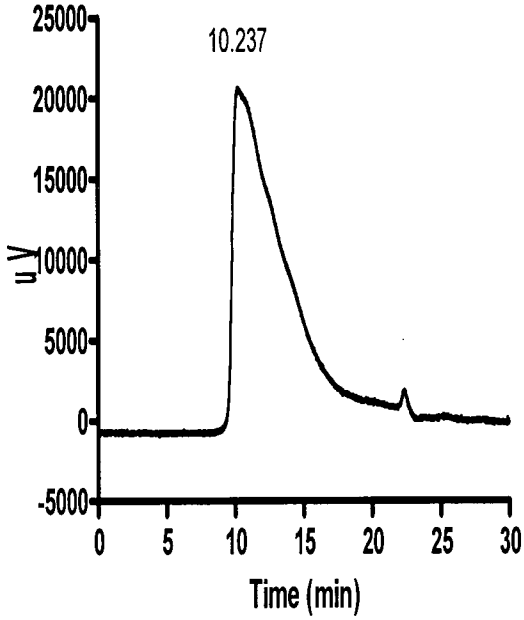


Figure 3B



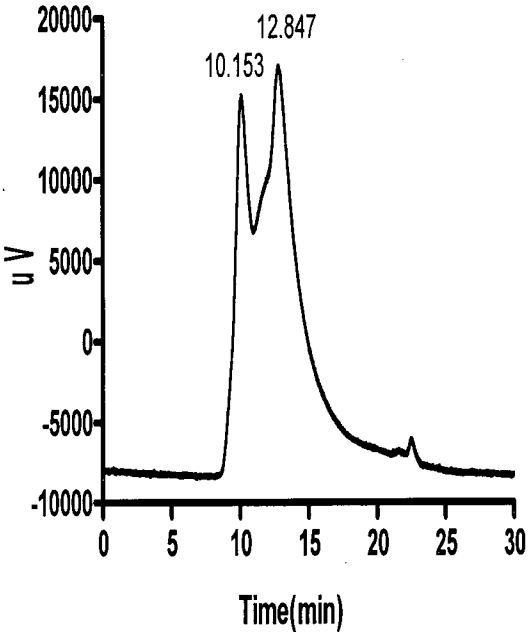


Figure 3C

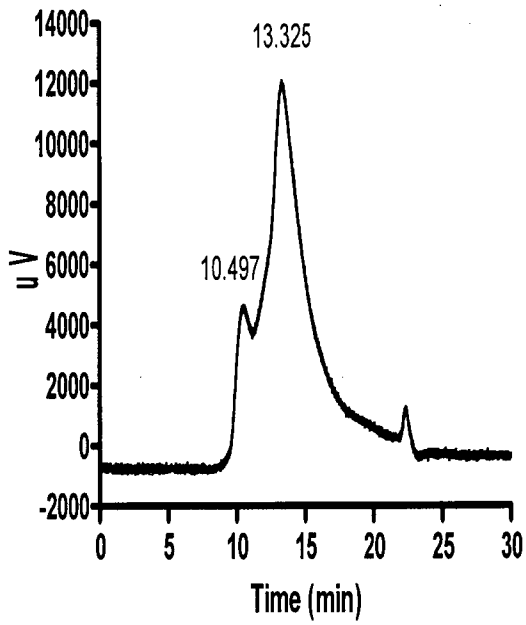


Figure 3D

Figure 4A

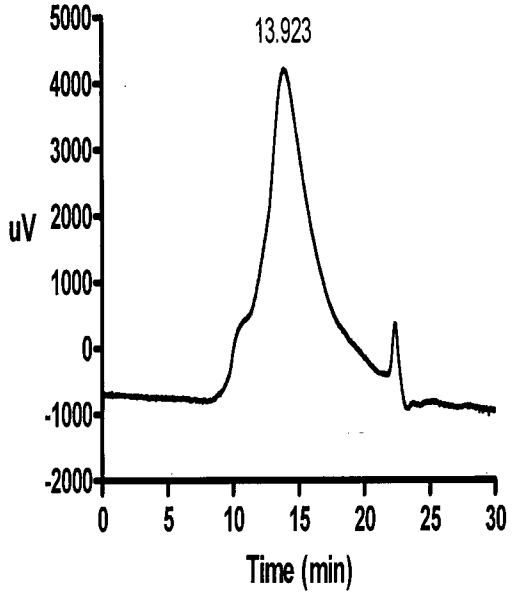
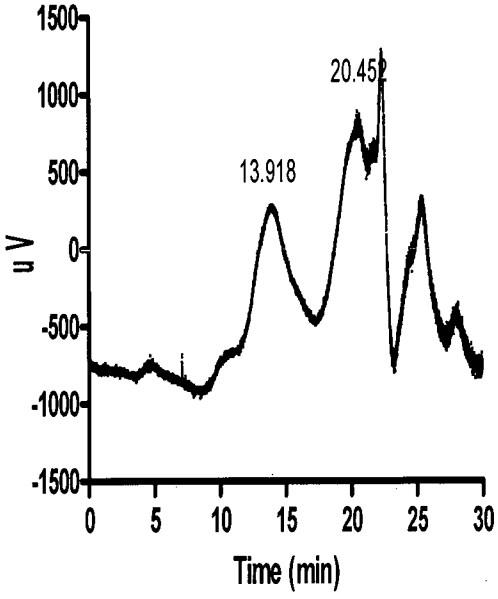


Figure 4B



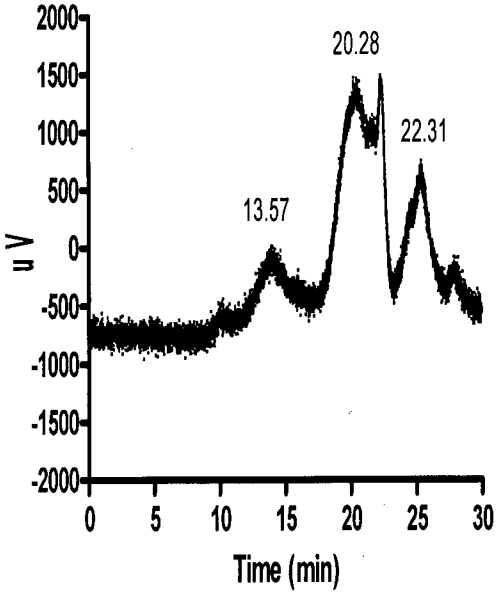


Figure 4C

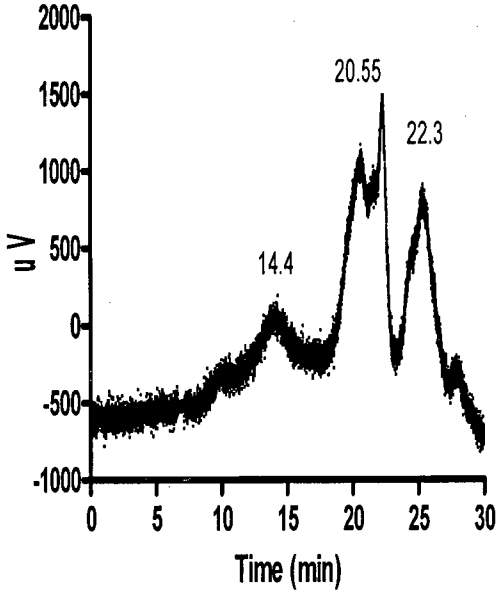


Figure 4D

Figure 5

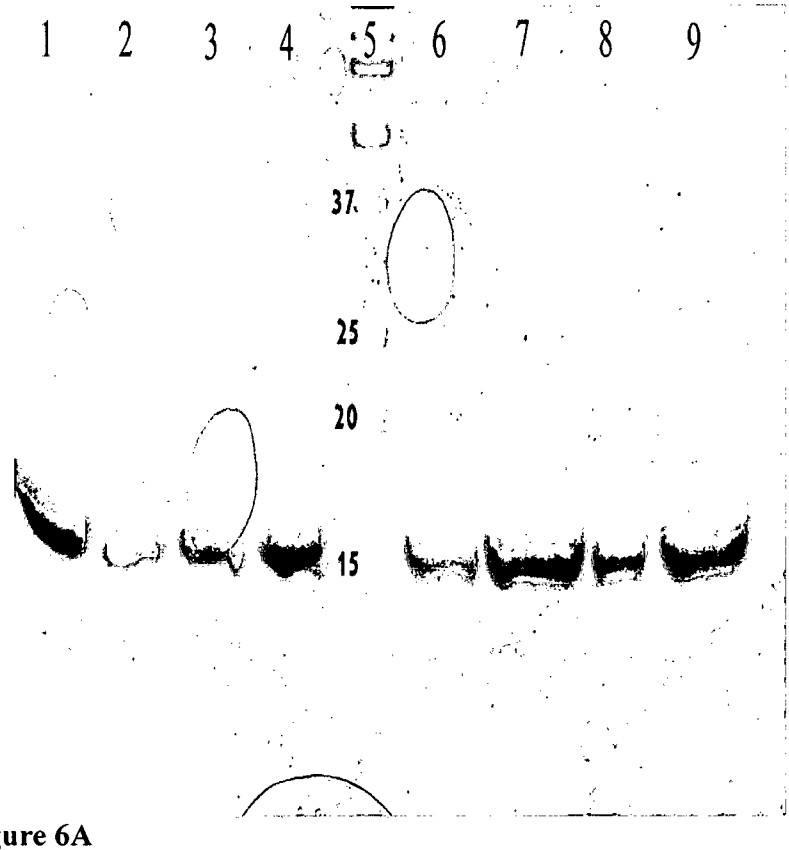


Figure 6A

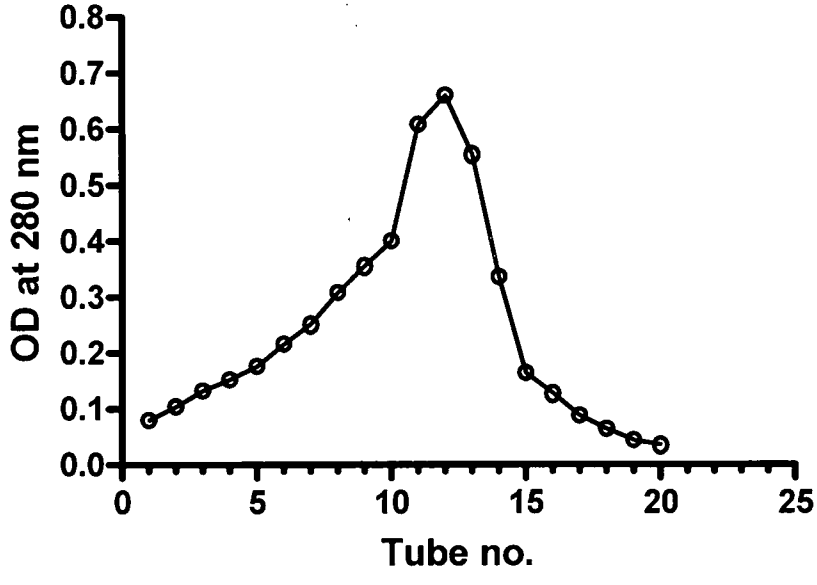


Figure 6B

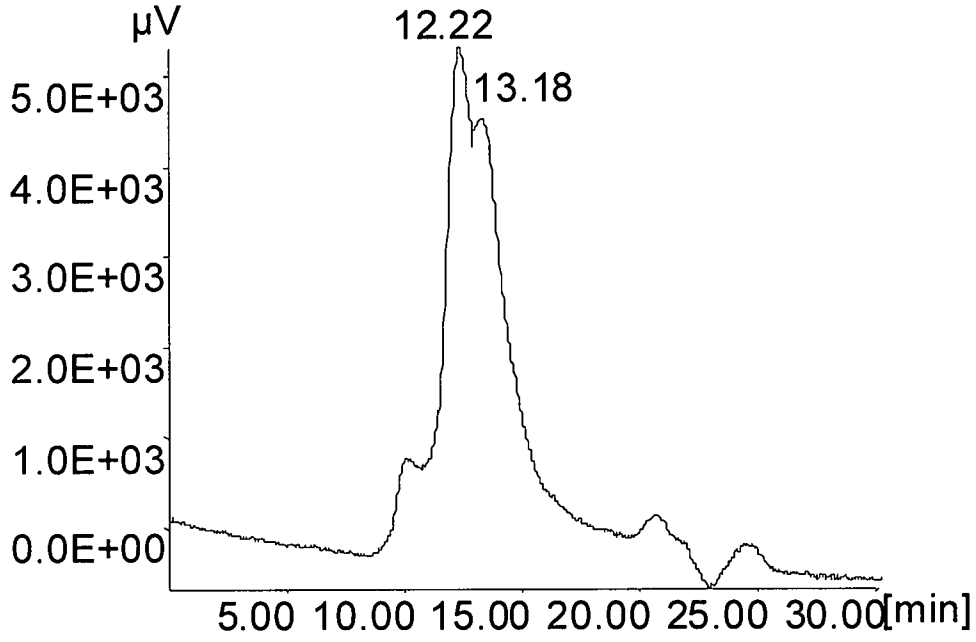


Figure 6C

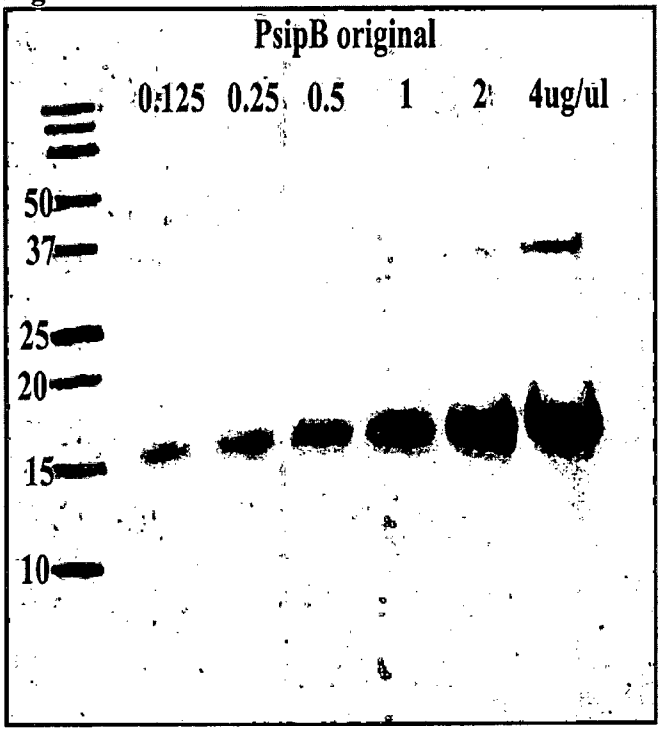


Figure 7A

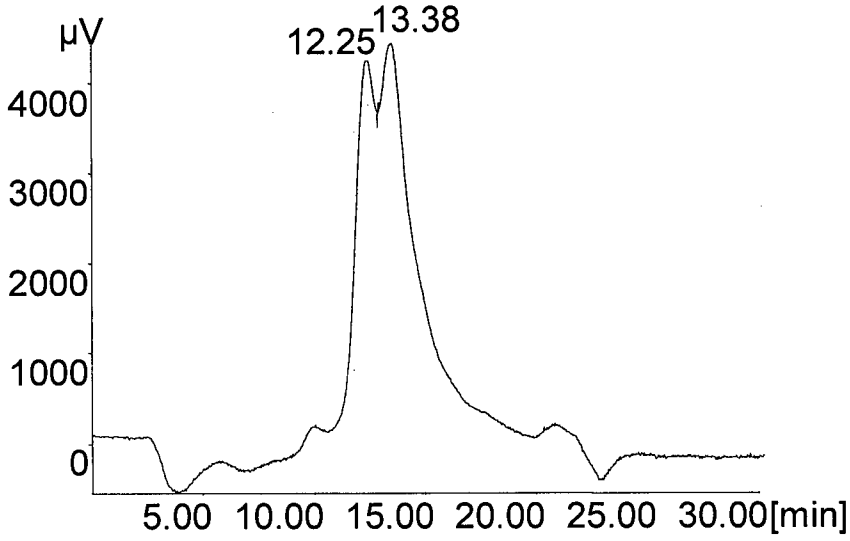


Figure 7B

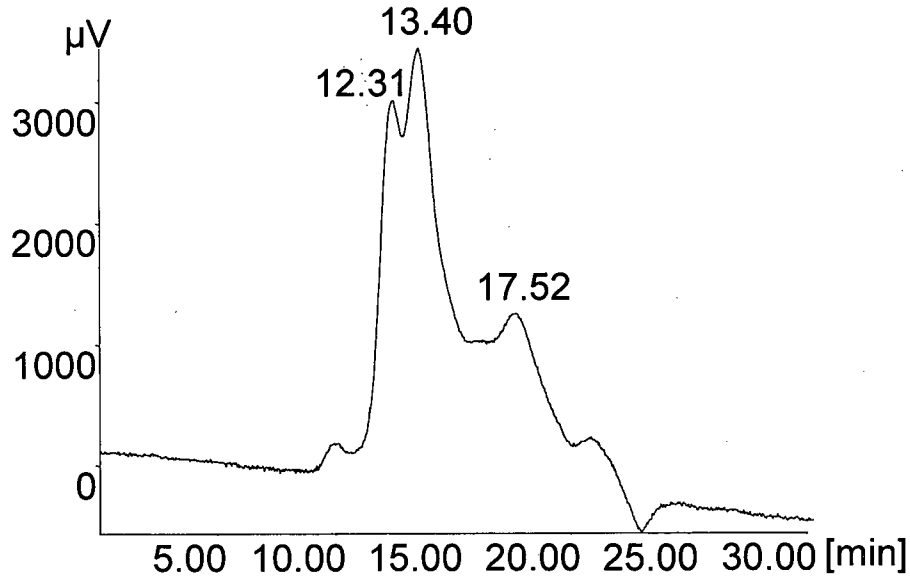


Figure 8

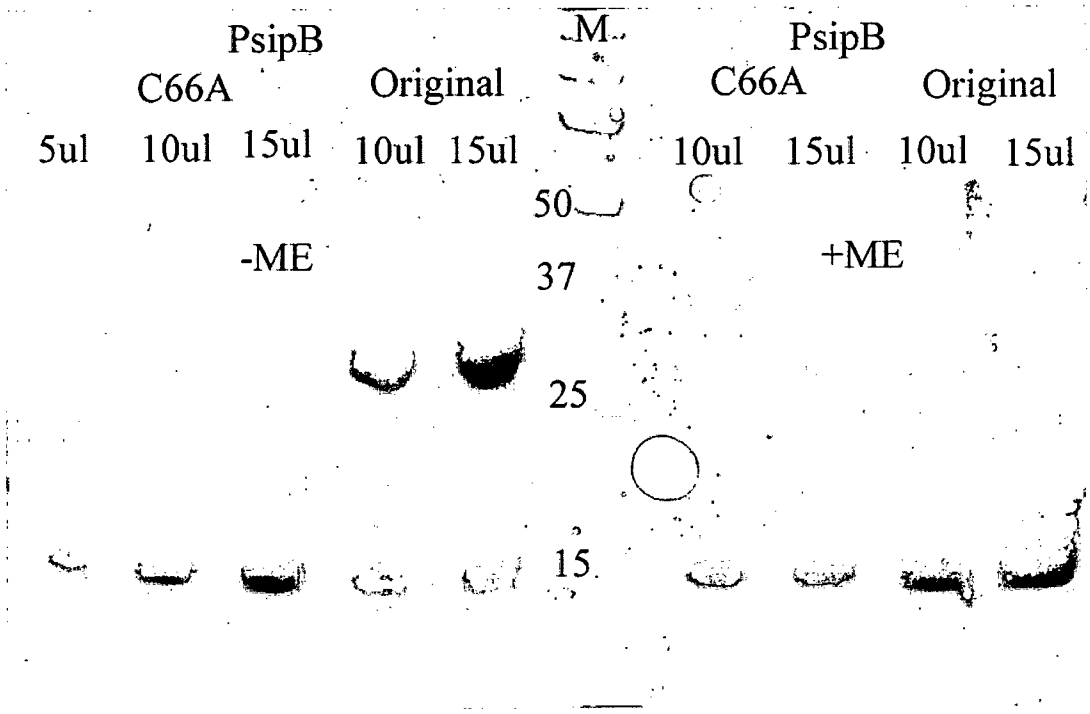


Figure 9A

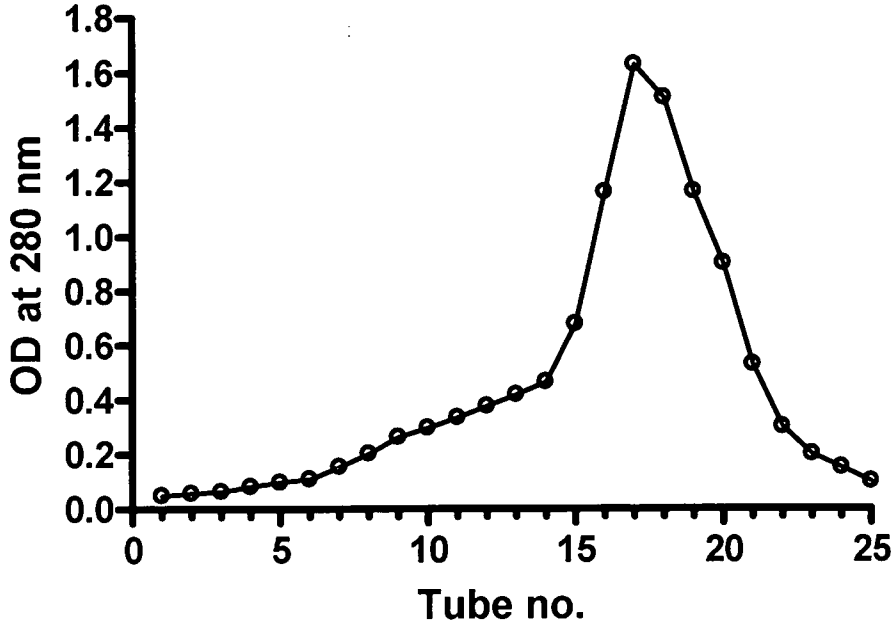


Figure 9B

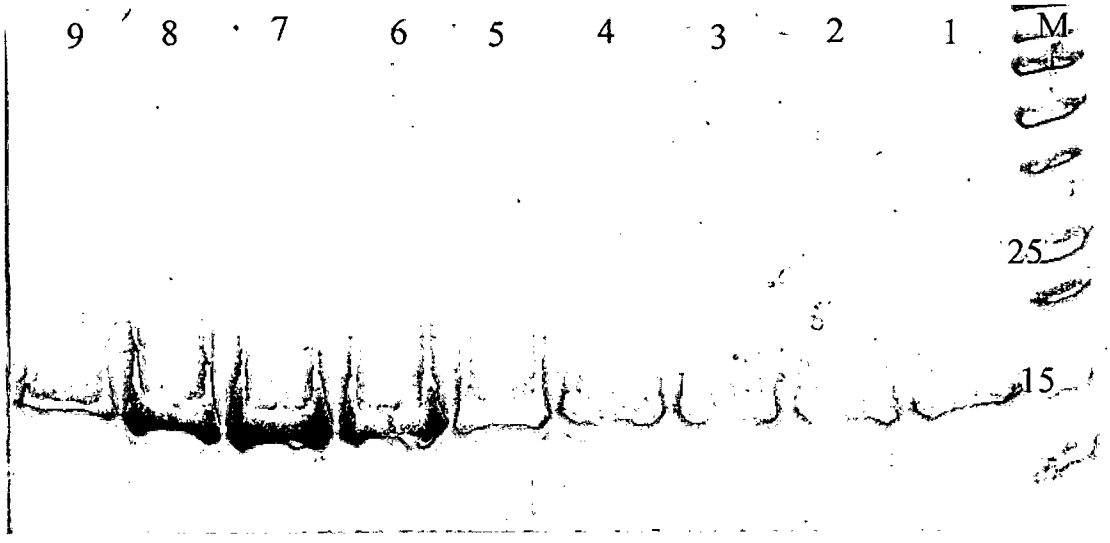


Figure10A

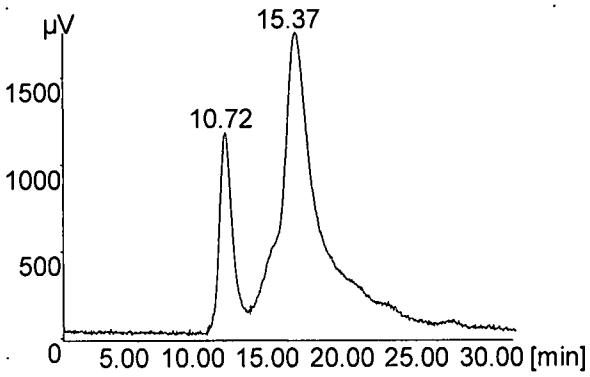
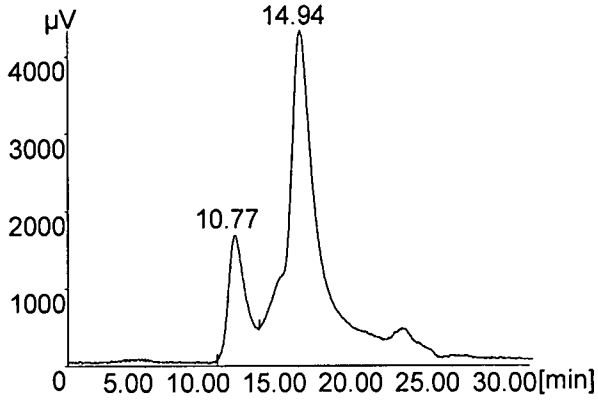


Figure10B



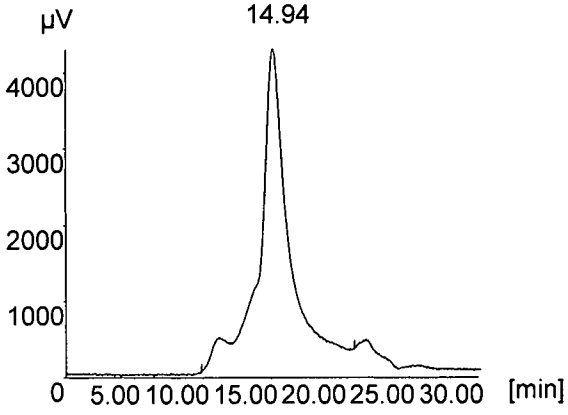


Figure10C

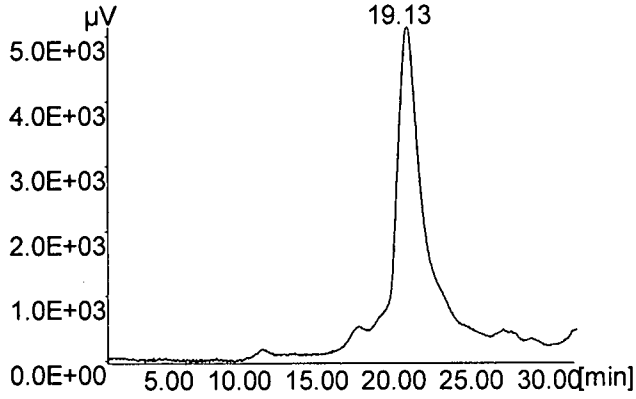


Figure10D

Figure 11

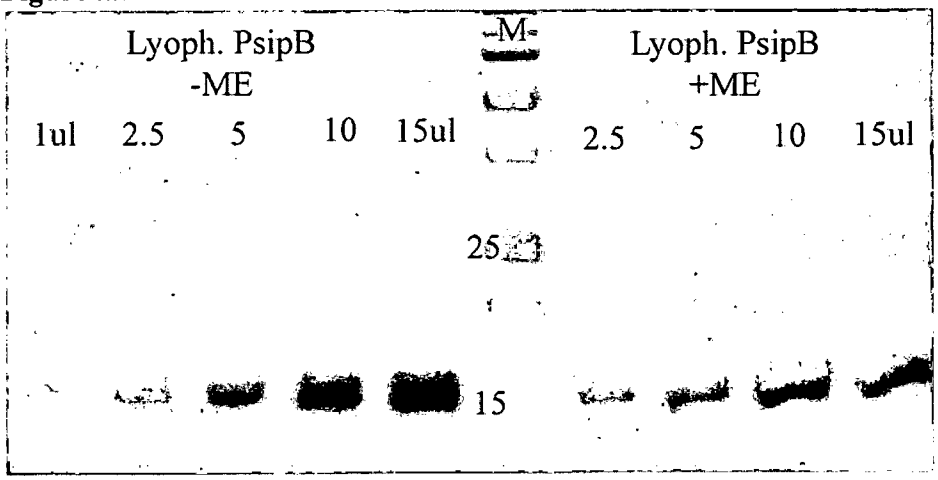


Figure 12

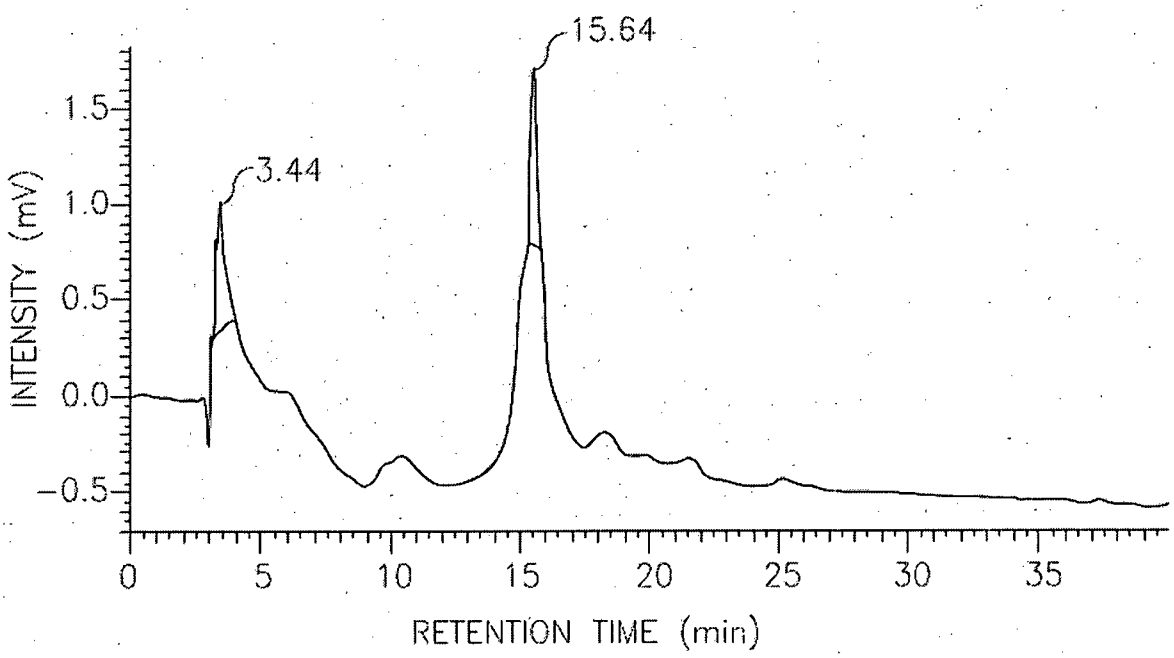


Figure 13

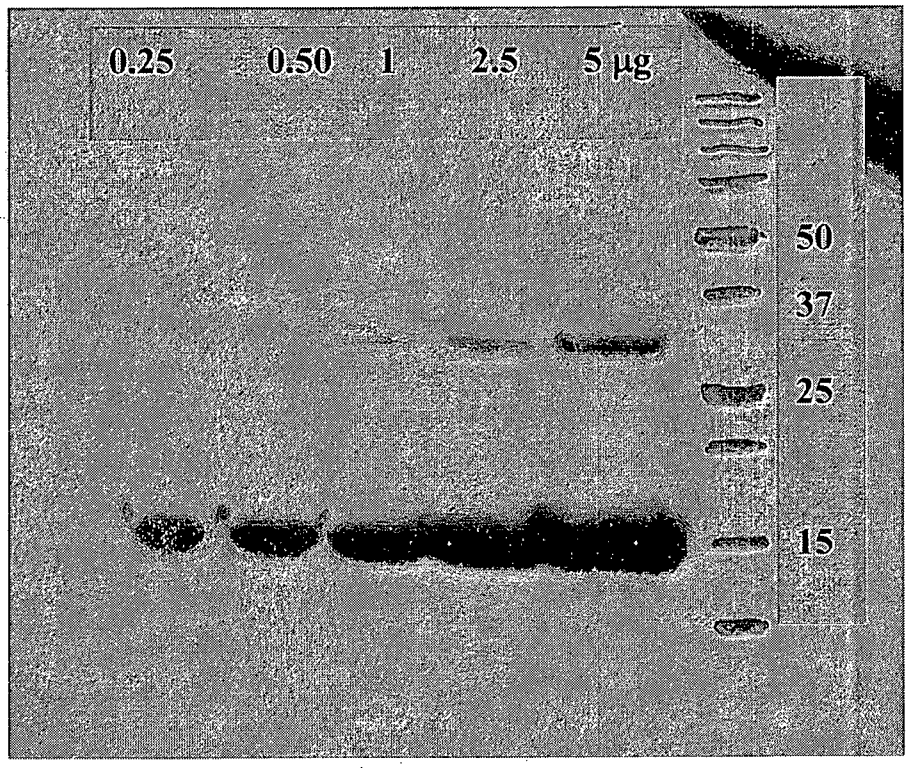


Figure 14A

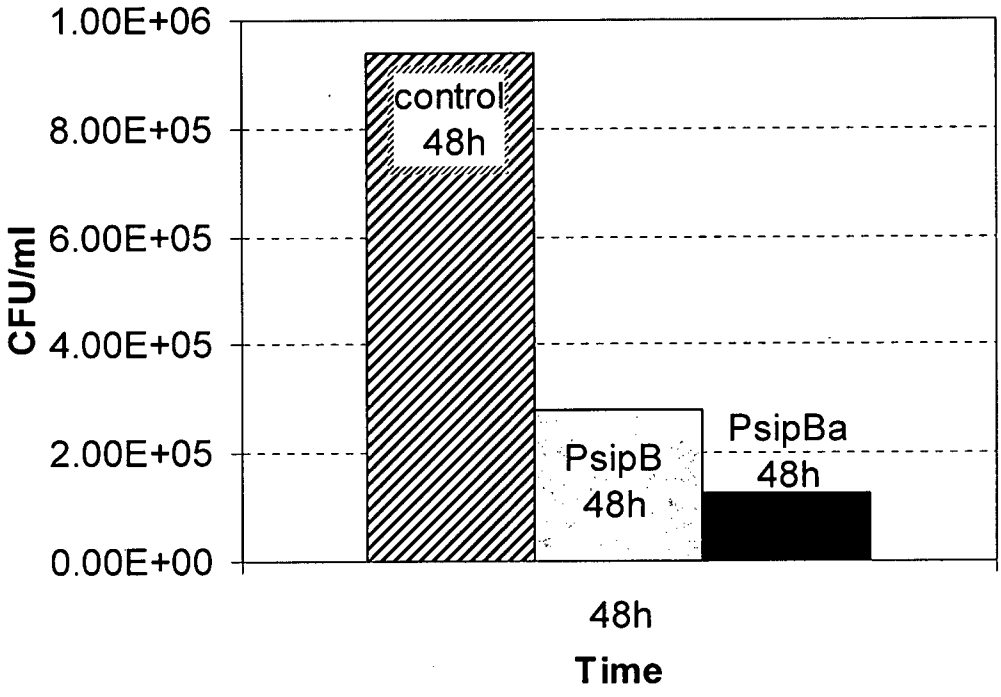


Figure 14B

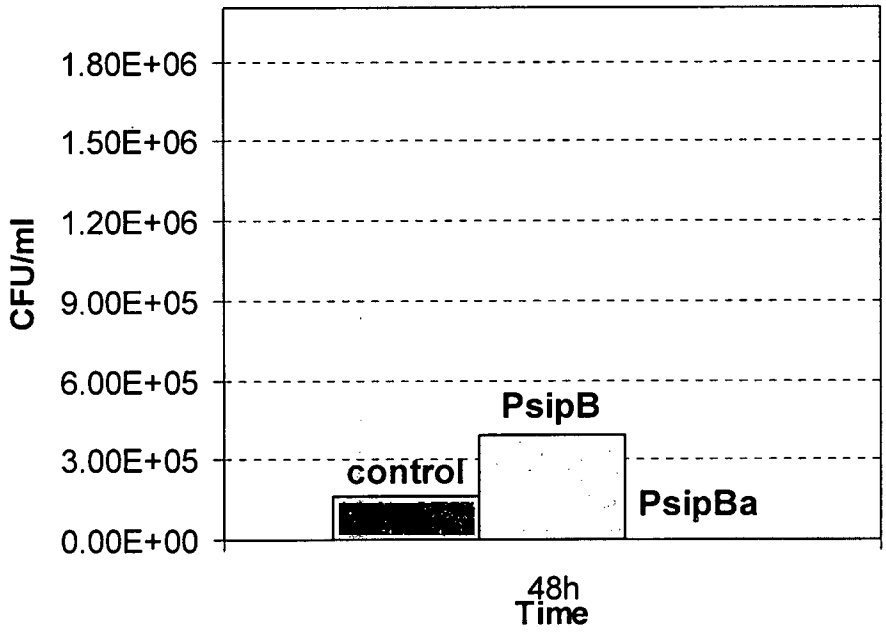


Figure 15

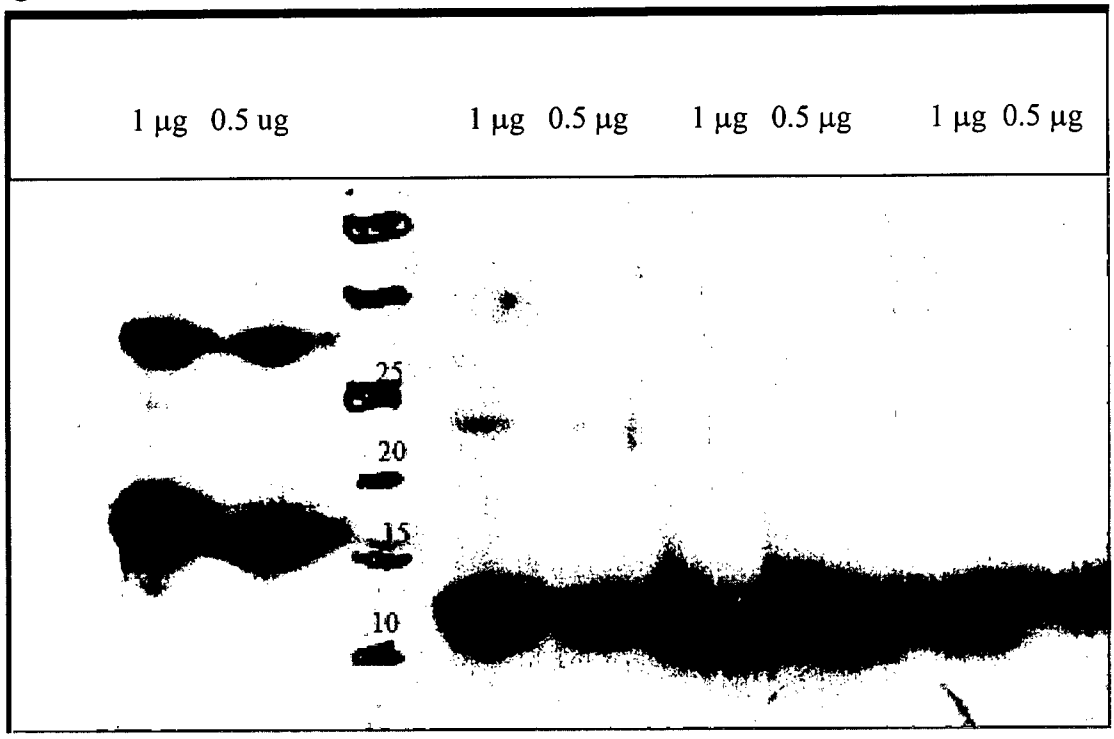


Figure 16

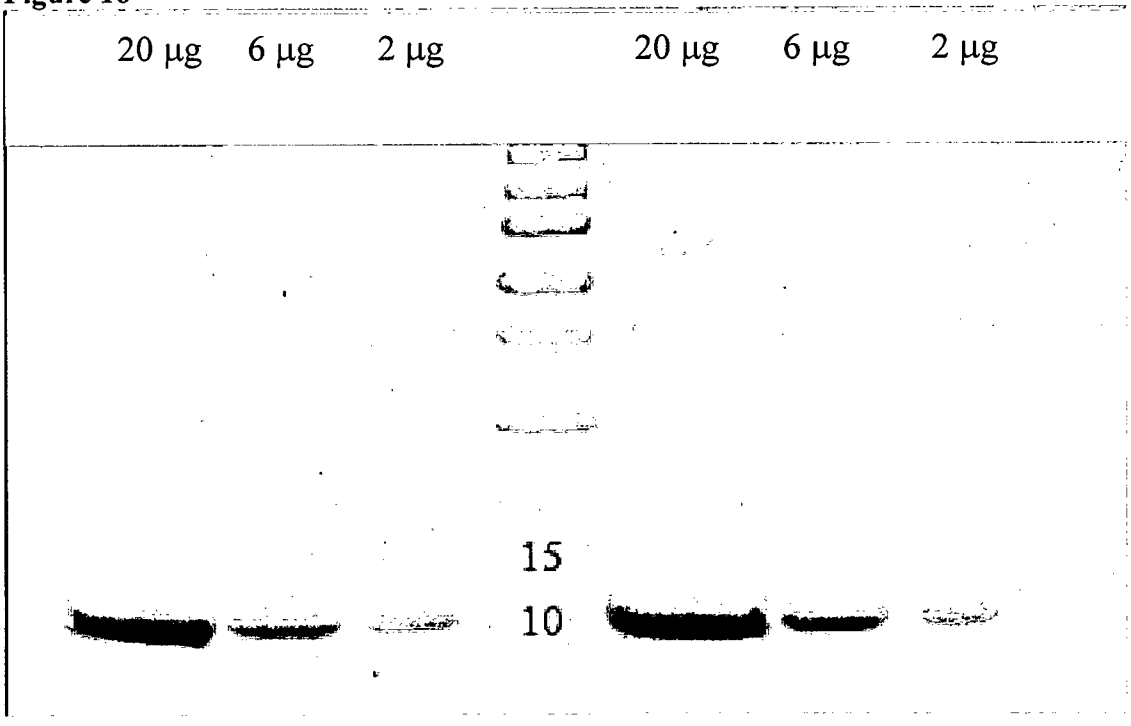


Figure 17

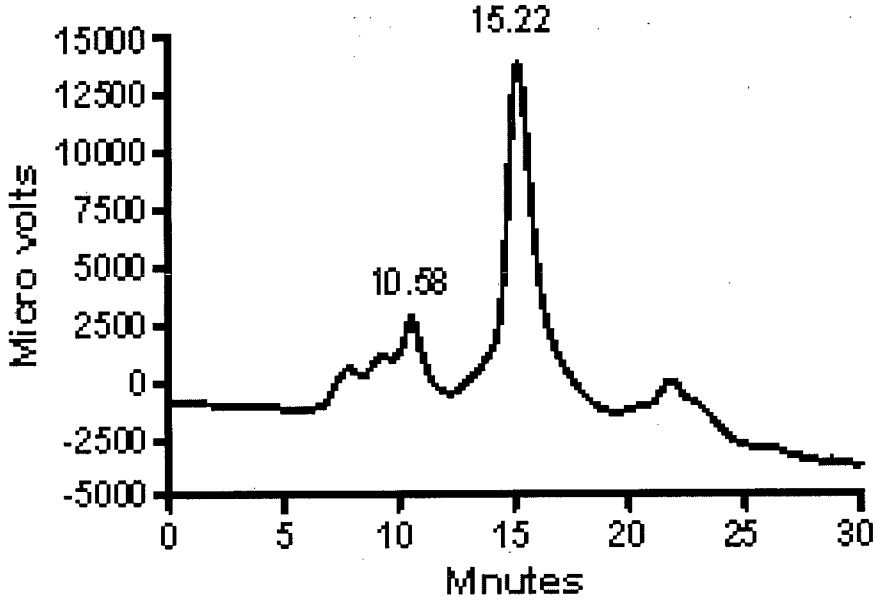


Figure 18

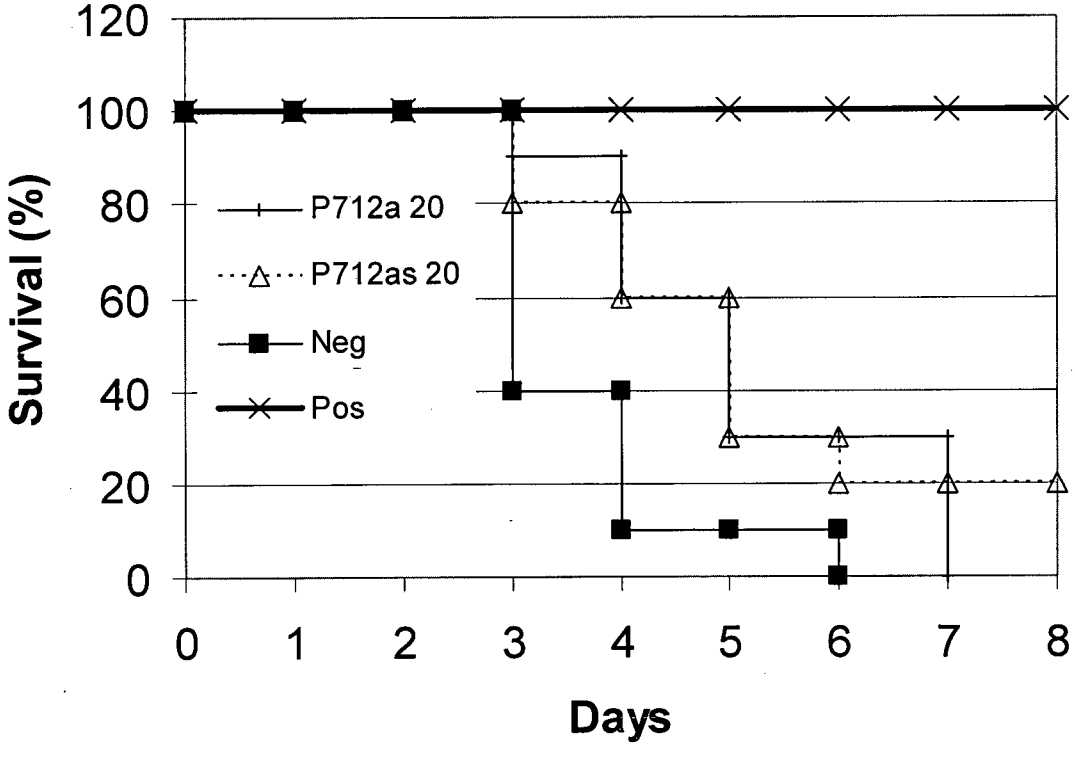
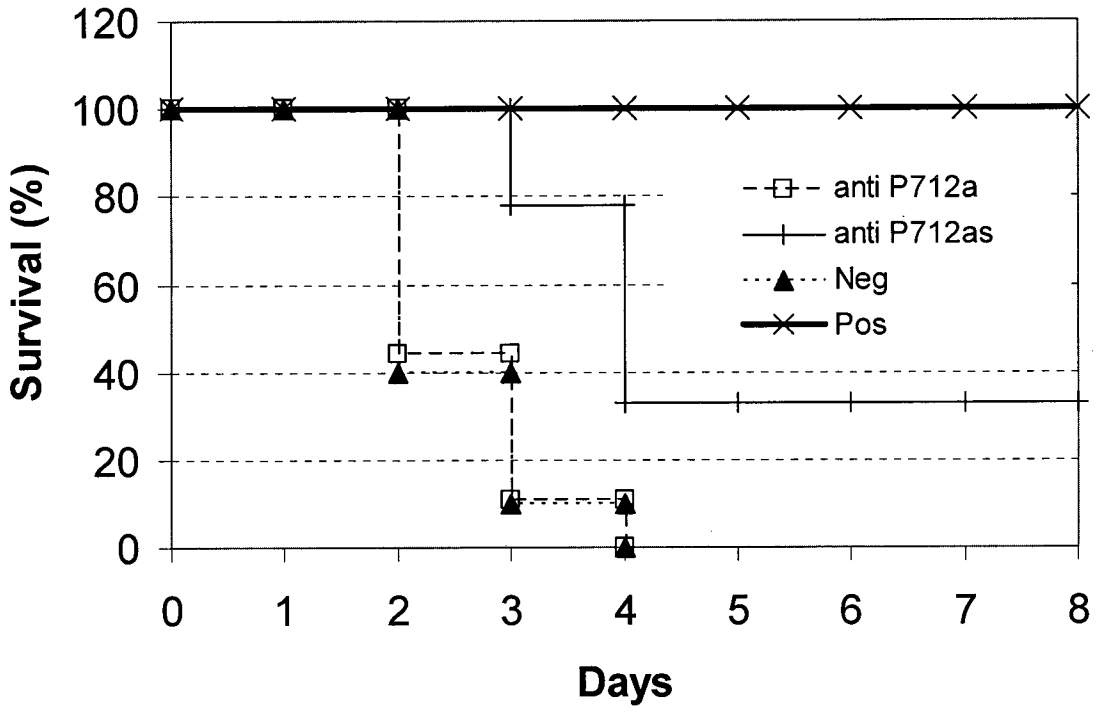


Figure 19


120
100
80
60
40
20
0
Survival (%)
0
1
2
3
4
5
6
7
8
Days
anti P712a
anti P712as
Neg
Pos