

PSEUDOMONAS ANTIGENS AND ANTIGEN COMBINATIONS

This application claims the benefit of UK provisional application 1221638.8 filed November 30th, 2012, the complete contents of all of which are hereby incorporated herein by reference for all purposes.

TECHNICAL FIELD

- 5 This invention relates to antigens derived from *P. aeruginosa* and to their use in immunisation.

BACKGROUND ART

Pseudomonas aeruginosa, an opportunistic gram-negative bacterial pathogen found in most environments including water reservoirs and soil, is one of the leading nosocomial pathogen worldwide. This Gram-negative bacterium is best known for being the leading cause of morbidity and mortality in cystic fibrosis (CF) patients, with 80% of adult CF patients carrying *P. aeruginosa* in their lungs [1], and has recently gained notoriety by being classified as a ‘superbug’ by the media. The latter emanates from the intrinsic resistance that this opportunistic pathogen has against antibiotics [2], and its prominence as a cause of nosocomial infections (*i.e.* there are an estimated 10,000 cases each year in UK hospitals) [3].

Despite considerable advances in antimicrobial therapy, effective treatment and control of *P. aeruginosa* infections remains a persistent problem, primarily because of the natural resistance of the organism and its remarkable ability to acquire resistance to multiple antimicrobial agents by various mechanisms.

A vaccine against *P. aeruginosa* has long been sought after, but is so far not available. Several vaccine candidates have been assessed in experimental animals and humans, which include sub-cellular fractions, capsule components, purified and recombinant proteins.

- 20 Unique characteristics of the host and the pathogen have complicated the vaccine development.

Reference 4 reports a recombinant protein based vaccine approach on a single fusion polypeptide obtained by the fusion of two fragments of two outer membrane derived proteins, namely OprF and OprI. This vaccine is undergoing clinical trials [5], and further details are disclosed in ref. 6.

Thus there remains a need to identify further and improved antigens for use as single antigens or in combinations in *P. aeruginosa* vaccines, and in particular for vaccines which are useful against multiple *P. aeruginosa* pathologies, comprising e.g. cystic fibrosis. Summing up, there is still the need to obtain an effective vaccine against *P. aeruginosa*.

DISCLOSURE OF THE INVENTION

The inventors have identified various *P. aeruginosa* polypeptides that are useful for immunisation, either alone or in combination. These polypeptides may be combined with *P. aeruginosa* saccharides or other *P. aeruginosa* polypeptides or antigens derived from other pathogens (*i.e.* *S. aureus*, *E. coli*, etc). The antigens are useful in *P. aeruginosa* vaccines but may also be used as components in vaccines for immunising against multiple pathogens.

The inventors have identified in total the following polypeptides:

a PSE54 (PA5340) antigen; a PSE44-4 (PA3526) antigen; a PSE10-1 (PA1178) antigen; a PSE21-5 (PA5112) antigen; a PSE27-1 (PA0328) antigen; a PSE52-1 (PA4765) antigen; a PSE53-1 (PA5047) antigen; PSE11-3 (PA1248) antigen; a PSE41-5 (PA2407) antigen; a PSE47A-2 (PA4082); PSE5-1 (PA0595); PSE13-2 (PA1954); PSE17-1 (PA3692); PSE18-2 (PA4370); PSE20-1 (PA4735); PSE23-1 (PA3647); PSE24-1 (PA0126); PSE25-1 (PA0189); PSE26-1 (PA0274); PSE28-2 (PA0537); PSE31-2 (PA0737); PSE33-2 (PA1086); PSE42-1 (PA2793); PSE45-2 (PA3535); PSE50-1 (PA4578); PSE51-4 (PA4667); PSE19-1 (PA4710); PSE34-1 (PA1106); PSE36-3 (PA1324); PSE38-1 (PA1777).

Amongst the total set of selected antigens it can be distinguished a ‘first antigen group’ which is described as a group of antigens for which no prior attempts have been made to test them as vaccine antigens. The “first antigen group” comprises 25 out of the 30 selected antigens.

In particular the “first antigen group” comprises the following antigens: a PSE54 (PA5340) antigen; a PSE44-4 (PA3526) antigen; a PSE21-5 (PA5112) antigen; a PSE27-1 (PA0328) antigen; a PSE53-1 (PA5047) antigen; a PSE41-5 (PA2407) antigen; a PSE47A-2 (PA4082) antigen; a PSE5-1 (PA0595) antigen; a PSE13-2 (PA1954) antigen; a PSE17-1 (PA3692) antigen; a PSE18-2 (PA4370) antigen; a PSE20-1 (PA4735) antigen; a PSE23-1 (PA3647) antigen; a PSE24-1 (PA0126) antigen; a PSE25-1 (PA0189) antigen; a PSE26-1 (PA0274) antigen; a PSE28-2 (PA0537) antigen; a PSE31-2 (PA0737) antigen; a PSE33-2 (PA1086) antigen; a PSE42-1 (PA2793) antigen; a PSE45-2 (PA3535) antigen; a PSE50-1 (PA4578) antigen; a PSE51-4 (PA4667) antigen; a PSE34-1 (PA1106) antigen; and a PSE36-3 antigen (PA1324).

Thus the invention provides an immunogenic composition comprising one or more (i.e. 1, 2, 3, 4, 5 or more) antigens selected from the first antigen group.

Within the first antigen group, antigens are preferably selected from the list of a PSE54 (PA5340) antigen, PSE21-5 (PA5112) antigen; a PSE27-1 (PA0328) antigen; a PSE41-5 (PA2407) antigen; a PSE44-4 (PA3526) antigen; a PSE47A-2 (PA4082) antigen; and/or a PSE53-1 (PA5047) antigen.

Within the ‘first antigen group’, antigens are preferably selected from a subset of 5 polypeptides, and particularly useful in producing a protective immunogenic response *in vivo* if used as single antigens or in combinations are: a PSE54 (PA5340) antigen; a PSE44-4 (PA3526) antigen; a PSE21-5 (PA5112) antigen; a PSE53-1 (PA5047) antigen; PSE42-1 (PA2793).

Within the first antigen group, all the listed antigens can be selected as single antigens for use against *P. aeruginosa*, with the proviso that the PSE27-1 (PA0328) antigen can be usefully omitted from this list (‘first antigen group’).

A “second antigen group” is defined as a group of identified antigens which has already been proposed as possible immunogenic stand-alone vaccine antigen but never considered in combination of at least two (i.e. 2, 3, 4, 5, 6 or more) antigens in *in vivo* experiments. A subset of the “second antigen group” is defined as the “further antigenic polypeptides” group and comprises those antigenic polypeptides that have been extensively tested as vaccine antigens *in vivo*.

The second antigen group comprises the following list of antigens: PSE10-1 (PA1178) antigen; PSE11-3 (PA1248) antigen; PSE52-1 (PA4765) antigen; PSE19-1 (PA4710) antigen; and PSE38-1 (PA1777) antigen.

5 The subset of the second antigen group defined as “further antigenic polypeptides” group comprises the following list of antigens: PilA (PA4524), OprF-OprI, FliC (PA1092), FliD (PA1094) and/or Exoprotein A (PA1148). Hence, the “second antigen group” comprises 10 polypeptides in total.

Thus the invention provides an immunogenic composition comprising a combination of antigens, said combination comprising two or more (*i.e.* 2, 3, 4, 5, 6 or more) antigens selected from the group consisting of: a PSE54 (PA5340) antigen; a PSE44-4 (PA3526) antigen; a PSE10-1 (PA1178) antigen; a
10 PSE21-5 (PA5112) antigen; a PSE27-1 (PA0328) antigen; a PSE52-1 (PA4765) antigen; a PSE53-1 (PA5047) antigen; PSE11-3 (PA1248) antigen; a PSE41 (PA2407) antigen; a PSE47A-2 (PA4082); PSE5-1 (PA0595); PSE13-2 (PA1954); PSE17-1 (PA3692); PSE18-2 (PA4370); PSE20-1 (PA4735); PSE23-1 (PA3647); PSE24-1 (PA0126); PSE25-1 (PA0189); PSE26-1 (PA0274); PSE28-2 (PA0537); PSE31-2 (PA0737); PSE33-2 (PA1086); PSE42-1 (PA2793); PSE45-2 (PA3535); PSE50-1 (PA4578);
15 PSE51-4 (PA4667); PSE19-1 (PA4710); PSE34-1 (PA1106); PSE36-3 (PA1324); PSE38-1 (PA1777).

Within the first antigen group, antigens are preferably selected from a subset of 7 of 30 polypeptides, namely: PSE54 (PA5340), PSE47A-2 (PA4082), PSE41-5 (PA2407), PSE53-1 (PA5047), PSE21-5 (PA5112), PSE27-1 (PA0328) or PSE44-4 (PA3526) antigens and a subset of the “second antigen group”, namely: PSE52-1 (PA4765), PSE10-1 (PA1178), PSE11-3 (PA1248) and the OprF-OprI which is
20 selected from the subset of the ‘second antigen group’ defined as “further antigenic polypeptides” group. Thus the invention provides an immunogenic composition comprising a combination of antigens, said combination comprising two or more (*i.e.* 2, 3, 4, 5, 6 or more) antigens selected from the group consisting of these eleven antigens.

Within the 11 antigens selected from the first, the second and the further antigenic polypeptides group
25 there are 55 possible pairs of antigen combinations.

Within the ‘second antigen group’, comprising the subset of 5 polypeptides referred to herein as ‘the further antigenic polypeptides’, there are in total 10 polypeptides. The invention provides an immunogenic composition comprising a combination of antigens, said combination comprising a mixture of two or more (*i.e.* 2, 3, 4, 5, 6 or more) antigens selected from any of the preferred antigens of the “first
30 antigen group” with anyone of the “second antigen group” or “further antigenic polypeptides” group.

The invention provides an immunogenic composition comprising a combination of antigens, said combination comprising a mixture of two or more (*i.e.* 2, 3, 4, 5, 6 or more) antigens selected from any antigens from the first antigen group and the second antigen group.

Within the 30 antigens of the mixture of the first antigen group and second antigen group there are 435
35 possible pairs of different antigens. All such pairs are disclosed herein and are part of the invention. Thus the invention provides an immunogenic composition comprising a pair of antigens, wherein said pair is one of said 435 pairs.

Within the 35 antigens of the mixture of the first antigen group and second antigen group there are 595 possible pairs of different antigens. All such pairs are disclosed herein and are part of the invention. Thus the invention provides an immunogenic composition comprising a pair of antigens, wherein said pair is one of said 595 pairs.

- 5 In one embodiment, a composition includes at least one antigen (*i.e.* 1, 2, 3, 4, 5, 6 or more) selected from the first antigen group and at least one antigen (*i.e.* 1, 2, 3, 4, 5, 6 or more) selected from the second antigen group. Antigens from the first antigen group may be selected from the preferred subset of PSE54 (PA5340), PSE47A-2 (PA4082), PSE41-5 (PA2407), PSE53-1 (PA5047), PSE21-5 (PA5112), or PSE44-4 (PA3526) antigens, and antigens from the second antigen group can be selected from PSE52-1
10 (PA4765), PSE10-1 (PA1178) or from any of the further antigenic polypeptide sub-set of the second antigen group, preferring the fusion OprF-OprI. .

The invention also provides an immunogenic composition comprising a combination of antigens, said combination comprising two or more (*i.e.* 2, 3, 4 or 5) antigens selected from the group consisting of: (1) a PSE54 antigen; (2) a PSE10-1 antigen; (3) a PSE44-4 antigen; (4) a PSE52-1 antigen; (5) a PSE53-1
15 antigen; (6) a PSE21-5 antigen; (7) a PSE27-1 antigen; (8) a PSE47A-2 antigen; and/or (9) an OprF-OprI antigen.

Within the preferred 9 antigens selected from the first antigen group, the second antigen group and/or the further antigen group there are 36 possible pairs of different antigens. All such pairs are disclosed herein and are part of the invention. Thus the invention provides an immunogenic composition comprising a pair
20 of antigens, wherein said pair is one of said 36 pairs.

The composition may also include an adjuvant *e.g.* an aluminium hydroxide adjuvant.

Advantageous combinations of the invention are those in which two or more antigens act synergistically. Thus the protection against *P. aeruginosa* disease achieved by their combined administration exceeds that expected by mere addition of their individual protective efficacy.

25 Specific combinations of interest include, but are not limited to:

- (1) An immunogenic composition comprising a PSE54 antigen, a PSE27 antigen
- (2) An immunogenic composition comprising a PSE54 antigen and OprF-OprI antigen
- (3) An immunogenic composition comprising a PSE54 antigen, a PSE27 antigen and/or a OprF-OprI antigen
- 30 (4) An immunogenic composition comprising PSE54 antigen and/or a PSE44 antigen
- (5) An immunogenic composition comprising PSE54 antigen and/or PSE21-5 antigen
- (6) An immunogenic composition comprising PSE54 antigen and/or PSE52-1 antigen
- (7) An immunogenic composition comprising PSE47A-2 antigen and/or PSE53-1 antigen
- (8) An immunogenic composition comprising PSE54 antigen and/or PSE10-1 antigen

- (9) An immunogenic composition comprising PSE54 and PSE53-1 antigen
- (10) An immunogenic composition comprising PSE47A-2 and PSE52-1 antigen
- (11) An immunogenic composition comprising PSE54 antigen and/or PSE44-4 antigen and/or PSE47A-2 antigen
- 5 (12) An immunogenic composition comprising a PSE47A-2 antigen, a PSE53-1 antigen, or a PSE54 antigen and/or a PSE27 antigen.
- (13) An immunogenic composition comprising (a) a PSE47A-2 antigen combined with a PSE53-1 antigen, or (b) a PSE54 antigen combined with a PSE21-5 antigen.
- (14) An immunogenic composition comprising a PSE47A-2 antigen and/or PSE52 antigen.
- 10 In some embodiments, any of these immunogenic and protective compositions may include additional pseudomonas antigens, and these further antigens can be polypeptides and/or saccharides. For example, they can useful also include one or more Pseudomonas antigens belonging to the “second antigen group” which includes the “further antigenic polypeptides” group, which include the fusion polypeptide OprF-OprI in a synergistic manner.
- 15 The immunogenic composition may also include an adjuvant.

Further polypeptide antigens group

In additions to antigens from the various antigen groups of the invention, immunogenic compositions may include one or more of the following *P. aeruginosa* antigens (or antigens comprising immunogenic fragment(s) thereof to enhance the efficacy against *P. aeruginosa* of an immune response elicited by the composition:

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- OprF-OprI [4]
- PA4525, known also as PilA
- PA1092, known also as FliC
- PA1094, known also as FliD
- 25 • PA1148, Exoprotein A or Exotoxin A

The “further antigenic polypeptides” group is defined as a subgroup of the second antigen group. This group of known antigens can be useful used in combination with 1, 2 or more other useful antigens of the first antigen group or the second antigen group.

Combinations with other P. aeruginosa derived antigens

30 The individual antigens identified in the antigen groups of the invention may be used in combination with other antigens from *P. aeruginosa*. In some embodiments the other antigens from *P. aeruginosa* can be in the form of saccharides conjugated with a carrier protein. Thus the invention provides an immunogenic composition comprising a combination of:

- (1) one or more antigen(s) selected from the first, second, or further antigen groups (as defined above); and/or their combination or admixture and
- 35

(2) one or more conjugates of a saccharide moiety, and a carrier protein.

A conjugate used in component (2) of this combination includes a saccharide moiety and a carrier moiety.

In embodiments of the invention, the composition further comprises the *P. aeruginosa* 5-hexose Psl polysaccharide, which can be present as free polysaccharide and/or conjugated to a carrier protein.

5 Optionally, one or more flagellin adjuvants and/or fusion proteins of the invention act as the carrier protein and have Psl polysaccharide conjugated thereto. For example, monomers and/or dimers of the *P. aeruginosa* polysaccharide can be conjugated to one or more of the flagellin adjuvants and/or fusion proteins. See reference 7.

A conjugate used in component (2) of this combination includes a saccharide moiety and a carrier moiety.

10 The saccharide moiety is from the exopolysaccharide of a *P. aeruginosa*. The saccharide may be a polysaccharide having the size that arises during purification from bacteria, or it may be an oligosaccharide achieved by fragmentation of such a polysaccharide.

The invention also provides an immunogenic composition comprising a combination of:

- (1) one or more antigen(s) selected from the first, second, or further antigen groups;
- 15 (2) one or more conjugates of a *P. aeruginosa* exopolysaccharide and a carrier protein.

The carrier moiety in these conjugates will usually be a protein, but usually not one of the antigens of (1).

Typical carrier proteins are bacterial toxins, such as diphtheria or tetanus toxins, or toxoids or mutants or fragments thereof. The CRM197 diphtheria toxin mutant [8] is useful. Other suitable carrier proteins
20 include the *N.meningitidis* outer membrane protein complex [9], synthetic peptides [10], heat shock proteins [11], pertussis proteins [12], cytokines [13], lymphokines [13], growth factors [13], artificial proteins comprising multiple human CD4⁺ T cell epitopes from various pathogen-derived antigens [14] such as N19 [15], protein D from *H.influenzae* 16, pneumolysin [17] or its non-toxic derivatives [18], pneumococcal surface protein PspA [19], iron-uptake proteins [20], toxin A or B from *C.difficile* [21],
25 recombinant *P.aeruginosa* exoprotein A (rEPA) [22], etc. In some embodiments the carrier protein is a *P. aeruginosa* protein, such as an antigen selected from the first, second, or further antigen groups.

Where a composition includes more than one conjugate, each conjugate may use the same carrier protein or a different carrier protein.

Conjugates may have excess carrier (w/w) or excess saccharide (w/w). In some embodiments, a conjugate
30 may include substantially equal weights of each.

The carrier molecule may be covalently conjugated to the carrier directly or via a linker. Direct linkages to the protein may be achieved by, for instance, reductive amination between the saccharide and the carrier, as described in, for example, references 23 and 24. The saccharide may first need to be activated e.g. by oxidation. Linkages via a linker group may be made using any known procedure, for example, the
35 procedures described in references 25 and 26. A preferred type of linkage is an adipic acid linker, which may be formed by coupling a free -NH₂ group (e.g. introduced to a glucan by amination) with adipic acid

(using, for example, diimide activation), and then coupling a protein to the resulting saccharide-adipic acid intermediate [27]. Another preferred type of linkage is a carbonyl linker, which may be formed by reaction of a free hydroxyl group of a saccharide CDI [28] followed by reaction with a protein to form a carbamate linkage. Other linkers include β -propionamido [29], nitrophenyl-ethylamine [30], haloacyl halides [31], glycosidic linkages [32], 6-aminocaproic acid [33], ADH [34], C₄ to C₁₂ moieties [35], *etc.* Carbodiimide condensation can also be used [36].

The individual antigens identified in the antigen groups of the invention may be used as carrier proteins for exopolysaccharides, to form a covalent conjugate. Thus the invention provides an immunogenic composition comprising a conjugate of (1) an antigen selected from the first, second, and further antigen groups and (2) a *P. aeruginosa* exopolysaccharide. These conjugates may be combined with any of the antigens disclosed herein.

Combinations with other pathogens derived (non-pseudomonas) antigens

The individual antigens identified in the antigen groups of the invention may be used also in combination with other pathogens derived antigens, *i.e.* non-pseudomonas antigens, and in particular with antigens from bacteria associated with nosocomial infections. Thus the invention provides an immunogenic composition comprising a combination of:

(1) one or more antigen(s) selected from the first, second, and further antigen groups (as defined above); and

(2) one or more antigen(s) selected from the pathogen group consisting of: *S. aureus* (including one or more conjugates of (i) a *S.aureus* exopolysaccharide; and/or one or more protein antigens of *S.aureus*); *Burkholderia cenocepacia* (*e.g.* O antigen lipopolysaccharide), *Clostridium difficile*; *Candida albicans*; and/or extraintestinal pathogenic *Escherichia coli*.

First antigen group

PA0328 or PSE27-1

The 'PA0328' antigen is annotated as 'outer membrane autotransporter'. In the PAO1 strain is annotated as 'hypothetical protein' and has amino acid sequence SEQ ID NO: 1 and described as PA0328 in reference 37. This sequence is annotated in NCBI as GI: 15595525. It has been recently demonstrated to be an autotransporter protein relevant in the virulence strategy adopted by *Pseudomonas aeruginosa* through its arginine-specific aminopeptidase activity, as in reference 38. Sometimes, PA0328 is referred to herein as 'PSE27-1' or as 'PSE27'.

Useful PA0328 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 1 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 1; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 1, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PA0328 proteins include variants of SEQ ID NO: 1. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 1. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1,

2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 27, 28, 29, 30, 35, 40, 45, 49, 50 or more) from the N-terminus of SEQ ID NO: 1 while retaining at least one epitope of SEQ ID NO: 1. The final 40-50 C-terminal amino acids of SEQ ID NO: 1 can usefully be omitted. The first 22 N-terminal amino acids of SEQ ID NO: 1 can usefully be omitted. Other fragments omit one or more protein domains.

- 5 SEQ ID NO: 36 is a useful fragment of SEQ ID NO: 1 ('PA0328₂₂₋₆₄₇'). This fragment omits the leader peptide at the N- terminal portion to enable expression and purification.

PA5112 or PSE21-5

- The 'PSE21-5' antigen is annotated as 'Esterase or EstA' in the PAO1 strain. In the PAO1 strain PSE21-5 is described as 'PA5112' and has amino acid sequence SEQ ID NO: 3. In the PAO1 strain its identifier in
10 NCBI is GI: 15600305 See Ref.37. Sometimes, PA5112 is referred to herein as 'PSE21-5' or 'PSE21'.

- Useful PSE21-5 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 3 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 3; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 3,
15 wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE21-5 proteins include variants of SEQ ID NO: 3. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 3. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 3 while retaining at least
20 one epitope of SEQ ID NO: 3. The final 40 C-terminal amino acids of SEQ ID NO: 3 can usefully be omitted. The first 24 N-terminal amino acids of SEQ ID NO: 3 can usefully be omitted. Other fragments omit one or more protein domains. PSE21-5 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

- SEQ ID NO: 38 is a useful fragment of SEQ ID NO: 3 ('PSE21-5₂₅₋₆₄₆'). This fragment includes the most
25 exposed domain of PSE21-5 and is more easily used at an industrial scale.

PA2407 or PSE41-5

- The 'PSE41-5' antigen is annotated as 'probable adhesion protein'. In the PAO1 strain PSE41-5 is named PA2407 and has amino acid sequence SEQ ID NO: 5 (GI: 15597603). See Ref.37. Sometimes, PA2407 is referred to herein as 'PSE41-5' or 'PSE41'. Sometimes, PA2407 is referred to herein as 'PSE41-5' or
30 'PSE41'.

- Useful 'PSE41-5' antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 5 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 5; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 5,
35 wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These 'PSE41-5' proteins include variants of SEQ ID NO: 5. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 5. Other preferred fragments lack one or more amino acids (*e.g.* 1,

2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 5 while retaining at least one epitope of SEQ ID NO: 5. The final 40 C-terminal amino acids of SEQ ID NO: 5 can usefully be omitted. The first 37 N-terminal amino acids of SEQ ID NO: 5 can usefully be omitted. Other fragments omit one or more protein domains. 'PSE41-5' is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 40 is a useful fragment of SEQ ID NO: 5 ('PSE41-5'₃₈₋₃₁₇). This fragment includes the most exposed domain of 'PSE41-5' and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins.

10 ***PA3526 or PSE44-4***

The PSE44-4 antigen is annotated as 'probable outer membrane protein precursor'. In the PAO1 strain PSE44-4 is PA3526 and has amino acid sequence SEQ ID NO: 6 (GI: 15598722). See Ref 37.. Sometimes, PA3526 is referred to herein as 'PSE44-4' or 'PSE44'.

Useful PSE44-4 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 6 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 6; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 6, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE44-4 proteins include variants of SEQ ID NO: 6. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 6. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 6 while retaining at least one epitope of SEQ ID NO: 6. The first 19 N-terminal amino acids of SEQ ID NO: 6 can usefully be omitted. Other fragments omit one or more protein domains. PSE44-4 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 41 is a useful fragment of SEQ ID NO: 6 ('PSE44-4'₂₀₋₃₂₁). This fragment includes the most exposed domain of PSE44-4 and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins.

PA4082 or PSE47A-2

30 The PSE47A-2 antigen is annotated as 'adhesive protein CupB5' or as "Serine protease". In the PAO1 strain PSE47A-2 is named PA4082 and has amino acid sequence SEQ ID NO: 7 (GI: 15599277). See Ref. 37. Sometimes, PA4082 is referred to herein as 'PSE47A' or 'PSE47A-2' (fragment).

Useful PSE47A-2 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 7 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 7; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 7, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200,

250 or more). These PSE47A-2 proteins include variants of SEQ ID NO: 7. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 7. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 7 while retaining at least one epitope of SEQ ID NO: 7. Since the C- terminal portion of this protein is corresponding to the translocator domain, which is totally embedded in the outer membrane and therefore totally inaccessible to antibodies can be useful omitted. Hence, the final 435 C-terminal amino acids of SEQ ID NO: 7 can usefully be omitted. The first 53 N-terminal amino acids of SEQ ID NO: 7 can usefully be omitted. Other fragments omit one or more protein domains. PSE47A-2 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 42 is a useful fragment of SEQ ID NO: 7 ('PSE47A-2₅₄₋₅₈₃'). This fragment includes the most exposed domain of PSE47A-2 and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins.

PA5047 or PSE53-1

The PSE53-1 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE53-1 is PA5047 and has amino acid sequence SEQ ID NO: 9 (GI: 15600240). See Ref.37. Sometimes, PA5047 is referred to herein as 'PSE53-1 or 'PSE53'.

Useful PSE53-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 9 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 9; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 9, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE53-1 proteins include variants of SEQ ID NO: 9. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 9. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 9 while retaining at least one epitope of SEQ ID NO: 9. The final 40 C-terminal amino acids of SEQ ID NO: 9 can usefully be omitted. The first 18 N-terminal amino acids of SEQ ID NO: 9 can usefully be omitted. Other fragments omit one or more protein domains. PSE53-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 44 is a useful fragment of SEQ ID NO: 9 ('PSE53-1₁₉₋₄₇₉'). This fragment includes the most exposed domain of PSE53-1 and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins.

PA5340 or PSE54

The PSE54 antigen is annotated as 'probable outer membrane protein precursor' and as 'hypothetical protein'. In the PAO1 strain PSE54 is PA3526 and has amino acid sequence SEQ ID NO: 10 (GI: 15598722). See Ref.37. Sometimes, PA5340 is referred to herein as 'PSE54'.

- Useful PSE54 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 10 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 10; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 10, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE54 proteins include variants of SEQ ID NO: 10. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 10. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 10 while retaining at least one epitope of SEQ ID NO: 10. The final 40 C-terminal amino acids of SEQ ID NO: 10 can usefully be omitted. The first 16 N-terminal amino acids of SEQ ID NO: 10 can usefully be omitted. Other fragments omit one or more protein domains. PSE54 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*
- SEQ ID NO: 45 is a useful fragment of SEQ ID NO: 10 ('PSE54₁₇₋₂₄₃'). This fragment includes the most exposed domain of PSE54 and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins.

PA0595 or PSE5-1

- The PSE5-1 antigen is annotated as 'organic solvent tolerance protein OstA precursor'. In the PAO1 strain PSE5-1 is PA0595 and has amino acid sequence SEQ ID NO: 11 (GI: 15595792). See Ref.37. Sometimes, PA0595 is referred to herein as 'PSE5-1 or 'PSE5'.

- Useful PSE5-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 11 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 11; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 11, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE5-1 proteins include variants of SEQ ID NO: 11. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 11. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 11 while retaining at least one epitope of SEQ ID NO: 11. The final 40 C-terminal amino acids of SEQ ID NO: 11 can usefully be omitted. The first 33 N-terminal amino acids of SEQ ID NO: 11 can usefully be omitted. Other fragments omit one or more protein domains. PSE5-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*
- SEQ ID NO: 46 is a useful fragment of SEQ ID NO: 11 ('PSE5-1₃₄₋₉₂₄'). This fragment includes the most exposed domain of PSE5-1 and is more easily used at an industrial scale.

PA1954 or PSE13-2

The PSE13-2 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE13-2 is PA1954 and has amino acid sequence SEQ ID NO: 12 (GI: 15597150). See Ref.37. Sometimes, PA1954 is referred to herein as 'PSE13-2' or 'PSE13'.

- 5 Useful PSE13-2 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 12 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 12; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 12, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 10 250 or more). These PSE13-2 proteins include variants of SEQ ID NO: 12. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 12. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 12 while retaining at least one epitope of SEQ ID NO: 12. The final 40 C-terminal amino acids of SEQ ID NO: 12 can usefully 15 be omitted. The first 24 N-terminal amino acids of SEQ ID NO: 12 can usefully be omitted. Other fragments omit one or more protein domains. PSE13-2 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 47 is a useful fragment of SEQ ID NO: 12 ('PSE13-2₂₅₋₃₄₀'). This fragment includes the most exposed domain of PSE13-2 and is more easily used at an industrial scale.

PA3692 or PSE17-1

The PSE17-1 antigen is annotated as 'Lipotoxin F, LptF'. In the PAO1 strain PSE17-1 is PA3692 and has amino acid sequence SEQ ID NO: 13 (GI: 15598888). See Ref.37. It has been described as belonging to Outer membrane protein and related peptidoglycan-associated (lipo) proteins as shown in reference 39. Sometimes, PA3692 is referred to herein as 'PSE17-1' or 'PSE17'.

- 25 Useful PSE17-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 13 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 13; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 13, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 30 250 or more). These PSE17-1 proteins include variants of SEQ ID NO: 13. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 13. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 13 while retaining at least one epitope of SEQ ID NO: 13. The final 40 C-terminal amino acids of SEQ ID NO: 13 can usefully 35 be omitted. The first 19 N-terminal amino acids of SEQ ID NO: 13 can usefully be omitted. Other fragments omit one or more protein domains. PSE17-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 48 is a useful fragment of SEQ ID NO: 13 ('PSE17-1₂₀₋₂₆₁'). This fragment includes the most exposed domain of PSE17-1 and is more easily used at an industrial scale.

PA4370 or PSE18-2

The PSE18-2 antigen is annotated as 'Insulin-cleaving metalloproteinase outer membrane protein precursor'. In the PAO1 strain PSE18-2 is PA4370 and has amino acid sequence SEQ ID NO: 14 (GI: 15599566). See Ref.37. It has been described as belonging to Outer membrane protein and in particular as insulin-cleaving metalloproteinase outer membrane protein (IcmP) as shown in reference 40. Sometimes, PA4370 is referred to herein as 'PSE18-2' or 'PSE18'.

Useful PSE18-2 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 14 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 14; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 14, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE18-2 proteins include variants of SEQ ID NO: 14. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 14. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 14 while retaining at least one epitope of SEQ ID NO: 14. The final 40 C-terminal amino acids of SEQ ID NO: 14 can usefully be omitted. The first 20 N-terminal amino acids of SEQ ID NO: 14 can usefully be omitted. Other fragments omit one or more protein domains. PSE18-2 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 49 is a useful fragment of SEQ ID NO: 14 ('PSE18-2₂₁₋₄₄₆'). This fragment includes the most exposed domain of PSE18-2 and is more easily used at an industrial scale.

PA4735 or PSE20-1

The PSE20-1 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE20-1 is PA4735 and has amino acid sequence SEQ ID NO: 16 (GI: 15599929). See Ref. 37. Sometimes, PA4735 is referred to herein as 'PSE20-1' or 'PSE20'.

Useful PSE20-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 16 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 16; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 16, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE20-1 proteins include variants of SEQ ID NO: 16. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 16. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 16 while retaining at least one epitope of SEQ ID NO: 16. The final 40 C-terminal amino acids of SEQ ID NO: 16 can usefully be omitted. The first 19 N-terminal amino acids of SEQ ID NO: 16 can usefully be omitted. Other

fragments omit one or more protein domains. PSE20-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 51 is a useful fragment of SEQ ID NO: 16 ('PSE20-1₂₀₋₁₀₈₈'). This fragment includes the most exposed domain of PSE20-1 and is more easily used at an industrial scale.

5 **PA3647 or PSE23-1**

The PSE23-1 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE23-1 is PA3647 and has amino acid sequence SEQ ID NO: 17 (GI: 15598843). See Ref. 37. It has been described as probable outer membrane protein precursor or as OmpH gene and it was described as contaminant during the purification process of OprI as shown in reference 41. Sometimes, PA3647 is referred to herein as
10 'PSE23-1' or 'PSE23'.

Useful PSE20-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 17 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 17; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO:
15 17, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE20-1 proteins include variants of SEQ ID NO: 17. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 17. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 17 while retaining at
20 least one epitope of SEQ ID NO: 17. The final 40 C-terminal amino acids of SEQ ID NO: 17 can usefully be omitted. The first 22 N-terminal amino acids of SEQ ID NO: 17 can usefully be omitted. Other fragments omit one or more protein domains. PSE23-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 52 is a useful fragment of SEQ ID NO: 17 ('PSE20-1₂₃₋₁₆₈'). This fragment includes the
25 most exposed domain of PSE23-1 and is more easily used at an industrial scale.

PA0126 or PSE24-1

The PSE24-1 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE24-1 is PA0126 and has amino acid sequence SEQ ID NO: 18 (GI: 15595324). See Ref. 37. Sometimes, PA0126 is referred to herein as 'PSE24-1' or 'PSE24'.

Useful PSE24-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 18 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 18; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO:
30 18, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE24-1 proteins include variants of SEQ ID NO: 18. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 18. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.*
35 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 18 while retaining at least one epitope of SEQ ID NO: 18. The final 40 C-terminal amino acids of SEQ ID NO: 18 can usefully be omitted. The first 22 N-terminal amino acids of SEQ ID NO: 18 can usefully be omitted. Other fragments omit one or more protein domains. PSE24-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 18 while retaining at least one epitope of SEQ ID NO: 18. The first 19 N-terminal amino acids of SEQ ID NO: 18 can usefully be omitted. Other fragments omit one or more protein domains. PSE24-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

- 5 SEQ ID NO: 53 is a useful fragment of SEQ ID NO: 18 ('PSE24-1₂₀₋₂₀₆'). This fragment includes the most exposed domain of PSE24-1 and is more easily used at an industrial scale.

PA0189 or PSE25-1

- The PSE25-1 antigen is annotated as 'probable porin'. In the PAO1 strain PSE25-1 is PA0189 and has amino acid sequence SEQ ID NO: 19 (GI: 15595387). See Ref. 37. Sometimes, PA0189 is referred to
10 herein as 'PSE25-1' or 'PSE25'.

- Useful PSE25-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 19 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 19; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO:
15 19, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE25-1 proteins include variants of SEQ ID NO: 19. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 19. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 19 while retaining at
20 least one epitope of SEQ ID NO: 19. The first 25 N-terminal amino acids of SEQ ID NO: 19 can usefully be omitted. Other fragments omit one or more protein domains. PSE25-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 54 is a useful fragment of SEQ ID NO: 19 ('PSE25-1₂₆₋₄₅₂'). This fragment includes the most exposed domain of PSE25-1 and is more easily used at an industrial scale.

PA0274 or PSE26-1

- 25 The PSE26-1 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE26-1 is PA0274 and has amino acid sequence SEQ ID NO: 20 (GI: 15595471). See Ref. 37. Sometimes, PA0274 is referred to herein as 'PSE26-1' or 'PSE26'.

- Useful PSE26-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 20 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 20; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO:
30 20, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE26-1 proteins include variants of SEQ ID NO: 20. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 20. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 20 while retaining at
35

least one epitope of SEQ ID NO: 20. The first 23 N-terminal amino acids of SEQ ID NO: 20 can usefully be omitted. Other fragments omit one or more protein domains. PSE26-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

5 SEQ ID NO: 55 is a useful fragment of SEQ ID NO: 20 ('PSE26-1₂₄₋₂₅₆'). This fragment includes the most exposed domain of PSE26-1 and is more easily used at an industrial scale.

PA0537 or PSE28-2

The PSE28-1 antigen is annotated as 'conserved hypothetical protein'. In the PAO1 strain PSE28-1 is PA0537 and has amino acid sequence SEQ ID NO: 21 (GI: 15595734). See Ref. 37. Sometimes, PA0537 is referred to herein as 'PSE28-1' or 'PSE28'.

10 Useful PSE28-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 21 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 21; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 21, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 15 250 or more). These PSE28-1 proteins include variants of SEQ ID NO: 21. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 21. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 21 while retaining at least one epitope of SEQ ID NO: 21. The first 19 N-terminal amino acids of SEQ ID NO: 21 can usefully 20 be omitted. Other fragments omit one or more protein domains. PSE28-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 56 is a useful fragment of SEQ ID NO: 21 ('PSE28-1₂₀₋₂₀₂'). This fragment includes the most exposed domain of PSE28-1 and is more easily used at an industrial scale.

PA0737 or PSE31-2

25 The PSE31-2 antigen is annotated as 'conserved hypothetical protein'. In the PAO1 strain PSE31-2 is PA0737 and has amino acid sequence SEQ ID NO: 22 (GI: 15595934). See Ref. 37. It has been described as up-regulated lipoproteins. See Ref. 42. Sometimes, PA0737 is referred to herein as 'PSE31-2' or 'PSE31'.

Useful PSE31-2 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ 30 ID NO: 22 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 22; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 22, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE31-2 proteins include variants of SEQ ID NO: 22. Preferred fragments of (b) 35 comprise an epitope from SEQ ID NO: 22. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 22 while retaining at

least one epitope of SEQ ID NO: 22. The first 19 N-terminal amino acids of SEQ ID NO: 22 can usefully be omitted. Other fragments omit one or more protein domains. PSE31-2 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

5 SEQ ID NO: 57 is a useful fragment of SEQ ID NO: 22 ('PSE31-2' 20-151'). This fragment includes the most exposed domain of PSE31-2 and is more easily used at an industrial scale.

PA1086 or PSE33-2

The PSE33-2 antigen is annotated as 'flagellar hook-associated protein 1 FlgK'. In the PAO1 strain PSE33-2 is PA1086 and has amino acid sequence SEQ ID NO: 23 (GI: 15596283). See Ref. 37. Sometimes, PA1086 is referred to herein as 'PSE33-2' or 'PSE33'.

10 Useful PSE33-2 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 23 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 23; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 23, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 15 250 or more). These PSE33-2 proteins include variants of SEQ ID NO: 23. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 23. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 23 while retaining at least one epitope of SEQ ID NO: 23. The first N-terminal amino acid of SEQ ID NO: 23 can usefully be 20 omitted. Other fragments omit one or more protein domains. PSE33-2 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 58 is a useful fragment of SEQ ID NO: 23, wherein only the Met at position 1 of the polypeptide has been removed to allow proper cloning and expression in commonly known expression systems *i.e.* PET vector system. This fragment includes the most exposed domain of PSE31-2 and is more 25 easily used at an industrial scale.

PA2793 or PSE42-1

The PSE42-1 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE42-1 is PA2793 and has amino acid sequence SEQ ID NO: 27 (GI: 15597989). See Ref. 37. Sometimes, PA2793 is referred to 30 herein as 'PSE42-1' or 'PSE42'.

PSORT available program has predicted this protein as lipoprotein and a Type II (lipoprotein) export signal predicted by LipoP by a cleavage after residue 20. See Ref. 37.

Useful PSE42-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 27 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 35 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 27; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 27, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200,

250 or more). These PSE42-1 proteins include variants of SEQ ID NO: 27. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 27. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 27 while retaining at least one epitope of SEQ ID NO: 27. The first 20 N-terminal amino acids of SEQ ID NO: 27 can usefully be omitted. Other fragments omit one or more protein domains. PSE42-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 62 is a useful fragment of SEQ ID NO: 27 ('PSE42-1₂₁₋₃₄₄'). This fragment includes the most exposed domain of PSE42-1 and is more easily used at an industrial scale.

10 **PA3535 or PSE45-2**

The PSE45-2 antigen is annotated as 'probable outer membrane protein precursor'. In the PAO1 strain PSE45-2 is PA3535 and has amino acid sequence SEQ ID NO: 28 (GI: 15598731). See Ref. 37. Sometimes, PA3535 is referred to herein as 'PSE45-2' or 'PSE45'.

Useful PSE45-2 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 28 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 28; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 28, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE45-2 proteins include variants of SEQ ID NO: 28. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 28. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 28 while retaining at least one epitope of SEQ ID NO: 28. The first 30 N-terminal amino acids of SEQ ID NO: 28 can usefully be omitted. Other fragments omit one or more protein domains. PSE45-2 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 63 is a useful fragment of SEQ ID NO: 28 ('PSE45-2₃₁₋₉₉₅'). This fragment includes the most exposed domain of PSE45-2 and is more easily used at an industrial scale.

PA4578 or PSE50-1

The PSE50-1 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE50-1 is PA4578 and has amino acid sequence SEQ ID NO: 29 (GI: 15599774). See Ref. 37. Sometimes, PA4578 is referred to herein as 'PSE50-1' or 'PSE50'.

Useful PSE50-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 29 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 29; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 29, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE50-1 proteins include variants of SEQ ID NO: 29. Preferred fragments of (b)

comprise an epitope from SEQ ID NO: 29. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 19, 20, 25 or more) from the N-terminus of SEQ ID NO: 29 while retaining at least one epitope of SEQ ID NO: 29. The first 19 N-terminal amino acids of SEQ ID NO: 29 can usefully be omitted. Other fragments omit one or more protein domains. PSE45-2 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 64 is a useful fragment of SEQ ID NO: 29 ('PSE50-1₂₀₋₁₆₂'). This fragment includes the most exposed domain of PSE50-1 and is more easily used at an industrial scale.

PA4667 or PSE51-4

- 10 The PSE51-4 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE51-4 is PA4667 and has amino acid sequence SEQ ID NO: 30 (GI: 15599862). See Ref. 37. Sometimes, PA4667 is referred to herein as 'PSE51-4' or 'PSE51'.

Useful PSE51-4 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 30 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 30; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 30, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE51-4 proteins include variants of SEQ ID NO: 30. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 30. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 19, 20, 25, 30 or more) from the N-terminus of SEQ ID NO: 30 while retaining at least one epitope of SEQ ID NO: 30. The first 31 N-terminal amino acids of SEQ ID NO: 30 can usefully be omitted. Other fragments omit one or more protein domains. PSE51-4 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

- 25 SEQ ID NO: 65 is a useful fragment of SEQ ID NO: 30 ('PSE51-4₃₂₋₅₉₀'). This fragment includes the most exposed domain of PSE51-4 and is more easily used at an industrial scale.

PA1106 or PSE34-1

- 30 The PSE34-1 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE34-1 is PA1106 and has amino acid sequence SEQ ID NO: 24 (GI: 15596303). See Ref. 37. Sometimes, PA1106 is referred to herein as 'PSE34-1' or 'PSE34'.

Useful PSE34-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 24 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 24; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 24, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE34-1 proteins include variants of SEQ ID NO: 24. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 24. Other preferred fragments lack one or more amino acids (*e.g.*

1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 24 while retaining at least one epitope of SEQ ID NO: 24. The first 20 N-terminal amino acids of SEQ ID NO: 24 can usefully be omitted. Other fragments omit one or more protein domains. PSE34-1 is naturally a long protein and so the use of fragments is helpful e.g. for purification, handling, fusion, expression, etc.

SEQ ID NO: 59 is a useful fragment of SEQ ID NO: 24 ('PSE34-1₂₁₋₂₃₇'). This fragment includes the most exposed domain of PSE34-1 and is more easily used at an industrial scale.

PA1324 or PSE36-3

The PSE36-3 antigen is annotated as 'hypothetical protein'. In the PAO1 strain PSE36-3 is PA1324 and has amino acid sequence SEQ ID NO: 25 (GI: 15596521). See Ref. 37. Sometimes, PA1324 is referred to herein as 'PSE36-3' or 'PSE36'.

PA1324 is postulated to be involved in the binding and transport of sugars or polysaccharides associated with the peptidoglycan matrix during biofilm formation. [43]

Useful PSE36-3 antigens can elicit an antibody (e.g. when administered to a human) that recognises SEQ ID NO: 25 and/or may comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 25; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 25, wherein 'n' is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE36-3 proteins include variants of SEQ ID NO: 25. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 25. Other preferred fragments lack one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 25 while retaining at least one epitope of SEQ ID NO: 25. The first 19 N-terminal amino acids of SEQ ID NO: 25 can usefully be omitted. Other fragments omit one or more protein domains. PSE36-3 is naturally a long protein and so the use of fragments is helpful e.g. for purification, handling, fusion, expression, etc.

SEQ ID NO: 60 is a useful fragment of SEQ ID NO: 25 ('PSE36-3₂₀₋₁₇₀'). This fragment includes the most exposed domain of PSE36-3 and is more easily used at an industrial scale.

Second antigen group

PA1178 or PSE10

The 'PSE10' antigen is annotated as 'PhoP/Q and low Mg²⁺ inducible outer membrane protein'. In the PAO1 strain PSE10 is called also as OprH [44] and has amino acid sequence SEQ ID NO: 2. In the PAO1 strain PSE10 is annotated as PA1178 and its NCBI identifier is GI: 15596375. See Ref. 37. Sometimes, PA1178 is referred to herein as 'PSE10-1' or 'PSE10'.

Useful PSE10 antigens can elicit an antibody (e.g. when administered to a human) that recognises SEQ ID NO: 2 and/or may comprise an amino acid sequence: (a) having 50% or more identity (e.g. 60%, 65%,

70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 2; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 2, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE10 proteins include variants of SEQ ID NO: 2. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 2. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50 or more) from the N-terminus of SEQ ID NO: 2 while retaining at least one epitope of SEQ ID NO: 2. The first 21 N-terminal amino acids of SEQ ID NO: 2 can usefully be omitted. Other fragments omit one or more protein domains. The use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 37 is a useful fragment of SEQ ID NO: 2 ('PSE10₂₂₋₂₀₀'). This fragment includes the most exposed domain of PSE10 and is more easily used at an industrial scale.

PA1248 or PSE11-3

The 'PSE11-3' antigen is annotated as 'Alkaline protease secretion outer membrane protein AprF precursor'. In the PAO1 strain PSE11-3 is PA1248 and has amino acid sequence SEQ ID NO: 4. In the PAO1 strain the NCBI identifier is GI: 15596445. See reference 37 and 45.

Sometimes, PA1248 is referred to herein as 'PSE11-3' or 'PSE11'.

Useful PSE11-3 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 4 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 4; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 4, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE11-3 proteins include variants of SEQ ID NO: 4. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 4. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 4 while retaining at least one epitope of SEQ ID NO: 4. The first 18 N-terminal amino acids of SEQ ID NO: 4 can usefully be omitted. Other fragments omit one or more protein domains. PSE11-3 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.* In reference 45 this antigen is described as a known virulence factor and tested as antigen.

SEQ ID NO: 39 is a useful fragment of SEQ ID NO: 4 ('PSE11-3₁₉₋₄₈₁'). This fragment includes the most exposed domain of PSE11-3 and is more easily used at an industrial scale.

PA4765 or PSE52-1

The PSE52-1 antigen is annotated as 'Outer membrane lipoprotein OmlA precursor'. In the PAO1 strain PSE52-1 is PA4765 and has amino acid sequence SEQ ID NO: 8 (GI: 15599959). See Ref. 37. Sometimes, PA4765 is referred to herein as 'PSE52-1' or 'PSE52'.

It has been described since 1999 as belonging to outer membrane protein family as in reference 46.

Useful PSE52-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 8 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 8; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 8, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE52-1 proteins include variants of SEQ ID NO: 8. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 8. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 8 while retaining at least one epitope of SEQ ID NO: 8. The final 40 C-terminal amino acids of SEQ ID NO: 8 can usefully be omitted. The first 21 N-terminal amino acids of SEQ ID NO: 8 can usefully be omitted. Other fragments omit one or more protein domains. PSE52-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 43 is a useful fragment of SEQ ID NO: 8 ('PSE52-1₂₂₋₁₇₆'). This fragment includes the most exposed domain of PSE52-1 and is more easily used at an industrial scale.

PA4710 or PSE19-1

The PSE19-1 antigen is annotated as 'Heme/Hemoglobin uptake outer membrane receptor PhuR precursor'. In the PAO1 strain PSE19-1 is PA4710 and has amino acid sequence SEQ ID NO: 15 (GI: 15599904). See Ref. 37. Short peptides derived from said antigen have been proposed to show certain immunogenicity, however this antigen has not been tested as vaccine antigen in combination [47]. Sometimes, PA4710 is referred to herein as 'PSE19-1' or 'PSE19'.

Useful PSE19-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 15 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 15; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 15, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE19-2 proteins include variants of SEQ ID NO: 15. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 15. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 15 while retaining at least one epitope of SEQ ID NO: 15. The final 40 C-terminal amino acids of SEQ ID NO: 15 can usefully be omitted. The first 25 N-terminal amino acids of SEQ ID NO: 15 can usefully be omitted. Other fragments omit one or more protein domains. PSE19-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 50 is a useful fragment of SEQ ID NO: 15 ('PSE19-1₂₆₋₇₆₄'). This fragment includes the most exposed domain of PSE19-1 and is more easily used at an industrial scale.

PA1777 or PSE38-1

The PSE38-1 antigen is annotated as 'Major porin and structural outer membrane porin OprF precursor'. In the PAO1 strain PSE38-1 is PA1777 and has amino acid sequence SEQ ID NO: 26 (GI: 15596974). See Ref. 37 and 48. EP0297291 described for the first time this protein as useful antigen. Sometimes, PA1777 is referred to herein as 'PSE38-1' or 'PSE38'.

Useful PSE38-1 antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 26 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 26; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 26, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PSE38-1 proteins include variants of SEQ ID NO: 26. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 26. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 26 while retaining at least one epitope of SEQ ID NO: 26. The first 24 N-terminal amino acids of SEQ ID NO: 26 can usefully be omitted. Other fragments omit one or more protein domains. PSE38-1 is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

SEQ ID NO: 61 is a useful fragment of SEQ ID NO: 26 ('PSE38-1₂₅₋₃₅₀'). This fragment includes the most exposed domain of PSE38-1 and is more easily used at an industrial scale.

Further antigenic polypeptides

PA4525 or PilA

The PilA antigen is annotated as 'type 4 fimbrial precursor PilA'. In the PAO1 strain PilA is PA4525 and has amino acid sequence SEQ ID NO: 31 (GI: 15599721). See Ref. 37. Useful PilA antigens can elicit an antibody (*e.g.* when administered to a human) that recognises SEQ ID NO: 31 and/or may comprise an amino acid sequence: (a) having 50% or more identity (*e.g.* 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) to SEQ ID NO: 31; and/or (b) comprising a fragment of at least 'n' consecutive amino acids of SEQ ID NO: 31, wherein 'n' is 7 or more (*e.g.* 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250 or more). These PilA proteins include variants of SEQ ID NO: 31. Preferred fragments of (b) comprise an epitope from SEQ ID NO: 31. Other preferred fragments lack one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the C-terminus and/or one or more amino acids (*e.g.* 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25 or more) from the N-terminus of SEQ ID NO: 31 while retaining at least one epitope of SEQ ID NO: 31. Other fragments omit one or more protein domains. PilA is naturally a long protein and so the use of fragments is helpful *e.g.* for purification, handling, fusion, expression, *etc.*

Useful fragment include the most exposed domain of PilA and is more easily used at an industrial scale. It also reduces the antigen's similarity with human proteins. Vaccines and immunotherapy using this antigen have been attempted as shown in reference 49, and in reference 50.

OprF-OprI

The OprF/I antigen is a fusion protein consisting of a hybrid protein [Met-Ala-(His)₆OprF (190-342)-OprI (21-83)], ("His)₆" disclosed as SEQ ID NO: 70), resulting by the fusion of the mature outer membrane protein I (OprI) and amino acids 190 to 342 of OprF of *Pseudomonas aeruginosa* expressed in *Escherichia coli* and purified.

The fusion protein has been described in reference 4 as SEQ ID NO 006. For reference purposes, a full-length amino acid sequence of the fusion protein described herein is given as SEQ ID NO: 32. This antigen can be usefully used as positive control as single antigen, or showing a surprising positive effect increasing vaccine efficacy in in vivo experiments when used in combination with specific pseudomonas antigens.

PA1092 or FliC (Flagellar protein)

Flagella and main flagella proteins like FliC (PA1092) or FliD (PA1094) have been extensively characterized and used as single vaccine antigens in the past as shown in reference 51. For reference purposes, a full-length amino acid sequence of FliC is given as SEQ ID NO: 33 herein.

PA1092 antigen and/or PA1094 antigen may be usefully combined with any of the "first antigen group" or the "second antigen group".

PA1094 or FliD (Flagellar protein)

Flagella and main flagella proteins like FliD (PA1094) have been extensively characterized and used as vaccine antigens in the past as shown in reference 51. For reference purposes, a full-length amino acid sequence of FliD is given as SEQ ID NO: 34 herein.

PA1094 may be usefully combined with any of the "first antigen group" or any of the "second antigen group".

PA1148 or Exoprotein A or Exotoxin A

The Exoprotein A known also as Exotoxin A is an exoprotein which has been extensively characterized and used primarily as carrier protein in polysaccharide conjugate vaccine approach, e.g. reference 22. It is known as PA1148 in the PAO1PAO1 strain. See Ref. 37.

PA1148 antigen may be usefully combined with any of the "first antigen group" or any of the "second antigen group".

Hybrid polypeptides

Antigens used in the invention may be present in the composition as individual separate polypeptides. Where more than one antigen is used, however, they do not have to be present as separate polypeptides. Instead, at least two (e.g. 2, 3, 4, 5, or more) antigens can be expressed as a single polypeptide chain (a 'hybrid' polypeptide). Hybrid polypeptides offer two main advantages: first, a polypeptide that may be unstable or poorly expressed on its own can be assisted by adding a suitable hybrid partner that overcomes the problem; second, commercial manufacture is simplified as only one expression and purification need be employed in order to produce two polypeptides which are both antigenically useful.

The hybrid polypeptide may comprise two or more polypeptide sequences from the first antigen group. The hybrid polypeptide may comprise one or more polypeptide sequences from the first antigen group and one or more polypeptide sequences from the second antigen group. Moreover, the hybrid polypeptide may comprise two or more polypeptide sequences from each of the antigens listed above, or two or more
5 variants of the same antigen in the cases in which the sequence has partial variability across strains.

Hybrids consisting of amino acid sequences from two, three, four, five, six, seven, eight, nine, or ten antigens are useful. In particular, hybrids consisting of amino acid sequences from two, three, four, or five antigens are preferred, such as two or three antigens.

Different hybrid polypeptides may be mixed together in a single formulation. Hybrids may be combined
10 with non-hybrid antigens selected from the first, second or third antigen groups. Within such combinations, an antigen may be present in more than one hybrid polypeptide and/or as a non-hybrid polypeptide. It is preferred, however, that an antigen is present either as a hybrid or as a non-hybrid, but not as both.

The hybrid polypeptides can also be combined with conjugates or non-*P.aeruginosa* antigens as described
15 above.

Hybrid polypeptides can be represented by the formula $\text{NH}_2\text{-A-}\{-\text{X-L-}\}_n\text{-B-COOH}$, wherein: X is an amino acid sequence of a *P. aeruginosa* antigen, as described above; L is an optional linker amino acid sequence; A is an optional N-terminal amino acid sequence; B is an optional C-terminal amino acid sequence; n is an integer of 2 or more (*e.g.* 2, 3, 4, 5, 6, *etc.*). Usually n is 2 or 3.

20 If a -X- moiety has a leader peptide sequence in its wild-type form, this may be included or omitted in the hybrid protein. In some embodiments, the leader peptides will be deleted except for that of the -X- moiety located at the N-terminus of the hybrid protein *i.e.* the leader peptide of X_1 will be retained, but the leader peptides of $X_2 \dots X_n$ will be omitted. This is equivalent to deleting all leader peptides and using the leader peptide of X_1 as moiety -A-.

25 For each n instances of $\{-\text{X-L-}\}$, linker amino acid sequence -L- may be present or absent. For instance, when $n=2$ the hybrid may be $\text{NH}_2\text{-X}_1\text{-L}_1\text{-X}_2\text{-L}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-X}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-L}_1\text{-X}_2\text{-COOH}$, $\text{NH}_2\text{-X}_1\text{-X}_2\text{-L}_2\text{-COOH}$, *etc.* Linker amino acid sequence(s) -L- will typically be short (*e.g.* 20 or fewer amino acids *i.e.* 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples comprise short peptide sequences which facilitate cloning, poly-glycine linkers (*i.e.* comprising Gly_n where $n = 2, 3, 4, 5,$
30 6, 7, 8, 9, 10 or more) (SEQ ID NO: 71), and histidine tags (*i.e.* His_n where $n = 3, 4, 5, 6, 7, 8, 9, 10$ or more) (SEQ ID NO: 72). Other suitable linker amino acid sequences will be apparent to those skilled in the art. A useful linker is GSGGGG (SEQ ID NO: 67) or GSGSGGGG (SEQ ID NO: 68), with the Gly-Ser dipeptide being formed from a *Bam*HI restriction site, thus aiding cloning and manipulation, and the $(\text{Gly})_4$ (SEQ ID NO: 73) tetrapeptide being a typical poly-glycine linker. Other suitable linkers,
35 particularly for use as the final L_n are ASGGGS (SEQ ID NO: 69) or a Leu-Glu dipeptide.

-A- is an optional N-terminal amino acid sequence. This will typically be short (*e.g.* 40 or fewer amino acids *i.e.* 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16,

15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1). Examples include leader sequences to direct protein trafficking, or short peptide sequences which facilitate cloning or purification (e.g. histidine tags *i.e.* His_n where *n* = 3, 4, 5, 6, 7, 8, 9, 10 or more) (SEQ ID NO: 72). A useful tag contains a sequence of 6 consecutive Histidine (SEQ ID NO: 70), having at its start a homologue or heterologous start Methionine and/or an Alanine, *i.e.* SEQ ID NO 66. Other suitable N-terminal amino acid sequences will be apparent to those skilled in the art. If X₁ lacks its own N-terminus methionine, -A- is preferably an oligopeptide (e.g. with 1, 2, 3, 4, 5, 6, 7 or 8 amino acids) which provides a N-terminus methionine *e.g.* Met-Ala-Ser, or a single Met residue.

-B- is an optional C-terminal amino acid sequence. This will typically be short (e.g. 40 or fewer amino acids *i.e.* 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1).

Polypeptides used with the invention

Polypeptides used with the invention can take various forms (e.g. native, fusions, glycosylated, non-glycosylated, lipidated, non-lipidated, phosphorylated, non-phosphorylated, myristoylated, non-myristoylated, monomeric, multimeric, particulate, denatured, *etc.*).

Polypeptides used with the invention can be prepared by various means (e.g. recombinant expression, purification from cell culture, chemical synthesis, isolated from a natural biological source *etc.*). Recombinantly-expressed proteins are preferred, particularly for hybrid polypeptides.

Polypeptides used with the invention are preferably provided in purified or substantially purified form *i.e.* substantially free from other polypeptides (e.g. free from naturally-occurring polypeptides), particularly from other pseudomonas or host cell polypeptides, and are generally at least about 50% pure (by weight), and usually at least about 90% pure *i.e.* less than about 50%, and more preferably less than about 10% (e.g. 5%) of a composition is made up of other expressed polypeptides. Thus the antigens in the compositions are separated from the whole organism with which the molecule is expressed.

Polypeptides used with the invention are preferably pseudomonas polypeptides.

The term "polypeptide" refers to amino acid polymers of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labelling component. Also included are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art. Polypeptides can occur as single chains or associated chains.

The invention provides polypeptides comprising a sequence -P-Q- or -Q-P-, wherein: -P- is an amino acid sequence as defined above and -Q- is not a sequence as defined above *i.e.* the invention provides fusion proteins. Where the N-terminus codon of -P- is not ATG, but this codon is not present at the N-terminus of a polypeptide, it will be translated as the standard amino acid for that codon rather than as a Met.

Where this codon is at the N-terminus of a polypeptide, however, it will be translated as Met. Examples of -Q- moieties include, but are not limited to, histidine tags (*i.e.* His_n where *n* = 3, 4, 5, 6, 7, 8, 9, 10 or more) (SEQ ID NO: 72), maltose-binding protein, or glutathione-S-transferase (GST).

The invention also provides a process for producing a polypeptide of the invention, comprising the step of culturing a host cell transformed with nucleic acid of the invention under conditions which induce polypeptide expression.

Although expression of the polypeptides of the invention may take place in a *Pseudomonas*, the invention will usually use a heterologous host for expression (recombinant expression). The heterologous host may be prokaryotic (*e.g.* a bacterium) or eukaryotic. It may be *E.coli*, but other suitable hosts include *Bacillus subtilis*, *Vibrio cholerae*, *Salmonella typhi*, *Salmonella typhimurium*, *Neisseria lactamica*, *Neisseria cinerea*, *Mycobacteria* (*e.g.* *M.tuberculosis*), yeasts, *etc.* Compared to the wild-type *P. aeruginosa* genes encoding polypeptides of the invention, it is helpful to change codons to optimise expression efficiency in such hosts without affecting the encoded amino acids.

The invention provides a process for producing a polypeptide of the invention, comprising the step of synthesising at least part of the polypeptide by chemical means.

Nucleic acids

The invention also provides nucleic acid encoding polypeptides and hybrid polypeptides of the invention. It also provides nucleic acid comprising a nucleotide sequence that encodes one or more polypeptides or hybrid polypeptides of the invention.

The invention also provides nucleic acid comprising nucleotide sequences having sequence identity to such nucleotide sequences. Identity between sequences is preferably determined by the Smith-Waterman homology search algorithm as described above. Such nucleic acids include those using alternative codons to encode the same amino acid.

The invention also provides nucleic acid which can hybridize to these nucleic acids. Hybridization reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridization reaction are widely known and published in the art. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37°C, 50°C, 55°C and 68°C; buffer concentrations of 10 x SSC, 6 x SSC, 1 x SSC, 0.1 x SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 x SSC, 1 x SSC, 0.1 x SSC, or de-ionized water. Hybridization techniques and their optimization are well known in the art.

In some embodiments, nucleic acid of the invention hybridizes to a target under low stringency conditions; in other embodiments it hybridizes under intermediate stringency conditions; in preferred embodiments, it hybridizes under high stringency conditions. An exemplary set of low stringency hybridization conditions is 50°C and 10 x SSC. An exemplary set of intermediate stringency

hybridization conditions is 55°C and 1 x SSC. An exemplary set of high stringency hybridization conditions is 68°C and 0.1 x SSC.

The invention includes nucleic acid comprising sequences complementary to these sequences (*e.g.* for antisense or probing, or for use as primers).

- 5 Nucleic acids of the invention can be used in hybridisation reactions (*e.g.* Northern or Southern blots, or in nucleic acid microarrays or ‘gene chips’) and amplification reactions (*e.g.* PCR, SDA, SSSR, LCR, TMA, NASBA, *etc.*) and other nucleic acid techniques.

Nucleic acid according to the invention can take various forms (*e.g.* single-stranded, double-stranded, vectors, primers, probes, labelled *etc.*). Nucleic acids of the invention may be circular or branched, but
10 will generally be linear. Unless otherwise specified or required, any embodiment of the invention that utilizes a nucleic acid may utilize both the double-stranded form and each of two complementary single-stranded forms which make up the double-stranded form. Primers and probes are generally single-stranded, as are antisense nucleic acids.

Nucleic acids of the invention are preferably provided in purified or substantially purified form *i.e.*
15 substantially free from other nucleic acids (*e.g.* free from naturally-occurring nucleic acids), particularly from other pseudomonas or host cell nucleic acids, generally being at least about 50% pure (by weight), and usually at least about 90% pure. Nucleic acids of the invention are preferably pseudomonas nucleic acids.

Nucleic acids of the invention may be prepared in many ways *e.g.* by chemical synthesis (*e.g.*
20 phosphoramidite synthesis of DNA) in whole or in part, by digesting longer nucleic acids using nucleases (*e.g.* restriction enzymes), by joining shorter nucleic acids or nucleotides (*e.g.* using ligases or polymerases), from genomic or cDNA libraries, *etc.*

Nucleic acid of the invention may be attached to a solid support (*e.g.* a bead, plate, filter, film, slide, microarray support, resin, *etc.*). Nucleic acid of the invention may be labelled *e.g.* with a radioactive or
25 fluorescent label, or a biotin label. This is particularly useful where the nucleic acid is to be used in detection techniques *e.g.* where the nucleic acid is a primer or as a probe.

The term “nucleic acid” includes in general means a polymeric form of nucleotides of any length, which contain deoxyribonucleotides, ribonucleotides, and/or their analogs. It includes DNA, RNA, DNA/RNA hybrids. It also includes DNA or RNA analogs, such as those containing modified backbones (*e.g.*
30 peptide nucleic acids (PNAs) or phosphorothioates) or modified bases. Thus the invention includes mRNA, tRNA, rRNA, ribozymes, DNA, cDNA, recombinant nucleic acids, branched nucleic acids, plasmids, vectors, probes, primers, *etc.* Where nucleic acid of the invention takes the form of RNA, it may or may not have a 5' cap.

Nucleic acids of the invention may be part of a vector *i.e.* part of a nucleic acid construct designed for
35 transduction/transfection of one or more cell types. Vectors may be, for example, “cloning vectors” which are designed for isolation, propagation and replication of inserted nucleotides, “expression vectors” which are designed for expression of a nucleotide sequence in a host cell, “viral vectors” which is designed to

result in the production of a recombinant virus or virus-like particle, or “shuttle vectors”, which comprise the attributes of more than one type of vector. Preferred vectors are plasmids. A “host cell” includes an individual cell or cell culture which can be or has been a recipient of exogenous nucleic acid. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. Host cells include cells transfected or infected *in vivo* or *in vitro* with nucleic acid of the invention.

Where a nucleic acid is DNA, it will be appreciated that “U” in a RNA sequence will be replaced by “T” in the DNA. Similarly, where a nucleic acid is RNA, it will be appreciated that “T” in a DNA sequence will be replaced by “U” in the RNA.

The term “complement” or “complementary” when used in relation to nucleic acids refers to Watson-Crick base pairing. Thus the complement of C is G, the complement of G is C, the complement of A is T (or U), and the complement of T (or U) is A. It is also possible to use bases such as I (the purine inosine) *e.g.* to complement pyrimidines (C or T).

Nucleic acids of the invention can be used, for example: to produce polypeptides; as hybridization probes for the detection of nucleic acid in biological samples; to generate additional copies of the nucleic acids; to generate ribozymes or antisense oligonucleotides; as single-stranded DNA primers or probes; or as triple-strand forming oligonucleotides.

The invention provides a process for producing nucleic acid of the invention, wherein the nucleic acid is synthesised in part or in whole using chemical means.

The invention provides vectors comprising nucleotide sequences of the invention (*e.g.* cloning or expression vectors) and host cells transformed with such vectors.

Nucleic acid amplification according to the invention may be quantitative and/or real-time.

For certain embodiments of the invention, nucleic acids are preferably at least 7 nucleotides in length (*e.g.* 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300 nucleotides or longer).

For certain embodiments of the invention, nucleic acids are preferably at most 500 nucleotides in length (*e.g.* 450, 400, 350, 300, 250, 200, 150, 140, 130, 120, 110, 100, 90, 80, 75, 70, 65, 60, 55, 50, 45, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15 nucleotides or shorter).

Primers and probes of the invention, and other nucleic acids used for hybridization, are preferably between 10 and 30 nucleotides in length (*e.g.* 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 40 or more nucleotides).

Strains and variants

Antigens are defined above by reference to existing nomenclature (e.g. “PA0328”), to “PSE52” or to “PSE followed by a natural number, indicating the clone number, i.e. PSE52-1, etc” or to the respective SEQ ID NOs numbers.

- 5 Table 1 below associates these three naming/numbering systems to existing PAO1 public available numbering.

PAO1 numbering refers to the genome of *P. aeruginosa* strain PAO1 which is extensively described in terms of genomic analysis in reference 37.

Functional annotations for each antigen are also given in the databases.

- 10 Thus an exemplary amino acid and nucleotide sequence for any of these antigens can easily be found in public sequence databases from the PAO1 strain, but the invention is not limited to sequences from the PAO1 strains. Standard search and alignment techniques can be used to identify in any of these (or other) further genome sequences the homolog of any particular sequence from the PAO1 strain. Moreover, the available sequences from the PAO1 strain can be used to design primers for amplification of homologous
15 sequences from other strains. Thus the invention is not limited to this strain, but rather encompasses such variants and homologs from other strains of *P. aeruginosa*, as well as non-natural variants. In general, suitable variants of a particular SEQ ID NO include its allelic variants, its polymorphic forms, its homologs, its orthologs, its paralogs, its mutants, etc.

- Thus, for instance, polypeptides used with the invention may, compared to the SEQ ID NO herein,
20 include one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, etc.) amino acid substitutions, such as conservative substitutions (i.e. substitutions of one amino acid with another which has a related side chain). Genetically-encoded amino acids are generally divided into four families: (1) acidic i.e. aspartate, glutamate; (2) basic i.e. lysine, arginine, histidine; (3) non-polar i.e. alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar i.e. glycine, asparagine,
25 glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In general, substitution of single amino acids within these families does not have a major effect on the biological activity. The polypeptides may also include one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, etc.) single amino acid deletions relative to the SEQ ID NO sequences. The polypeptides may also include one or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, etc.) insertions (e.g. each of 1,
30 2, 3, 4 or 5 amino acids) relative to the SEQ ID NO sequences.

Similarly, a polypeptide used with the invention may comprise an amino acid sequence that:

- is identical (i.e. 100% identical) to a sequence disclosed in the sequence listing;
- shares sequence identity (e.g. 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, 99.5% or more) with a sequence disclosed in the sequence listing;
- 35 • has 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 (or more) single amino acid alterations (deletions, insertions, substitutions), which may be at separate locations or may be contiguous, as compared to the sequences of (a) or (b);

- when aligned with a particular sequence from the sequence listing using a pairwise alignment algorithm, each moving window of x amino acids from N-terminus to C-terminus (such that for an alignment that extends to p amino acids, where $p > x$, there are $p-x+1$ such windows) has at least $x \cdot y$ identical aligned amino acids, where: x is selected from 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 150, 200; y is selected from 0.50, 0.60, 0.70, 0.75, 0.80, 0.85, 0.90, 0.91, 0.92, 0.93, 0.94, 0.95, 0.96, 0.97, 0.98, 0.99; and if $x \cdot y$ is not an integer then it is rounded up to the nearest integer. The preferred pairwise alignment algorithm is the Needleman-Wunsch global alignment algorithm [52], using default parameters (e.g. with Gap opening penalty = 10.0, and with Gap extension penalty = 0.5, using the EBLOSUM62 scoring matrix). This algorithm is conveniently implemented in the *needle* tool in the EMBOSS package [53].

Where hybrid polypeptides are used, the individual antigens within the hybrid (i.e. individual -X-moieties) may be from one or more strains. Where $n=2$, for instance, X_2 may be from the same strain as X_1 or from a different strain. Where $n=3$, the strains might be (i) $X_1=X_2=X_3$ (ii) $X_1=X_2 \neq X_3$ (iii) $X_1 \neq X_2=X_3$ (iv) $X_1 \neq X_2 \neq X_3$ or (v) $X_1=X_3 \neq X_2$, etc.

Within group (c), deletions or substitutions may be at the N-terminus and/or C-terminus, or may be between the two termini. Thus a truncation is an example of a deletion. Truncations may involve deletion of up to 40 (or more) amino acids at the N-terminus and/or C-terminus. N-terminus truncation can remove leader peptides e.g. to facilitate recombinant expression in a heterologous host. C-terminus truncation can remove anchor sequences e.g. to facilitate recombinant expression in a heterologous host.

In general, when an antigen comprises a sequence that is not identical to a complete *P. aeruginosa* sequence from the sequence listing (e.g. when it comprises a sequence listing with <100% sequence identity thereto, or when it comprises a fragment thereof) it is preferred in each individual instance that the antigen can elicit an antibody which recognises the respective complete *P. aeruginosa* sequence.

Mutant bacteria

Present invention, also provides a *P. aeruginosa* bacterium in which one or more of the antigens from the various antigen groups of the invention has/have been knocked out (see Ref. 46). Techniques for producing knockout bacteria are well known, and knockout of genes from *P. aeruginosa* strains have been reported i.e. in Ref. 54. A knockout mutation may be situated in the coding region of the gene or may lie within its transcriptional control regions (e.g. within its promoter). A knockout mutation will reduce the level of mRNA encoding the antigen to <1% of that produced by the wild-type bacterium, preferably <0.5%, more preferably <0.1%, and most preferably to 0%.

The invention also provides a *P. aeruginosa* in which one or more of the antigens from the various antigen groups of the invention has a mutation which inhibits its activity. The gene encoding the antigen will have a mutation that changes the encoded amino acid sequence. Mutation may involve deletion, substitution, and/or insertion, any of which may be involve one or more amino acids.

The invention also provides a bacterium, such as a *P. aeruginosa* bacterium, which hyper-expresses an antigen of the invention.

The invention also provides a bacterium, such as a *P. aeruginosa* bacterium, that constitutively expresses an antigen of the invention. The invention also provides a *E. coli* comprising a gene encoding an antigen of the invention, wherein the gene is under the control of an inducible promoter.

5 Mutant bacteria are particularly useful for preparing bacterial outer membrane vesicles which include *P. aeruginosa* antigens (*e.g.* antigens of the invention), which can be used as immunogens [55-57].

Immunogenic compositions and medicaments

Immunogenic compositions of the invention may be useful as vaccines. Vaccines according to the invention may either be prophylactic (*i.e.* to prevent infection) or therapeutic (*i.e.* to treat infection), but will typically be prophylactic.

10 Compositions may thus be pharmaceutically acceptable. They will usually include components in addition to the antigens *e.g.* they typically include one or more pharmaceutical carrier(s) and/or excipient(s).

Compositions will generally be administered to a mammal in aqueous form. Prior to administration, however, the composition may have been in a non-aqueous form. For instance, although some vaccines
15 are manufactured in aqueous form, then filled and distributed and administered also in aqueous form, other vaccines are lyophilised during manufacture and are reconstituted into an aqueous form at the time of use. Thus a composition of the invention may be dried, such as a lyophilised formulation.

The composition may include preservatives such as thiomersal or 2-phenoxyethanol. It is preferred, however, that the vaccine should be substantially free from (*i.e.* less than 5µg/ml) mercurial material *e.g.*
20 thiomersal-free. Vaccines containing no mercury are more preferred. Preservative-free vaccines are particularly preferred.

To improve thermal stability, a composition may include a temperature protective agent. Further details of such agents are provided below.

To control tonicity, it is preferred to include a physiological salt, such as a sodium salt. Sodium chloride
25 (NaCl) is preferred, which may be present at between 1 and 20 mg/ml *e.g.* about 10±2mg/ml NaCl. Other salts that may be present include potassium chloride, potassium dihydrogen phosphate, disodium phosphate dehydrate, magnesium chloride, calcium chloride, *etc.*

Compositions will generally have an osmolality of between 200 mOsm/kg and 400 mOsm/kg, preferably between 240-360 mOsm/kg, and will more preferably fall within the range of 290-310 mOsm/kg.

30 Compositions may include one or more buffers. Typical buffers include: a phosphate buffer; a Tris buffer; a borate buffer; a succinate buffer; a histidine buffer (particularly with an aluminum hydroxide adjuvant); or a citrate buffer. Buffers will typically be included in the 5-20mM range.

The pH of a composition will generally be between 5.0 and 8.1, and more typically between 6.0 and 8.0 *e.g.* 6.5 and 7.5, or between 7.0 and 7.8.

The composition is preferably sterile. The composition is preferably non-pyrogenic *e.g.* containing <1 EU (endotoxin unit, a standard measure) per dose, and preferably <0.1 EU per dose. The composition is preferably gluten free.

5 The composition may include material for a single immunisation, or may include material for multiple immunisations (*i.e.* a ‘multidose’ kit). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to including a preservative in multidose compositions, the compositions may be contained in a container having an aseptic adaptor for removal of material.

Human vaccines are typically administered in a dosage volume of about 0.5ml, although a half dose (*i.e.* about 0.25ml) may be administered to children.

10 Immunogenic compositions of the invention may also comprise one or more immunoregulatory agents. Preferably, one or more of the immunoregulatory agents include one or more adjuvants. The adjuvants may include a TH1 adjuvant and/or a TH2 adjuvant, or a TLR7 agonist further discussed below.

Thus the invention provides an immunogenic composition comprising a combination of:

- 15 (1) one or more antigen(s) selected from the first, second, and further antigen group (as defined above); and
- (2) an adjuvant, such as an aluminium hydroxide adjuvant (for example, one or more antigens may be adsorbed to aluminium hydroxide).

For instance, the invention provides an immunogenic composition comprising a combination of a sta006 antigen and an adjuvant, such as an aluminium hydroxide adjuvant. Similarly, the invention provides an immunogenic composition comprising a combination of a sta011 antigen and an adjuvant, such as an aluminium hydroxide adjuvant. These compositions are ideally buffered *e.g.* with a histidine buffer.

20

Adjuvants which may be used in compositions of the invention include, but are not limited to:

A. Mineral-containing compositions

Mineral containing compositions suitable for use as adjuvants in the invention include mineral salts, such as aluminium salts and calcium salts (or mixtures thereof). Calcium salts include calcium phosphate (*e.g.* the “CAP” particles disclosed in ref. 58). Aluminum salts include hydroxides, phosphates, sulfates, *etc.*, with the salts taking any suitable form (*e.g.* gel, crystalline, amorphous, *etc.*). Adsorption to these salts is preferred (*e.g.* all antigens may be adsorbed). The mineral containing compositions may also be formulated as a particle of metal salt [59].

25

30 The adjuvants known as aluminum hydroxide and aluminum phosphate may be used. The invention can use any of the “hydroxide” or “phosphate” adjuvants that are in general use as adjuvants. The adjuvants known as “aluminium hydroxide” are typically aluminium oxyhydroxide salts, which are usually at least partially crystalline. The adjuvants known as “aluminium phosphate” are typically aluminium hydroxyphosphates, often also containing a small amount of sulfate (*i.e.* aluminium hydroxyphosphate sulfate). They may be obtained by precipitation, and the reaction conditions and concentrations during precipitation influence the degree of substitution of phosphate for hydroxyl in the salt.

35

A fibrous morphology (*e.g.* as seen in transmission electron micrographs) is typical for aluminium hydroxide adjuvants. The pI of aluminium hydroxide adjuvants is typically about 11 *i.e.* the adjuvant itself has a positive surface charge at physiological pH. Adsorptive capacities of between 1.8-2.6 mg protein per mg Al⁺⁺⁺ at pH 7.4 have been reported for aluminium hydroxide adjuvants.

- 5 Aluminium phosphate adjuvants generally have a PO₄/Al molar ratio between 0.3 and 1.2, preferably between 0.8 and 1.2, and more preferably 0.95±0.1. The aluminium phosphate will generally be amorphous, particularly for hydroxyphosphate salts. A typical adjuvant is amorphous aluminium hydroxyphosphate with PO₄/Al molar ratio between 0.84 and 0.92, included at 0.6mg Al³⁺/ml. The aluminium phosphate will generally be particulate (*e.g.* plate-like morphology as seen in transmission
10 electron micrographs). Typical diameters of the particles are in the range 0.5-20µm (*e.g.* about 5-10µm) after any antigen adsorption. Adsorptive capacities of between 0.7-1.5 mg protein per mg Al⁺⁺⁺ at pH 7.4 have been reported for aluminium phosphate adjuvants.

- The point of zero charge (PZC) of aluminium phosphate is inversely related to the degree of substitution of phosphate for hydroxyl, and this degree of substitution can vary depending on reaction conditions and
15 concentration of reactants used for preparing the salt by precipitation. PZC is also altered by changing the concentration of free phosphate ions in solution (more phosphate = more acidic PZC) or by adding a buffer such as a histidine buffer (makes PZC more basic). Aluminium phosphates used according to the invention will generally have a PZC of between 4.0 and 7.0, more preferably between 5.0 and 6.5 *e.g.* about 5.7.

- 20 Suspensions of aluminium salts used to prepare compositions of the invention may contain a buffer (*e.g.* a phosphate or a histidine or a Tris buffer), but this is not always necessary. The suspensions are preferably sterile and pyrogen-free. A suspension may include free aqueous phosphate ions *e.g.* present at a concentration between 1.0 and 20 mM, preferably between 5 and 15 mM, and more preferably about 10 mM. The suspensions may also comprise sodium chloride.

- 25 The invention can use a mixture of both an aluminium hydroxide and an aluminium phosphate. In this case there may be more aluminium phosphate than hydroxide *e.g.* a weight ratio of at least 2:1 *e.g.* ≥5:1, ≥6:1, ≥7:1, ≥8:1, ≥9:1, *etc.*

- The concentration of Al⁺⁺⁺ in a composition for administration to a patient is preferably less than 10mg/ml *e.g.* ≤5 mg/ml, ≤4 mg/ml, ≤3 mg/ml, ≤2 mg/ml, ≤1 mg/ml, *etc.* A preferred range is between 0.3
30 and 1mg/ml. A maximum of 0.85mg/dose is preferred.

B. Oil Emulsions

- Oil emulsion compositions suitable for use as adjuvants in the invention include squalene-water emulsions, such as MF59 [Chapter 10 of ref. 63; see also ref. 60] (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer). Complete Freund's adjuvant
35 (CFA) and incomplete Freund's adjuvant (IFA) may also be used.

Various oil-in-water emulsion adjuvants are known, and they typically include at least one oil and at least one surfactant, with the oil(s) and surfactant(s) being biodegradable (metabolizable) and biocompatible.

The oil droplets in the emulsion are generally less than 5 μ m in diameter, and ideally have a sub-micron diameter, with these small sizes being achieved with a microfluidiser to provide stable emulsions. Droplets with a size less than 220nm are preferred as they can be subjected to filter sterilization.

The emulsion can comprise oils such as those from an animal (such as fish) or vegetable source. Sources for vegetable oils include nuts, seeds and grains. Peanut oil, soybean oil, coconut oil, and olive oil, the most commonly available, exemplify the nut oils. Jojoba oil can be used *e.g.* obtained from the jojoba bean. Seed oils include safflower oil, cottonseed oil, sunflower seed oil, sesame seed oil and the like. In the grain group, corn oil is the most readily available, but the oil of other cereal grains such as wheat, oats, rye, rice, teff, triticale and the like may also be used. 6-10 carbon fatty acid esters of glycerol and 1,2-propanediol, while not occurring naturally in seed oils, may be prepared by hydrolysis, separation and esterification of the appropriate materials starting from the nut and seed oils. Fats and oils from mammalian milk are metabolizable and may therefore be used in the practice of this invention. The procedures for separation, purification, saponification and other means necessary for obtaining pure oils from animal sources are well known in the art. Most fish contain metabolizable oils which may be readily recovered. For example, cod liver oil, shark liver oils, and whale oil such as spermaceti exemplify several of the fish oils which may be used herein. A number of branched chain oils are synthesized biochemically in 5-carbon isoprene units and are generally referred to as terpenoids. Shark liver oil contains a branched, unsaturated terpenoids known as squalene, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene, which is particularly preferred herein. Squalane, the saturated analog to squalene, is also a preferred oil. Fish oils, including squalene and squalane, are readily available from commercial sources or may be obtained by methods known in the art. Other preferred oils are the tocopherols (see below). Mixtures of oils can be used.

Surfactants can be classified by their 'HLB' (hydrophile/lipophile balance). Preferred surfactants of the invention have a HLB of at least 10, preferably at least 15, and more preferably at least 16. The invention can be used with surfactants including, but not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially polysorbate 20 and polysorbate 80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAXTM tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the TergitolTM NP series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. Non-ionic surfactants are preferred. Preferred surfactants for including in the emulsion are Tween 80 (polyoxyethylene sorbitan monooleate), Span 85 (sorbitan trioleate), lecithin and Triton X-100.

Mixtures of surfactants can be used *e.g.* Tween 80/Span 85 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (Tween 80) and an

octoxynol such as t-octylphenoxypolyethoxyethanol (Triton X-100) is also suitable. Another useful combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol.

Preferred amounts of surfactants (% by weight) are: polyoxyethylene sorbitan esters (such as Tween 80) 0.01 to 1%, in particular about 0.1 %; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100, or other detergents in the Triton series) 0.001 to 0.1 %, in particular 0.005 to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1 to 20 %, preferably 0.1 to 10 % and in particular 0.1 to 1 % or about 0.5%.

Preferred emulsion adjuvants have an average droplets size of $<1\mu\text{m}$ *e.g.* $\leq 750\text{nm}$, $\leq 500\text{nm}$, $\leq 400\text{nm}$, $\leq 300\text{nm}$, $\leq 250\text{nm}$, $\leq 220\text{nm}$, $\leq 200\text{nm}$, or smaller. These droplet sizes can conveniently be achieved by techniques such as microfluidisation.

Specific oil-in-water emulsion adjuvants useful with the invention include, but are not limited to:

- A submicron emulsion of squalene, Tween 80, and Span 85. The composition of the emulsion by volume can be about 5% squalene, about 0.5% polysorbate 80 and about 0.5% Span 85. In weight terms, these ratios become 4.3% squalene, 0.5% polysorbate 80 and 0.48% Span 85. This adjuvant is known as ‘MF59’ [61-], as described in more detail in Chapter 10 of ref. 63 and chapter 12 of ref. 64. The MF59 emulsion advantageously includes citrate ions *e.g.* 10mM sodium citrate buffer.
- An emulsion of squalene, a tocopherol, and polysorbate 80 (Tween 80). The emulsion may include phosphate buffered saline. It may also include Span 85 (*e.g.* at 1%) and/or lecithin. These emulsions may have from 2 to 10% squalene, from 2 to 10% tocopherol and from 0.3 to 3% Tween 80, and the weight ratio of squalene:tocopherol is preferably ≤ 1 as this provides a more stable emulsion. Squalene and Tween 80 may be present volume ratio of about 5:2 or at a weight ratio of about 11:5. One such emulsion can be made by dissolving Tween 80 in PBS to give a 2% solution, then mixing 90ml of this solution with a mixture of (5g of DL- α -tocopherol and 5ml squalene), then microfluidising the mixture. The resulting emulsion may have submicron oil droplets *e.g.* with an average diameter of between 100 and 250nm, preferably about 180nm. The emulsion may also include a 3-de-O-acylated monophosphoryl lipid A (3d-MPL). Another useful emulsion of this type may comprise, per human dose, 0.5-10 mg squalene, 0.5-11 mg tocopherol, and 0.1-4 mg polysorbate 80 [65].
- An emulsion of squalene, a tocopherol, and a Triton detergent (*e.g.* Triton X-100). The emulsion may also include a 3d-MPL (see below). The emulsion may contain a phosphate buffer.
- An emulsion comprising a polysorbate (*e.g.* polysorbate 80), a Triton detergent (*e.g.* Triton X-100) and a tocopherol (*e.g.* an α -tocopherol succinate). The emulsion may include these three components at a mass ratio of about 75:11:10 (*e.g.* 750 $\mu\text{g}/\text{ml}$ polysorbate 80, 110 $\mu\text{g}/\text{ml}$ Triton X-100 and 100 $\mu\text{g}/\text{ml}$ α -tocopherol succinate), and these concentrations should include any contribution of these components from antigens. The emulsion may also include squalene. The emulsion may also include a 3d-MPL (see below). The aqueous phase may contain a phosphate buffer.
- An emulsion of squalene, polysorbate 80 and poloxamer 401 (“Pluronic™ L121”). The emulsion can be formulated in phosphate buffered saline, pH 7.4. This emulsion is a useful delivery vehicle

for muramyl dipeptides, and has been used with threonyl-MDP in the “SAF-1” adjuvant [66] (0.05-1% Thr-MDP, 5% squalene, 2.5% Pluronic L121 and 0.2% polysorbate 80). It can also be used without the Thr-MDP, as in the “AF” adjuvant [67] (5% squalene, 1.25% Pluronic L121 and 0.2% polysorbate 80). Microfluidisation is preferred.

- 5 • An emulsion comprising squalene, an aqueous solvent, a polyoxyethylene alkyl ether hydrophilic nonionic surfactant (*e.g.* polyoxyethylene (12) cetostearyl ether) and a hydrophobic nonionic surfactant (*e.g.* a sorbitan ester or mannide ester, such as sorbitan monoleate or ‘Span 80’). The emulsion is preferably thermoreversible and/or has at least 90% of the oil droplets (by volume) with a size less than 200 nm [68]. The emulsion may also include one or more of: alditol; a
10 cryoprotective agent (*e.g.* a sugar, such as dodecylmaltoside and/or sucrose); and/or an alkylpolyglycoside. The emulsion may include a TLR4 agonist [69]. Such emulsions may be lyophilized.
- 15 • An emulsion of squalene, poloxamer 105 and Abil-Care [70]. The final concentration (weight) of these components in adjuvanted vaccines are 5% squalene, 4% poloxamer 105 (pluronic polyol) and 2% Abil-Care 85 (Bis-PEG/PPG-16/16 PEG/PPG-16/16 dimethicone; caprylic/capric triglyceride).
- 20 • An emulsion having from 0.5-50% of an oil, 0.1-10% of a phospholipid, and 0.05-5% of a non-ionic surfactant. As described in reference 71, preferred phospholipid components are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidic acid, sphingomyelin and cardiolipin. Submicron droplet sizes are advantageous.
- 25 • A submicron oil-in-water emulsion of a non-metabolizable oil (such as light mineral oil) and at least one surfactant (such as lecithin, Tween 80 or Span 80). Additives may be included, such as QuilA saponin, cholesterol, a saponin-lipophile conjugate (such as GPI-0100, described in reference 72, produced by addition of aliphatic amine to desacylsaponin via the carboxyl group of glucuronic acid), dimethyldioctadecylammonium bromide and/or N,N-dioctadecyl-N,N-bis (2-hydroxyethyl)propanediamine.
- 30 • An emulsion in which a saponin (*e.g.* QuilA or QS21) and a sterol (*e.g.* a cholesterol) are associated as helical micelles [73].
- 30 • An emulsion comprising a mineral oil, a non-ionic lipophilic ethoxylated fatty alcohol, and a non-ionic hydrophilic surfactant (*e.g.* an ethoxylated fatty alcohol and/or polyoxyethylene-polyoxypropylene block copolymer) [74].

In some embodiments an emulsion may be mixed with antigen extemporaneously, at the time of delivery, and thus the adjuvant and antigen may be kept separately in a packaged or distributed vaccine, ready for
35 final formulation at the time of use. In other embodiments an emulsion is mixed with antigen during manufacture, and thus the composition is packaged in a liquid adjuvanted form,. The antigen will generally be in an aqueous form, such that the vaccine is finally prepared by mixing two liquids. The volume ratio of the two liquids for mixing can vary (*e.g.* between 5:1 and 1:5) but is generally about 1:1. Where concentrations of components are given in the above descriptions of specific emulsions, these

concentrations are typically for an undiluted composition, and the concentration after mixing with an antigen solution will thus decrease.

Where a composition includes a tocopherol, any of the α , β , γ , δ , ϵ or ξ tocopherols can be used, but α -tocopherols are preferred. The tocopherol can take several forms *e.g.* different salts and/or isomers.

5 Salts include organic salts, such as succinate, acetate, nicotinate, *etc.* D- α -tocopherol and DL- α -tocopherol can both be used. Tocopherols are advantageously included in vaccines for use in elderly patients (*e.g.* aged 60 years or older) because vitamin E has been reported to have a positive effect on the immune response in this patient group [75]. They also have antioxidant properties that may help to stabilize the emulsions [76]. A preferred α -tocopherol is DL- α -tocopherol, and the preferred salt of this
10 tocopherol is the succinate. The succinate salt has been found to cooperate with TNF-related ligands *in vivo*.

C. Saponin formulations

Saponin formulations may also be used as adjuvants in the invention. Saponins are a heterogeneous group of sterol glycosides and triterpenoid glycosides that are found in the bark, leaves, stems, roots and even
15 flowers of a wide range of plant species. Saponin from the bark of the *Quillaia saponaria* Molina tree has been widely studied as adjuvant. Saponin can also be commercially obtained from *Smilax ornata* (sarsapilla), *Gypsophilla paniculata* (brides veil), and *Saponaria officianalis* (soap root). Saponin adjuvant formulations include purified formulations, such as QS21, as well as lipid formulations, such as ISCOMs. QS21 is marketed as Stimulon™.

20 Saponin compositions have been purified using HPLC and RP-HPLC. Specific purified fractions using these techniques have been identified, including QS7, QS17, QS18, QS21, QH-A, QH-B and QH-C. Preferably, the saponin is QS21. A method of production of QS21 is disclosed in ref. 77. Saponin formulations may also comprise a sterol, such as cholesterol [78].

Combinations of saponins and cholesterol can be used to form unique particles called
25 immunostimulating complexes (ISCOMs) [chapter 23 of ref. 63]. ISCOMs typically also include a phospholipid such as phosphatidylethanolamine or phosphatidylcholine. Any known saponin can be used in ISCOMs. Preferably, the ISCOM includes one or more of QuilA, QHA & QHC. ISCOMs are further described in refs. 78-. Optionally, the ISCOMS may be devoid of additional detergent [80].

A review of the development of saponin based adjuvants can be found in ref. 81.

30 E. Bacterial or microbial derivatives

Adjuvants suitable for use in the invention include bacterial or microbial derivatives such as non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), Lipid A derivatives, immunostimulatory oligonucleotides and ADP-ribosylating toxins and detoxified derivatives thereof.

Non-toxic derivatives of LPS include monophosphoryl lipid A (MPL) and 3-O-deacylated MPL
35 (3dMPL). 3dMPL is a mixture of 3 de-O-acylated monophosphoryl lipid A with 4, 5 or 6 acylated chains. A preferred “small particle” form of 3 De-O-acylated monophosphoryl lipid A is disclosed in ref. 82. Such “small particles” of 3dMPL are small enough to be sterile filtered through a 0.22 μ m membrane [82].

Other non-toxic LPS derivatives include monophosphoryl lipid A mimics, such as aminoalkyl glucosaminide phosphate derivatives *e.g.* RC-529 [83].

Lipid A derivatives include derivatives of lipid A from *Escherichia coli* such as OM-174. OM-174 is described for example in refs. 84 & 85.

- 5 Immunostimulatory oligonucleotides suitable for use as adjuvants in the invention include nucleotide sequences containing a CpG motif (a dinucleotide sequence containing an unmethylated cytosine linked by a phosphate bond to a guanosine). Double-stranded RNAs and oligonucleotides containing palindromic or poly(dG) sequences have also been shown to be immunostimulatory.

- 10 The CpG's can include nucleotide modifications/analogs such as phosphorothioate modifications and can be double-stranded or single-stranded. References 86 and 87 disclose possible analog substitutions *e.g.* replacement of guanosine with 2'-deoxy-7-deazaguanosine. The adjuvant effect of CpG oligonucleotides is further discussed in refs. 88.

- 15 The CpG sequence may be directed to TLR9, such as the motif GTCGTT or TTCGTT [89]. The CpG sequence may be specific for inducing a Th1 immune response, such as a CpG-A ODN, or it may be more specific for inducing a B cell response, such a CpG-B ODN. CpG-A and CpG-B ODNs are discussed in refs. 90-. Preferably, the CpG is a CpG-A ODN.

Preferably, the CpG oligonucleotide is constructed so that the 5' end is accessible for receptor recognition. Optionally, two CpG oligonucleotide sequences may be attached at their 3' ends to form "immunomers". See, for example, refs. 92-.

- 20 A useful CpG adjuvant is CpG7909, also known as ProMune™ (Coley Pharmaceutical Group, Inc.). Another is CpG1826. As an alternative, or in addition, to using CpG sequences, TpG sequences can be used [94], and these oligonucleotides may be free from unmethylated CpG motifs. The immunostimulatory oligonucleotide may be pyrimidine-rich. For example, it may comprise more than one consecutive thymidine nucleotide (*e.g.* TTTT, as disclosed in ref. 94), and/or it may have a nucleotide composition with >25% thymidine (*e.g.* >35%, >40%, >50%, >60%, >80%, *etc.*). For example, it may comprise more than one consecutive cytosine nucleotide (*e.g.* CCCC, as disclosed in ref. 94), and/or it may have a nucleotide composition with >25% cytosine (*e.g.* >35%, >40%, >50%, >60%, >80%, *etc.*). These oligonucleotides may be free from unmethylated CpG motifs. Immunostimulatory oligonucleotides will typically comprise at least 20 nucleotides. They may comprise fewer than 100 nucleotides.

- 30 A particularly useful adjuvant based around immunostimulatory oligonucleotides is known as IC-31™ [95]. Thus an adjuvant used with the invention may comprise a mixture of (i) an oligonucleotide (*e.g.* between 15-40 nucleotides) including at least one (and preferably multiple) CpI motifs (*i.e.* a cytosine linked to an inosine to form a dinucleotide), and (ii) a polycationic polymer, such as an oligopeptide (*e.g.* between 5-20 amino acids) including at least one (and preferably multiple) Lys-Arg-Lys tripeptide sequence(s). The oligonucleotide may be a deoxynucleotide comprising 26-mer sequence 5'-(IC)₃₁-3'.

Bacterial ADP-ribosylating toxins and detoxified derivatives thereof may be used as adjuvants in the invention. Preferably, the protein is derived from *E.coli* (*E.coli* heat labile enterotoxin "LT"), cholera

5 (“CT”), or pertussis (“PT”). The use of detoxified ADP-ribosylating toxins as mucosal adjuvants is described in ref. 96 and as parenteral adjuvants in ref. 97. The toxin or toxoid is preferably in the form of a holotoxin, comprising both A and B subunits. Preferably, the A subunit contains a detoxifying mutation; preferably the B subunit is not mutated. Preferably, the adjuvant is a detoxified LT mutant such as LT-K63, LT-R72, and LT-G192. The use of ADP-ribosylating toxins and detoxified derivatives thereof, particularly LT-K63 and LT-R72, as adjuvants can be found in refs. 98-101. A useful CT mutant is or CT-E29H [102]. Numerical reference for amino acid substitutions is preferably based on the alignments of the A and B subunits of ADP-ribosylating toxins set forth in ref. 103, specifically incorporated herein by reference in its entirety.

10 *F. Human immunomodulators*

Human immunomodulators suitable for use as adjuvants in the invention include cytokines, such as interleukins (*e.g.* IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12 [104], *etc.*), interferons (*e.g.* interferon- γ), macrophage colony stimulating factor, and tumor necrosis factor. A preferred immunomodulator is IL-12.

G. Bioadhesives and Mucoadhesives

15 Bioadhesives and mucoadhesives may also be used as adjuvants in the invention. Suitable bioadhesives include esterified hyaluronic acid microspheres [105] or mucoadhesives such as cross-linked derivatives of poly(acrylic acid), polyvinyl alcohol, polyvinyl pyrrolidone, polysaccharides and carboxymethylcellulose. Chitosan and derivatives thereof may also be used as adjuvants in the invention [106].

20 *H. Microparticles*

Microparticles may also be used as adjuvants in the invention. Microparticles (*i.e.* a particle of ~100nm to ~150 μ m in diameter, more preferably ~200nm to ~30 μ m in diameter, and most preferably ~500nm to ~10 μ m in diameter) formed from materials that are biodegradable and non-toxic (*e.g.* a poly(α -hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone, *etc.*), with poly(lactide-co-glycolide) are preferred, optionally treated to have a negatively-charged surface (*e.g.* with SDS) or a positively-charged surface (*e.g.* with a cationic detergent, such as CTAB).

I. Liposomes

Examples of liposome formulations suitable for use as adjuvants are described in refs. 107-.

J. Polyoxyethylene ether and polyoxyethylene ester formulations

30 Adjuvants suitable for use in the invention include polyoxyethylene ethers and polyoxyethylene esters [109]. Such formulations further include polyoxyethylene sorbitan ester surfactants in combination with an octoxynol [110] as well as polyoxyethylene alkyl ethers or ester surfactants in combination with at least one additional non-ionic surfactant such as an octoxynol [111]. Preferred polyoxyethylene ethers are selected from the following group: polyoxyethylene-9-lauryl ether (laureth 9), polyoxyethylene-9-stearyl ether, polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether.

K. *Phosphazenes*

A phosphazene, such as poly[di(carboxylatophenoxy)phosphazene] (“PCPP”) as described, for example, in references 112 and 113, may be used.

L. *Muramyl peptides*

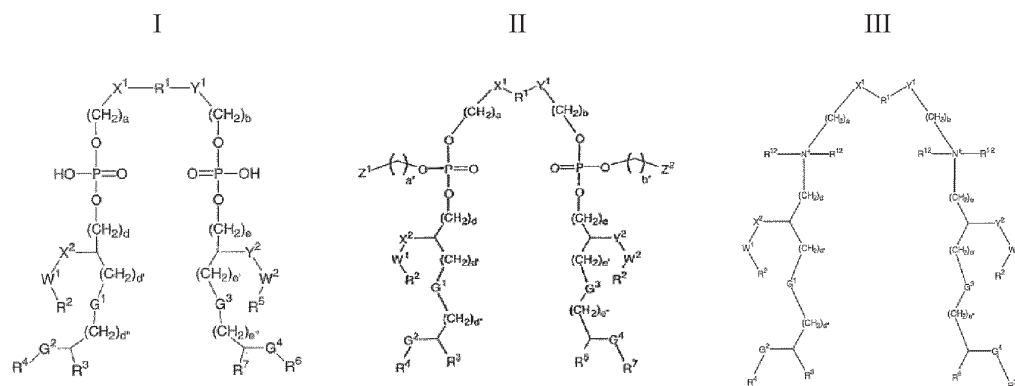
- 5 Examples of muramyl peptides suitable for use as adjuvants in the invention include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), and N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-*sn*-glycero-3-hydroxyphosphoryloxy)-ethylamine MTP-PE).

M. *Imidazoquinolone Compounds.*

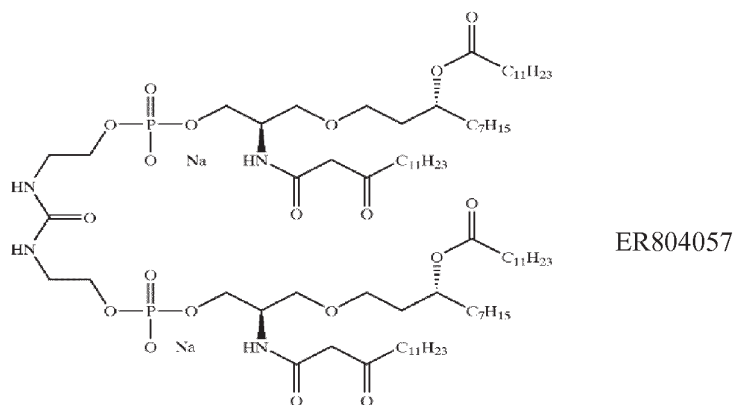
- 10 Examples of imidazoquinolone compounds suitable for use adjuvants in the invention include Imiquimod (“R-837”) [114], Resiquimod (“R-848”) [115], and their analogs; and salts thereof (*e.g.* the hydrochloride salts).

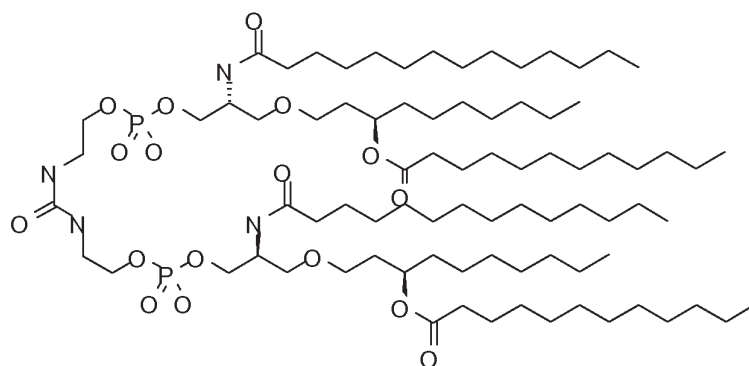
N. *Substituted ureas*

Substituted ureas useful as adjuvants include compounds of formula I, II or III, or salts thereof:



- 15 as defined in reference 116, such as ‘ER 803058’, ‘ER 803732’, ‘ER 804053’, ‘ER 804058’, ‘ER 804059’, ‘ER 804442’, ‘ER 804680’, ‘ER 804764’, ‘ER 803022 or ‘ER 804057’ *e.g.*:



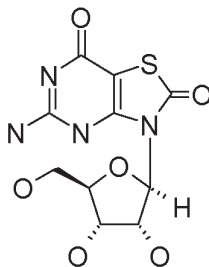


ER-803022:

O. Further adjuvants

Further adjuvants that may be used with the invention include:

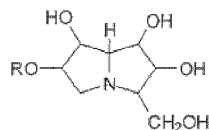
- An aminoalkyl glucosaminide phosphate derivative, such as RC-529 [117].
- A thiosemicarbazone compound, such as those disclosed in reference 118. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 118. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- α .
- A tryptanthrin compound, such as those disclosed in reference 119. Methods of formulating, manufacturing, and screening for active compounds are also described in reference 119. The thiosemicarbazones are particularly effective in the stimulation of human peripheral blood mononuclear cells for the production of cytokines, such as TNF- α .
- A nucleoside analog, such as: (a) Isatorabine (ANA-245; 7-thia-8-oxoguanosine):



and prodrugs thereof; (b) ANA975; (c) ANA-025-1; (d) ANA380; (e) the compounds disclosed in references 120 to Loxoribine (7-allyl-8-oxoguanosine) [122].

- Compounds disclosed in reference 123, including: Acylpiperazine compounds, Indoleione compounds, Tetrahydroisoquinoline (THIQ) compounds, Benzocyclodione compounds, Aminoazavinyl compounds, Aminobenzimidazole quinolinone (ABIQ) compounds [124], Hydraphtalamide compounds, Benzophenone compounds, Isoxazole compounds, Sterol compounds, Quinazolinone compounds, Pyrrole compounds [125], Anthraquinone compounds, Quinoxaline compounds, Triazine compounds, Pyrazalopyrimidine compounds, and Benzazole compounds [126].

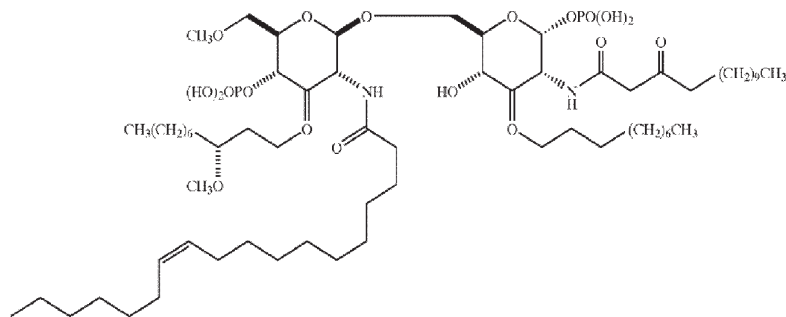
- Compounds containing lipids linked to a phosphate-containing acyclic backbone, such as the TLR4 antagonist E5564 [127];
 - A polyoxidonium polymer [128] or other N-oxidized polyethylene-piperazine derivative.
 - Methyl inosine 5'-monophosphate ("MIMP") [129].
- 5 • A polyhydroxylated pyrrolizidine compound [130], such as one having formula:



where R is selected from the group comprising hydrogen, straight or branched, unsubstituted or substituted, saturated or unsaturated acyl, alkyl (*e.g.* cycloalkyl), alkenyl, alkynyl and aryl groups, or a pharmaceutically acceptable salt or derivative thereof. Examples include, but are not limited to:

10 casuarine, casuarine-6- α -D-glucopyranose, 3-*epi*-casuarine, 7-*epi*-casuarine, 3,7-*diepi*-casuarine, *etc.*

- A CD1d ligand, such as an α -glycosylceramide [131-] (*e.g.* α -galactosylceramide), phytosphingosine-containing α -glycosylceramides, OCH, KRN7000 [(2S,3S,4R)-1-O-(α -D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol], CRONY-101, 3"-O-sulfo-
- 15 galactosylceramide, *etc.*
- A gamma inulin [133] or derivative thereof, such as algammulin.



Adjuvant combinations

The invention may also comprise combinations of one or more of the adjuvants identified above. For

20 example, the following adjuvant compositions may be used in the invention: (1) a saponin and an oil-in-water emulsion [134]; (2) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL); (3) a saponin (*e.g.* QS21) + a non-toxic LPS derivative (*e.g.* 3dMPL) + a cholesterol; (4) a saponin (*e.g.* QS21) + 3dMPL + IL-12 (optionally + a sterol); (5) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions [135]; (6) SAF, containing 10% squalane, 0.4% Tween 80™, 5% pluronic-block

25 polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion. (7) Ribi™ adjuvant system (RAS), (Ribi Immunochem) containing 2% squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphorylipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably

MPL + CWS (Detox™); and (8) one or more mineral salts (such as an aluminum salt) + a non-toxic derivative of LPS (such as 3dMPL).

The use of an aluminium hydroxide and/or aluminium phosphate adjuvant is particularly preferred, and antigens are generally adsorbed to these salts. Calcium phosphate is another preferred adjuvant. Other preferred adjuvant combinations include combinations of Th1 and Th2 adjuvants such as CpG & alum or resiquimod & alum. A combination of aluminium phosphate and 3dMPL may be used.

The compositions of the invention may elicit both a cell mediated immune response as well as a humoral immune response. This immune response will preferably induce long lasting (*e.g.* neutralising) antibodies and a cell mediated immunity that can quickly respond upon exposure to pseudomonas.

Two types of T cells, CD4 and CD8 cells, are generally thought necessary to initiate and/or enhance cell mediated immunity and humoral immunity. CD8 T cells can express a CD8 co-receptor and are commonly referred to as Cytotoxic T lymphocytes (CTLs). CD8 T cells are able to recognize or interact with antigens displayed on MHC Class I molecules.

CD4 T cells can express a CD4 co-receptor and are commonly referred to as T helper cells. CD4 T cells are able to recognize antigenic peptides bound to MHC class II molecules. Upon interaction with a MHC class II molecule, the CD4 cells can secrete factors such as cytokines. These secreted cytokines can activate B cells, cytotoxic T cells, macrophages, and other cells that participate in an immune response. Helper T cells or CD4+ cells can be further divided into two functionally distinct subsets: TH1 phenotype and TH2 phenotypes which differ in their cytokine and effector function.

Activated TH1 cells enhance cellular immunity (including an increase in antigen-specific CTL production) and are therefore of particular value in responding to intracellular infections. Activated TH1 cells may secrete one or more of IL-2, IFN- γ , and TNF- β . A TH1 immune response may result in local inflammatory reactions by activating macrophages, NK (natural killer) cells, and CD8 cytotoxic T cells (CTLs). A TH1 immune response may also act to expand the immune response by stimulating growth of B and T cells with IL-12. TH1 stimulated B cells may secrete IgG2a.

Activated TH2 cells enhance antibody production and are therefore of value in responding to extracellular infections. Activated TH2 cells may secrete one or more of IL-4, IL-5, IL-6, and IL-10. A TH2 immune response may result in the production of IgG1, IgE, IgA and memory B cells for future protection.

An enhanced immune response may include one or more of an enhanced TH1 immune response and a TH2 immune response.

A TH1 immune response may include one or more of an increase in CTLs, an increase in one or more of the cytokines associated with a TH1 immune response (such as IL-2, IFN- γ , and TNF- β), an increase in activated macrophages, an increase in NK activity, or an increase in the production of IgG2a. Preferably, the enhanced TH1 immune response will include an increase in IgG2a production.

A TH1 immune response may be elicited using a TH1 adjuvant. A TH1 adjuvant will generally elicit increased levels of IgG2a production relative to immunization of the antigen without adjuvant. TH1

adjuvants suitable for use in the invention may include for example saponin formulations, virosomes and virus like particles, non-toxic derivatives of enterobacterial lipopolysaccharide (LPS), immunostimulatory oligonucleotides. Immunostimulatory oligonucleotides, such as oligonucleotides containing a CpG motif, are preferred TH1 adjuvants for use in the invention.

- 5 A TH2 immune response may include one or more of an increase in one or more of the cytokines associated with a TH2 immune response (such as IL-4, IL-5, IL-6 and IL-10), or an increase in the production of IgG1, IgE, IgA and memory B cells. Preferably, the enhanced TH2 immune response will include an increase in IgG1 production.

- 10 A TH2 immune response may be elicited using a TH2 adjuvant. A TH2 adjuvant will generally elicit increased levels of IgG1 production relative to immunization of the antigen without adjuvant. TH2 adjuvants suitable for use in the invention include, for example, mineral containing compositions, oil-emulsions, and ADP-ribosylating toxins and detoxified derivatives thereof. Mineral containing compositions, such as aluminium salts are preferred TH2 adjuvants for use in the invention.

- 15 Preferably, the invention includes a composition comprising a combination of a TH1 adjuvant and a TH2 adjuvant. Preferably, such a composition elicits an enhanced TH1 and an enhanced TH2 response, i.e., an increase in the production of both IgG1 and IgG2a production relative to immunization without an adjuvant. Still more preferably, the composition comprising a combination of a TH1 and a TH2 adjuvant elicits an increased TH1 and/or an increased TH2 immune response relative to immunization with a single adjuvant (*i.e.*, relative to immunization with a TH1 adjuvant alone or immunization with a TH2 adjuvant alone).

The immune response may be one or both of a TH1 immune response and a TH2 response. Preferably, immune response provides for one or both of an enhanced TH1 response and an enhanced TH2 response.

- 25 The enhanced immune response may be one or both of a systemic and a mucosal immune response. Preferably, the immune response provides for one or both of an enhanced systemic and an enhanced mucosal immune response. Preferably the mucosal immune response is a TH2 immune response. Preferably, the mucosal immune response includes an increase in the production of IgA.

- 30 *P. aeruginosa* infections can affect various areas of the body and so the compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared (*e.g.* a lyophilised composition or a spray-freeze dried composition). The composition may be prepared for topical administration *e.g.* as an ointment, cream or powder. The composition may be prepared for oral administration *e.g.* as a tablet or capsule, as a spray, or as a syrup (optionally flavoured). The composition may be prepared for pulmonary administration *e.g.* as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary.
- 35 The composition may be prepared for nasal, aural or ocular administration *e.g.* as drops. The composition may be in kit form, designed such that a combined composition is reconstituted just prior to

administration to a patient. Such kits may comprise one or more antigens in liquid form and one or more lyophilised antigens.

Where a composition is to be prepared extemporaneously prior to use (*e.g.* where a component is presented in lyophilised form) and is presented as a kit, the kit may comprise two vials, or it may comprise one ready-filled syringe and one vial, with the contents of the syringe being used to reactivate the contents of the vial prior to injection.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of antigen(s), as well as any other components, as needed. By 'immunologically effective amount', it is meant 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. 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 synthesise 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. Where more than one antigen is included in a composition then two antigens may be present at the same dose as each other or at different doses.

As mentioned above, a composition may include a temperature protective agent, and this component may be particularly useful in adjuvanted compositions (particularly those containing a mineral adjuvant, such as an aluminium salt). As described in reference 136, a liquid temperature protective agent may be added to an aqueous vaccine composition to lower its freezing point *e.g.* to reduce the freezing point to below 0°C. Thus the composition can be stored below 0°C, but above its freezing point, to inhibit thermal breakdown. The temperature protective agent also permits freezing of the composition while protecting mineral salt adjuvants against agglomeration or sedimentation after freezing and thawing, and may also protect the composition at elevated temperatures *e.g.* above 40°C. A starting aqueous vaccine and the liquid temperature protective agent may be mixed such that the liquid temperature protective agent forms from 1-80% by volume of the final mixture. Suitable temperature protective agents should be safe for human administration, readily miscible/soluble in water, and should not damage other components (*e.g.* antigen and adjuvant) in the composition. Examples include glycerin, propylene glycol, and/or polyethylene glycol (PEG). Suitable PEGs may have an average molecular weight ranging from 200-20,000 Da. In a preferred embodiment, the polyethylene glycol can have an average molecular weight of about 300 Da ('PEG-300').

The invention provides an immunogenic composition comprising: (i) one or more antigen(s) selected from the first, second, third or fourth antigen groups; and (ii) a temperature protective agent. This composition may be formed by mixing (i) an aqueous composition comprising one or more antigen(s) selected from the first, second, third or fourth antigen groups, with (ii) a temperature protective agent. The mixture may then be stored *e.g.* below 0°C, from 0-20°C, from 20-35°C, from 35-55°C, or higher. It may be stored in liquid or frozen form. The mixture may be lyophilised. The composition may alternatively be formed by mixing (i) a dried composition comprising one or more antigen(s) selected

from the first, second, third or fourth antigen groups, with (ii) a liquid composition comprising the temperature protective agent. Thus component (ii) can be used to reconstitute component (i).

Methods of treatment, and administration of the vaccine

The invention also provides a method for raising an immune response in a mammal comprising the step of administering an effective amount of a composition of the invention. The immune response is preferably protective and preferably involves antibodies and/or cell-mediated immunity. The method may raise a booster response.

The invention also provides at least two antigens of the invention for combined use as a medicament *e.g.* for use in raising an immune response in a mammal.

- 10 The invention also provides the use of at least two antigens of the invention in the manufacture of a medicament for raising an immune response in a mammal.

By raising an immune response in the mammal by these uses and methods, the mammal can be protected against *P. aeruginosa* infection, including a nosocomial infection. More particularly, the mammal may be protected against a skin infection, including those of burns, trauma wounds and the eyes as shown in reference 137. pneumonia, meningitis and neonatal meningitis, osteomyelitis endocarditis, pseudomonas folliculitis, toxic shock syndrome, and/or septicaemia and cystic fibrosis.

The invention also provides a kit comprising a first component and a second component wherein neither the first component nor the second component is a composition of the invention as described above, but wherein the first component and the second component can be combined to provide a composition of the invention as described above. The kit may further include a third component comprising one or more of the following: instructions, syringe or other delivery device, adjuvant, or pharmaceutically acceptable formulating solution.

The invention also provides a delivery device pre-filled with an immunogenic composition of the invention.

- 25 The mammal is preferably a human. Where the vaccine is for prophylactic use, the human is preferably a child (*e.g.* a toddler or infant) or a teenager; where the vaccine is for therapeutic use, the human is preferably a teenager or an adult. A vaccine intended for children may also be administered to adults *e.g.* to assess safety, dosage, immunogenicity, *etc.* Other mammals which can usefully be immunised according to the invention are cows, dogs, horses, and pigs.

- 30 One way of checking efficacy of therapeutic treatment involves monitoring *P. aeruginosa* infection after administration of the compositions of the invention. One way of checking efficacy of prophylactic treatment involves monitoring immune responses, systemically (such as monitoring the level of IgG1 and IgG2a production) and/or mucosally (such as monitoring the level of IgA production), against the antigens in the compositions of the invention after administration of the composition. Typically, antigen-specific serum antibody responses are determined post-immunisation but pre-challenge whereas antigen-specific mucosal antibody responses are determined post-immunisation and post-challenge.
- 35

Another way of assessing the immunogenicity of the compositions of the present invention is to express the proteins recombinantly for screening patient sera or mucosal secretions by immunoblot and/or microarrays. A positive reaction between the protein and the patient sample indicates that the patient has mounted an immune response to the protein in question. This method may also be used to identify immunodominant antigens and/or epitopes within antigens.

The efficacy of vaccine compositions can also be determined *in vivo* by challenging animal models of *P. aeruginosa* infection, *e.g.*, guinea pigs or mice, with the vaccine compositions. In particular, there one useful animal model for the study of *P. aeruginosa* infectious disease, described in details in the chapter entitled “efficacy testing” The lethal infection model looks at the number of mice which survive after being infected by a normally-lethal dose of *P. aeruginosa* via intra-tracheal route. Different antigens, and different antigen combinations, may contribute to different aspects of an effective vaccine.

Compositions of the invention will generally be administered directly to a patient. Direct delivery may be accomplished by parenteral injection (*e.g.* subcutaneously, intraperitoneally, intravenously, intramuscularly, or to the interstitial space of a tissue), or mucosally, such as by rectal, oral (*e.g.* tablet, spray), vaginal, topical, transdermal or transcutaneous, intranasal, ocular, aural, pulmonary or other mucosal administration.

The invention may be used to elicit systemic and/or mucosal immunity, preferably to elicit an enhanced systemic and/or mucosal immunity.

Preferably the enhanced systemic and/or mucosal immunity is reflected in an enhanced TH1 and/or TH2 immune response. Preferably, the enhanced immune response includes an increase in the production of IgG1 and/or IgG2a and/or IgA.

Th17 cells are a recently described lineage of helper T cells that can enhance antibacterial mucosal defenses and can potentially mediate protective vaccine-induced response. See reference 138

Dosage can be by a single dose schedule or a multiple dose schedule. Multiple doses may be used in a primary immunisation schedule and/or in a booster immunisation schedule. In a multiple dose schedule the various doses may be given by the same or different routes *e.g.* a parenteral prime and mucosal boost, a mucosal prime and parenteral boost, *etc.* Multiple doses will typically be administered at least 1 week apart (*e.g.* about 2 weeks, about 3 weeks, about 4 weeks, about 6 weeks, about 8 weeks, about 10 weeks, about 12 weeks, about 16 weeks, *etc.*).

Vaccines prepared according to the invention may be used to treat both children and adults. Thus a human patient may be less than 1 year old, 1-5 years old, 5-15 years old, 15-55 years old, or at least 55 years old. Preferred patients for receiving the vaccines are the elderly (*e.g.* ≥ 50 years old, ≥ 60 years old, and preferably ≥ 65 years), the young (*e.g.* ≤ 5 years old), hospitalised patients, healthcare workers, armed service and military personnel, pregnant women, the chronically ill, or immunodeficient patients. The vaccines are not suitable solely for these groups, however, and may be used more generally in a population.

Vaccines produced by the invention may be administered to patients at substantially the same time as (e.g. during the same medical consultation or visit to a healthcare professional or vaccination centre) other vaccines e.g. at substantially the same time as an influenza vaccine, a measles vaccine, a mumps vaccine, a rubella vaccine, a MMR vaccine, a varicella vaccine, a MMRV vaccine, a diphtheria vaccine, a tetanus vaccine, a pertussis vaccine, a DTP vaccine, a conjugated *H.influenzae* type b vaccine, an inactivated poliovirus vaccine, a hepatitis B virus vaccine, a meningococcal conjugate vaccine (such as a tetravalent A-C-W135-Y vaccine), a respiratory syncytial virus vaccine, etc. Further non-pseudomonas vaccines suitable for co-administration may include one or more antigens.

Nucleic acid immunisation

The immunogenic compositions described above include polypeptide antigens from *P. aeruginosa*. In all cases, however, the polypeptide antigens can be replaced by nucleic acids (typically DNA) encoding those polypeptides, to give compositions, methods and uses based on nucleic acid immunisation. Nucleic acid immunisation is now a developed field.

The nucleic acid encoding the immunogen is expressed *in vivo* after delivery to a patient and the expressed immunogen then stimulates the immune system. The active ingredient will typically take the form of a nucleic acid vector comprising: (i) a promoter; (ii) a sequence encoding the immunogen, operably linked to the promoter; and optionally (iii) a selectable marker. Preferred vectors may further comprise (iv) an origin of replication; and (v) a transcription terminator downstream of and operably linked to (ii). In general, (i) & (v) will be eukaryotic and (iii) & (iv) will be prokaryotic.

Preferred promoters are viral promoters e.g. from cytomegalovirus (CMV). The vector may also include transcriptional regulatory sequences (e.g. enhancers) in addition to the promoter and which interact functionally with the promoter. Preferred vectors include the immediate-early CMV enhancer/promoter, and more preferred vectors also include CMV intron A. The promoter is operably linked to a downstream sequence encoding an immunogen, such that expression of the immunogen-encoding sequence is under the promoter's control.

Where a marker is used, it preferably functions in a microbial host (e.g. in a prokaryote, in a bacteria, in a yeast). The marker is preferably a prokaryotic selectable marker (e.g. transcribed under the control of a prokaryotic promoter). For convenience, typical markers are antibiotic resistance genes.

The vector of the invention is preferably an autonomously replicating episomal or extrachromosomal vector, such as a plasmid.

The vector of the invention preferably comprises an origin of replication. It is preferred that the origin of replication is active in prokaryotes but not in eukaryotes.

Preferred vectors thus include a prokaryotic marker for selection of the vector, a prokaryotic origin of replication, but a eukaryotic promoter for driving transcription of the immunogen-encoding sequence.

The vectors will therefore (a) be amplified and selected in prokaryotic hosts without polypeptide expression, but (b) be expressed in eukaryotic hosts without being amplified. This arrangement is ideal for nucleic acid immunization vectors.

The vector of the invention may comprise a eukaryotic transcriptional terminator sequence downstream of the coding sequence. This can enhance transcription levels. Where the coding sequence does not have its own, the vector of the invention preferably comprises a polyadenylation sequence. A preferred polyadenylation sequence is from bovine growth hormone.

- 5 The vector of the invention may comprise a multiple cloning site

In addition to sequences encoding the immunogen and a marker, the vector may comprise a second eukaryotic coding sequence. The vector may also comprise an IRES upstream of said second sequence in order to permit translation of a second eukaryotic polypeptide from the same transcript as the immunogen. Alternatively, the immunogen-coding sequence may be downstream of an IRES.

- 10 The vector of the invention may comprise unmethylated CpG motifs *e.g.* unmethylated DNA sequences which have in common a cytosine preceding a guanosine, flanked by two 5' purines and two 3' pyrimidines. In their unmethylated form these DNA motifs have been demonstrated to be potent stimulators of several types of immune cell.

- Vectors may be delivered in a targeted way. Receptor-mediated DNA delivery techniques are described in the known art. Therapeutic compositions containing a nucleic acid are administered in a range of about 100ng to about 200mg of DNA for local administration in a gene therapy protocol. Concentration ranges of about 500 ng to about 50 mg, about 1µg to about 2 mg, about 5µg to about 500µg, and about 20µg to about 100µg of DNA can also be used during a gene therapy protocol. Factors such as method of action (*e.g.* for enhancing or inhibiting levels of the encoded gene product) and efficacy of transformation and expression are considerations which will affect the dosage required for ultimate efficacy. Where greater expression is desired over a larger area of tissue, larger amounts of vector or the same amounts re-administered in a successive protocol of administrations, or several administrations to different adjacent or close tissue portions may be required to effect a positive therapeutic outcome. In all cases, routine experimentation in clinical trials will determine specific ranges for optimal therapeutic effect.

- 25 Vectors can be delivered using gene delivery vehicles. The gene delivery vehicle can be of viral or non-viral origin (see generally reference 139).

- Viral-based vectors for delivery of a desired nucleic acid and expression in a desired cell are well known in the art. Exemplary viral-based vehicles include, but are not limited to, recombinant retroviruses (*e.g.* references 140 to), alphavirus-based vectors (*e.g.* Sindbis virus vectors, Semliki forest virus (ATCC VR-67; ATCC VR-1247), Ross River virus (ATCC VR-373; ATCC VR-1246) and Venezuelan equine encephalitis virus (ATCC VR-923; ATCC VR-1250; ATCC VR 1249; ATCC VR-532); hybrids or chimeras of these viruses may also be used), poxvirus vectors (*e.g.* vaccinia, fowlpox, canarypox, modified vaccinia Ankara, *etc.*), adenovirus vectors, and adeno-associated virus (AAV) vectors (*e.g.* see refs. 142 to). Administration of DNA linked to killed adenovirus [144] can also be employed.

- 35 Non-viral delivery vehicles and methods can also be employed, including, but not limited to, polycationic condensed DNA linked or unlinked to killed adenovirus alone [*e.g.* 144], ligand-linked DNA [145], eukaryotic cell delivery vehicles cells [*e.g.* refs. 146 to] and nucleic charge neutralization or fusion with

cell membranes. Naked DNA can also be employed. Exemplary naked DNA introduction methods are described in ref. 148. Liposomes (e.g. immunoliposomes) that can act as gene delivery vehicles are described in refs. 149 to . Additional approaches are described in references 151-152.

Further non-viral delivery suitable for use includes mechanical delivery systems such as the approach described in ref. 152. Moreover, the coding sequence and the product of expression of such can be delivered through deposition of photopolymerized hydrogel materials or use of ionizing radiation (e.g. refs. 153). Other conventional methods for gene delivery that can be used for delivery of the coding sequence include, for example, use of hand-held gene transfer particle gun [154] or use of ionizing radiation for activating transferred genes.

- 10 Delivery DNA using PLG {poly(lactide-co-glycolide)} microparticles is a particularly preferred method e.g. by adsorption to the microparticles, which are optionally treated to have a negatively-charged surface (e.g. treated with SDS) or a positively-charged surface (e.g. treated with a cationic detergent, such as CTAB).

Antibodies

- 15 Antibodies against *P. aeruginosa* antigens can be used for passive immunisation. Thus the invention provides an antibody which is specific for an antigen in the first, second, third or fourth antigen groups. The invention also provides the use of such antibodies in therapy. The invention also provides the use of such antibodies in the manufacture of a medicament. The invention also provides a method for treating a mammal comprising the step of administering an effective amount of an antibody of the invention. As
20 described above for immunogenic compositions, these methods and uses allow a mammal to be protected against *P. aeruginosa* infection.

- The term “antibody” includes intact immunoglobulin molecules, as well as fragments thereof which are capable of binding an antigen. These include hybrid (chimeric) antibody molecules; F(ab')₂ and F(ab) fragments and Fv molecules; non-covalent heterodimers; single-chain Fv molecules (sFv); dimeric and
25 trimeric antibody fragment constructs; minibodies; humanized antibody molecules; and any functional fragments obtained from such molecules, as well as antibodies obtained through non-conventional processes such as phage display. Preferably, the antibodies are monoclonal antibodies. Methods of obtaining monoclonal antibodies are well known in the art. Humanised or fully-human antibodies are preferred.

General

30 The practice of the present invention will employ, unless otherwise indicated, conventional methods of chemistry, biochemistry, molecular biology, immunology and pharmacology, within the skill of the art. Such techniques are explained fully in the literature.

- “GI” numbering is used above. A GI number, or “GenInfo Identifier”, is a series of digits assigned
35 consecutively to each sequence record processed by NCBI when sequences are added to its databases. The GI number bears no resemblance to the accession number of the sequence record. When a sequence

is updated (*e.g.* for correction, or to add more annotation or information) then it receives a new GI number. Thus the sequence associated with a given GI number is never changed. See also reference 37.

Where the invention concerns an “epitope”, this epitope may be a B-cell epitope and/or a T-cell epitope. Such epitopes can be identified empirically (*e.g.* using PEPSCAN [155] or similar methods), or they can be predicted (*e.g.* using the Jameson-Wolf antigenic index [156], matrix-based approaches [157], MAPITOPE [158], TEPITOPE [159], OptiMer & EpiMer [160], ADEPT [161], Tsites, hydrophilicity, antigenic index or the methods known in the art. Epitopes are the parts of an antigen that are recognised by and bind to the antigen binding sites of antibodies or T-cell receptors, and they may also be referred to as “antigenic determinants”.

10 Where an antigen “domain” is omitted, this may involve omission of a signal peptide, of a cytoplasmic domain, of a transmembrane domain, of an extracellular domain, *etc.*

The term “comprising” encompasses “including” as well as “consisting” *e.g.* a composition “comprising” X may consist exclusively of X or may include something additional *e.g.* X + Y.

The term “about” in relation to a numerical value *x* is optional and means, for example, $x \pm 10\%$.

15 References to a percentage sequence identity between two amino acid sequences means that, when aligned, that percentage of amino acids are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in section 7.7.18 of ref. 162. A preferred alignment is determined by the Smith-Waterman homology search algorithm using an affine gap search with a gap open penalty of 12
20 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is disclosed in ref. 163.

MODES FOR CARRYING OUT THE INVENTION

Antigen selection

25 *P. aeruginosa* proteins have been selected for use as vaccine components based on the combination of various criteria which include the following ones:

- Cellular localization prediction, through which priority was attributed to proteins predicted as “outer membrane”, “periplasmic”, extracellular” and “unknown”. In relation to the latest definition proteins predicted as having an “unknown” cellular localization which are often composed of multiple domains, of which one could actually be surface exposed.
- 30 • Significant homology to known virulence factors, vaccine candidates from other species
- Lack of significant homology to human proteins encoded by the sequenced human genome, in order to limit the probability of generation of autoimmune response or vaccine induced autoimmunity.

- Lack of significant homology to *E. coli* proteins, considering that proteins having counterparts in many bacterial species, either pathogenic or non-pathogenic have higher probability to have house-keeping functions and therefore are less likely to be good antigens
- Conservation over a panel of at least 5 out of 7 fully sequenced *P. aeruginosa* genomes.
- 5 • Useful aminoacid sequence length which is considered to be of at least 150 aa
- Microarray data. *In vitro* expression of *P. aeruginosa* PAO1 derived proteins repertoire was tested to analyse changes in gene expression under anaerobic conditions as those found in the mucus of CF (cystic fibrosis) patients compared with aerobic conditions found in the environment. Priority was assigned to proteins whose expression was maintained in both aerobic and anaerobic cell culture conditions.

The protein can also adsorb reasonably well to aluminium hydroxide (see also below), which is useful for stable formulation for delivery to humans.

Strain coverage

In order to evaluate the conservation of the antigens selected, various *P. aeruginosa* clinical isolates were used. *P. aeruginosa* clinical strains were isolated from eight pancreatic-insufficient CF patients attending the CF clinic of the Medizinische Hochschule Hannover. *P. aeruginosa* strains from the first positive cultures are designated as “early” isolates, whereas intermediate isolates were collected 1 to 5 years thereafter and late isolates were collected 7 to 16 years after colonization or prior to death or lung transplantation. Strains tested are listed in the following Table.

20 ***Table on strain coverage***

Strain Genotype	Years of infection
SG1	0
SG57	15,8
SG58	15,8
BT2	0
BT72	15,8
BT73	16,3
AA2	0,5
AA43°	7,5
AA44°	7,5
TR1	0
TR66	12,8
TR67°	13,5
MF1	0
MF51°	10,1
KK1	0
KK71°	12,6
KK72°	12,6
BST2	0,9
BST44	15,8

The symbol ° indicates the last *P. aeruginosa* strain prior to death or lung transplantation. Genes encoding PSE54, PSE44-4, PSE10-1, PSE21-5, PSE27-1, PSE52-1, PSE53-1, PSE11, PSE41, PSE47-2, were present in all tested strains as confirmed by PCR (polymerase chain reaction).

Thus, considering the vaccine efficacy in terms of a broader cross-strain protection a vaccine based on any of the best combinations/cocktails as tested in table 2, can be a valid solution in order to extend vaccine coverage against pseudomonas derived infections.

Cloning and expression of *P. aeruginosa* recombinant proteins

5 Cloning and expression of antigens can be performed by standard methods.

Polypeptides antigens from PA strain PAO1 were PCR-amplified using specific oligonucleotides and PA chromosomal DNA as template. Resulting PCR products were cloned in pET15b (Novagen) using the PIPE method [164], consisting in the PCR amplification of the cloning vector (V-PCR) and in the PCR amplification of the insert (I-PCR). Then, 1 µl of V-PCR and 1 µl of I-PCR are mixed and transformed in
10 chemically competent HK100 cells [165]. I-PCR reactions were set up containing 1 µM each of the forward and reverse primers, 1x Cloned Pfu DNA Polymerase Reaction Buffer, 2.5 units of Pfu Turbo DNA polymerase (Stratagene), 200 µM of each dNTP (Invitrogen) and 50 ng of genomic DNA template. The reactions were conducted as follows: initial denaturation for 2 min at 95 °C, then 25 cycles of 95 °C for 30 s, 55 °C for 45 s, and 68 °C for 3 min followed by a final cool down to 4 °C. V-PCR reactions
15 were identical to the I-PCR reactions but the steps at 68 °C were lasting 14 min and 2 ng of pET15b plasmid were used as DNA template. Correct transformants were selected by PCR screening and DNA plasmid sequencing of the vector-insert junctions. The correct plasmid were then prepared from selected HK100 clones and used to transform BL21(DE3)T1^r cells (Sigma) in order to allow protein expression.

To express cloned proteins, BL21(DE3)T1^r clones containing pET15b constructs were grown in LB
20 medium containing 100 µg/ml Ampicillin at 37 °C until OD₆₀₀= 0.5. Protein expression was then induced by adding 1 mM IPTG and growing at the same temperature for additional 3 hrs. Conventional protein extractions and SDS-Page were performed to check protein expression. Western blot techniques known in the art were used to confirm proper expression of tested *P. aeruginosa* antigens. Specific antisera from immunized mice were used confirm protein expression. Immunofluorescence techniques known in the art
25 were used to confirm surface localization of tested *P. aeruginosa* antigens using anti-cell wall antibodies as co-localizator and/or a specific anti-antigen serum obtained after mice immunization.

Adjuvant formulation

Selected *P. aeruginosa* protein antigen candidates have been formulated with aluminium hydroxide, either individually or as a combination of proteins. The formulations have been optimized for pH and
30 osmolality.

The antigens were formulated as monovalent antigen or multivalent antigens combinations in Aluminium Hydroxide. Each antigen was used at 10 µg/formulation/animal. Aluminium hydroxide was used at 2 mg/ml final concentration, in a 10mM histidine buffer (pH 6.5). Sodium chloride was used to adjust osmolality to physiologic conditions. Formulations were given intratracheal in a final vaccine
35 composition volume of 200 µl/animal.

All monovalent and combination formulations, could be adjusted with respect to a desired pH and osmolality. The formulations had pH in the range 6.2-7.3, and osmolality in the range 248-360 mOsm/kg.

Most of the proteins tested, in various monovalent and combination formulations, adsorbed well to the aluminium hydroxide adjuvant.

The individual PSE54, PSE10-1, PSE21-5, PSE27-1, PSE44-4, PSE52-1, PSE53-1 proteins were completely adsorbed, and could be desorbed without altering their pre-adsorption electrophoretic profile.

- 5 Each antigen in a combination of was completely adsorbed, with no inter-antigen competition for the adjuvant. The antigens in a combination of PSE25+PSE54, PSE27-1+PSE44, PSE38-1+PSE11-3, PSE38-1+PSE11-3, PSE41-5+PSE47A-2, PSE41-5+PSE53-1, PSE47A-2+PSE53-1, PSE47A-2+PSE52-1, were also completely adsorbed.

- 10 All tested formulations were stable for their pH and osmolality. All antigens remained completely adsorbed to the adjuvant. All antigens maintained their desorption characteristics. There was no evidence of increased degradation or aggregation of antigens after desorption.

Efficacy testing

Individual antigens as listed in Table 2 were tested for their ability to protect against intra-tracheal (IT) lethal infection challenge by 5×10^6 cfu of planktonic PAO1 strain. Results are shown in Figure 1.

- 15 Recombinant proteins were used to immunize mice for protection studies against *P. aeruginosa*, using as reference strain PAO1. Groups of 10 mice (C57BL/6NCrlBR male 5 weeks old, Charles River Laboratories, Italy) were immunized at day 0, 21 and 35 with different antigens and at day 50 challenged with the homologous *P. aeruginosa* PAO1 referent strain by acute infection. In each boost every mouse received 10 µg or 20 µg of recombinant protein/s adsorbed with alum alone or with 10^7 cfu of heat inactivated PAO1. To obtain antisera mice of all groups were bleeding at day -1, day 34, and day 49. As negative control 10 mice per immunization round were injected with alum alone, while as positive control 10 mice per immunization round were boosted with 10^7 cfu of heat inactivated PAO1 strain. On day 50, mice were infected with 5×10^6 cfu (first lethal dose) of planktonic *P. aeruginosa* PAO1 via intra-tracheal (IT) route. Mice were anesthetized and the trachea directly visualized by a ventral midline incision, exposed and intubated with a sterile, flexible 22-g cannula attached to a 1 ml syringe. A 60 µl inoculum of planktonic bacteria were implanted via the cannula into the lung. Mice were monitored for survival for 120 hrs at intervals of twelve hours and compared with un-vaccinated and PAO1 vaccinated control groups.

- 30 Antigens showed to be able to give an incremental shift in the survival curves compared with the control were listed in table 2, and the best results were seen with PSE21-5, PSE47A-2, PSE52-1, PSE53-1 or PSE54, PSE10, PSE 11-3, PSE 27-1, PSE44-4 and PSE 41-5.

Further, individual antigens were tested in combination as reported in Table 2.

- 35 Table 2 gives a summary of results obtained with various antigens used alone or in combinations *in vivo* in the animal mouse model. Survival data are shown in Table 2. In the statistical significance column, the p value in Mantel-Cox test is calculated against negative control group. Survival curve of each protein was compared with the survival curve of the negative control of the same round in which the protein was

tested. Survival was measured for 120 hrs at intervals of twelve hours and compared with un-vaccinated and PAO1 vaccinated control groups. Percentage of mice survival after the 36 hours was evidence of positive immunization results *in vivo*.

Among the different rounds considering the 30 tested recombinant proteins (Table 2), three proteins (PSE10-1 (PA1178), PSE47A-2 (PA4082), and PSE52-1 (PA4765) had also a highly statistical significance different survival curves when compared with the negative control group (p value in Mantel-Cox test against negative control group 0,0261, 0,0364 and 0,0275 respectively).

On the basis of the analysis of the survival curves of seven additional recombinant proteins it can be predicted that results may also be significantly corroborated by increasing the numbers of animal tested *in vivo* further confirming the preliminary positive and surprising data of PSE11-3 (PA1248), PSE41-5 (PA2407), PSE44-4 (PA3526), PSE53-1 (PA5047), PSE21-5 (PA5112), PSE-54 (PA5340), PSE-27-1 (PA0328).

In addition when a known antigen, namely OprF-OprI, was used alone to immunize mice in the present animal model a 20% of survival, with a statistical significance of 0,0446 was obtained.

In order to further improve the survival in this *in vivo* model, specific combinations were also tested by combining together the most promising proteins in order to further increase vaccine efficacy.

Comparison of Combinations versus its individual polypeptides

Combinations alias known as cocktails of antigens in a single formulation were also used to immunise mice. The combinations were typically adjuvanted with aluminium hydroxide (see chapter “Adjuvant Formulation”) and were administered on days 0, 21 and 35. The immunisations were in C57BL/6NCrIBR male 5 weeks old mice, 10 per group. On day 50 the mice were challenged with a lethal dose of heat inactivated bacteria and survival was then followed for 120 hrs. For comparison, PBS was used as a negative control and PAO1 heat-inactivated as a positive control.

The increase in survival, during monitoring for 120 hrs after the immunization schedule and further infection with lethal dose of the *Pseudomonas* homologous strain, when compared to the negative control group, was surprisingly showing best result when the following combinations were tested (Table 2): PSE47A-2+PSE53-1, PSE47A-2+PSE52-1, PSE54+PSE44-4 and PSE54+PSE21-5 being the most promising; whereas PSE47A-2+PSE52-1 combination was less promising.

Various tests were performed to compare various combinations to its seven individual polypeptides (i.e PSE54 or PSE21-5 or PSE27-1, PSE44-4, PSE52-1, PSE53-1, PSE47A-2), as well as OprF/I as further positive control or to an antigen-free negative control.

Mice were immunized with cocktails of two different proteins. Eight cocktails had statistically different survival curves when compared to the negative control group (considering also the PSE54+OprF-OprI composition).

In the sixth round, different cocktails of proteins combined with the PSE47A-2 were tested: PSE47A-2+PSE52-1 and PSE47A-2+PSE53-1 were showing a surprising statistical difference respect to the

negative control group (p value in Mantel-Cox test against negative control group 0,0374 and 0,0373 respectively).

In particular, vaccination with the cocktail PSE47A-2+PSE53-1 resulted surprisingly in 30% of survival, maintain a good statistical significance.

- 5 In the tenth round, different cocktails of proteins combined with the PSE54 were tested. Five cocktails out of eight gave a significantly different mortality curve when compared with negative control group as reported in the Table 2. In particular, vaccination with the cocktails of PSE54+PSE44-4 and PSE54+PSE52-1 antigens gave a very good animal protection resulting in 40% and 33% of animal survival respectively (p value in Mantel-Cox test against negative control group 0,0076 and 0,0142
10 respectively). When re-tested in the eleventh round, the PSE54+PSE44-4 confirmed the positive result showing an increase of animal survival of 57%. Other cocktails showing better vaccine efficacy when used in combination were: PSE54+PSE21-5, PSE54+PSE53-1 and PSE54+PSE10-1 (p value in Mantel-Cox test against negative control group 0,0332, 0,0085 and 0,0025 respectively).

- Furthermore, this last cocktail, PSE54+PSE10-1, had comparable results in two different rounds of
15 immunization corroborating the positive and surprising result, resulting in the same survival rate of animals (20%) in both rounds.

Finally four additional cocktails of different proteins (PSE27-1+PSE44-4, PSE53-1+PSE41-5 and PSE53-1+PSE52-1 and PSE54+PSE27-1) may become even more significant further repeating the experiments in further confirmatory experiments increasing the number of animals.

- 20 In order to even further improve the capacity of protection and ultimately the vaccine efficacy of selected antigens, combination of three proteins were tested. Seven groups were tested maintaining two fixed proteins PSE54+PSE44-4 plus one variable protein, while others groups tested were with PSE47A-2+PSE52-1+PSE53-1, PSE54+PSE52-1+PSE53-1 and PSE54+PSE53-1+PSE 27-1. Some of these combination did not gave significant animal protection when compared with negative control group
25 whereas when considering e.g. the PSE54+PSE44-4+PSE47A-2 or PSE54+PSE53-1+PSE 27-1 they may provide significant protection simply through confirmatory experiments, obtained by increasing the number of animals (See table 2). Thus, some of the combination of three proteins did not improve protection rather it seemed worse when considering two proteins.

- However, when considering a further combination adding in the cocktail of antigens also the positive
30 control OprF-OprI fusion was included in the further combinations.

- When OprF-OprI was used as single fusion protein antigen it showed 20% of survival with a statistical significance of 0.0446, similarly to other single immunization with single promising antigens, whereas when used in combination with PSE54 surprisingly the survival percentage increased to 50% with a similar statistical significance. In addition, the immunization with the following antigens in combination
35 PSE54+PSE27+OprF-OprI showed surprisingly even 60% of survival with a comparable statistical significance, while considering the other combination, namely PSE54+PSE53+OprF-OprI did not show any additive effect or significant immunogenic vaccine efficacy than the single antigens immunization,

even in one single immunization using a decrease amount of each single antigen (Table 2). It is reasonable to expect that by increasing the number of animal tested also this specific combination might provide significative increase in immunological protection.

5 It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

TABLE 1: NOMENCLATURE CROSS-REFERENCE

SEQ ID NOs	Locus Tag PAO1 strain	Internal Name	NCBI definitions
1	PA0328	PSE27	outer membrane autotransporter
2	PA1178	PSE10	PhoP/Q low Mg ²⁺ inducible outer membrane protein H1 precursor
3	PA5112	PSE21	esterase EstA
4	PA1248	PSE11	alkaline protease secretion outer membrane protein AprF precursor
5	PA2407	PSE41-5	putative adhesion protein
6	PA3526	PSE44	outer membrane protein precursor
7	PA4082	PSE47	adhesive protein CupB5
8	PA4765	PSE52	Outer membrane lipoprotein OmlA precursor
9	PA5047	PSE53	lipoprotein, putative; peptidase
10	PA5340	PSE54	lipoprotein, putative
11	PA0595	PSE5	OstA precursor
12	PA1954	PSE13	hypothetical protein PA1954
13	PA3692	PSE17	Lipotoxin F
14	PA4370	PSE18	Metalloproteinase outer membrane protein precursor
15	PA4710	PSE19	receptor PhuR precursor
16	PA4735	PSE20	hypothetical protein PA4735
17	PA3647	PSE23	hypothetical protein PA3647
18	PA0126	PSE24	hypothetical protein PA0126
19	PA0189	PSE25	putative porin
20	PA0274	PSE26	hypothetical protein PA0274
21	PA0537	PSE28	putative lipoprotein
22	PA0737	PSE31	putative lipoprotein
23	PA1086	PSE33	flagellar hook-associated FlgK
24	PA1106	PSE34	hypothetical protein PA1106
25	PA1324	PSE36	putative lipoprotein
26	PA1777	PSE38	outer membrane OprF precursor
27	PA2793	PSE42	putative lipoprotein
28	PA3535	PSE45	putative serine protease
29	PA4578	PSE50	hypothetical protein PA4578
30	PA4667	PSE51	TPR domain protein
31	PA4525	PilA	type 4 fimbrial precursor PilA
32	Fusion	OprF/I	Fusion protein
33	PA1092	FliC	Flagellar protein
34	PA1094	FliD	Flagellar protein
35	PA1148	ExoA	Exotoxin A
36	PA0328	PSE27	Fragment without N-terminus
37	PA1178	PSE10	Fragment without N-terminus
38	PA5112	PSE21	Fragment without N-terminus
39	PA1248	PSE11	Fragment without N-terminus
40	PA2407	PSE41-5	Fragment without N-terminus
41	PA3526	PSE44	Fragment without N-terminus

42	PA4082	PSE47-A2	without N-term and translocator domain
43	PA4765	PSE52	Fragment without N-terminus
44	PA5047	PSE53	Fragment without N-terminus
45	PA5340	PSE54	Fragment without N-terminus
46	PA0595	PSE5	Fragment without N-terminus
47	PA1954	PSE13	Fragment without N-terminus
48	PA3692	PSE17	Fragment without N-terminus
49	PA4370	PSE18	Fragment without N-terminus
50	PA4710	PSE19	Fragment without N-terminus
51	PA4735	PSE20	Fragment without N-terminus
52	PA3647	PSE23	Fragment without N-terminus
53	PA0126	PSE24	Fragment without N-terminus
54	PA0189	PSE25	Fragment without N-terminus
55	PA0274	PSE26	Fragment without N-terminus
56	PA0537	PSE28	Fragment without N-terminus
57	PA0737	PSE31	Fragment without N-terminus
58	PA1086	PSE33	Fragment without N-terminus
59	PA1106	PSE34	Fragment without N-terminus
60	PA1324	PSE36	Fragment without N-terminus
61	PA1777	PSE38	Fragment without N-terminus
62	PA2793	PSE42	Fragment without N-terminus
63	PA3535	PSE45	Fragment without N-terminus
64	PA4578	PSE50	Fragment without N-terminus
65	PA4667	PSE51	Fragment without N-terminus
66	Histidine-Tag	N.A.	N.A.
67	Linker	N.A.	N.A.
68	Linker	N.A.	N.A.
69	Linker	N.A.	N.A.
70	His6	N.A.	Synthetic 6xHis tag
71	Glyn	N.A.	Synthetic peptide encompassing 2 to 10 residues, wherein some positions may be absent
72	His _n	N.A.	Synthetic peptide His _n where $n = 3, 4, 5, 6, 7, 8, 9, 10$ or more
73	(Gly) ₄	N.A.	Synthetic peptide

TABLE 2: MOUSE ANIMAL MODEL RESULTS SUMMARY

Ags	Round	ug Ags	Statistical significance [£]	Survival %
PSE5	11	10	0.10	0 (0/5)
PSE10	1	10	0.026	0 (0/9)
PSE10-1	8	20	0.56	0 (0/10)
PSE11-3	2	10	0.47*	14 (1/7)
	7	20	0.28*	22 (2/9)
PSE13	4	10	0.55	0 (0/10)
PSE17	11	10	0.35	0 (0/8)
PSE18-2	1	10	0.73	0 (0/8)
PSE19-1	4	10	0.17	0 (0/6)
PSE20	11	10	0.91	12.5 (1/8)
PSE21-5	2	10	0.08*	0 (0/10)
PSE21	11	10	0.19*	50 (5/10)
PSE23-1	1	10	0.61	0 (0/9)
PSE24-1	1	10	0.19	0 (0/8)
PSE25-1	4	10	0.10	0 (0/10)
	8	20	0.90	0 (0/10)
PSE26	3	10	0.32	0 (0/9)
PSE27-1	2	10	0.91	0 (0/10)
	4	10	0.21*	0 (0/10)
	7	20	0.80	11 (1/9)
PSE28-2	2	10	0.37	0 (0/10)
PSE31-2	2	10	1	0 (0/10)
	11	10	0.76	10 (1/10)
PSE33	11	10	0.61	0 (0/9)
PSE34	1	10	0.17	0 (0/9)
PSE36-3	1	10	1	0 (0/10)
PSE38-1	2	10	0.53	22 (2/9)
PSE41-5	1	10	0.34*	0 (0/10)
	6	20	0.25	0 (0/9)
	7	20	0.65*	0 (0/10)
PSE42-1	4	10	0.55	13 (1/8)
PSE44-4	2	10	0.08*	0 (0/9)
	7	20	0.28*	22 (2/9)
PSE45-2	4	10	0.32	0 (0/8)
PSE47-3	4	10	0.06	0 (0/5)
PSE47A-2	2	10	0.0364	10 (1/10)
	4	10	0.55	0 (0/10)

	6	20	0.26	20 (2/10)
	9	20	0.27	20 (2/10)
PSE50	3	10	0.96	0 (0/9)
PSE51	3	10	1	0 (0/8)
PSE52	3	10	0.0275	0 (0/9)
PSE52-1	6	20	0.0208	10 (1/10)
PSE53	3	10	0.07*	0 (0/9)
PSE53-1	6	20	0.22*	0 (0/8)
	7	20	0.25*	22 (2/9)
PSE54	3	10	0.17*	10 (1/10)
	8	20	0.88	0 (0/10)
	9	20	0.16*	0 (0/8)
	14	10	0.19*	60 (6/10)
PSE25+PSE54	8	10+10	0.49	0 (0/10)
PSE27-1+PSE44	7	10+10	0.38*	0 (0/10)
PSE38-1+PSE11-3	9	10+10	0.004 [#]	0 (0/9)
PSE41-5+PSE52-1	6	10+10	0.27	11 (1/9)
PSE41-5+PSE47A-2	6	10+10	0.98	0 (0/4)
PSE41-5+PSE53-1	7	10+10	0.55*	10 (1/10)
PSE47A-2+PSE53-1	6	10+10	0.0373	30 (3/10)
PSE47A-2+PSE53-1	9	10+10	0.09	0 (0/10)
PSE47A-2+PSE52-1	6	10+10	0.0374	0 (0/7)
PSE47A-2+PSE52-1	9	10+10	0.06	0 (0/9)
PAE47A-2+PSE21-5	9	10+10	0.28	11 (1/9)
PSE47A-2+PSE44-4	9	10+10	0.34	0 (0/9)
PSE47A-2+PSE38-1	9	10+10	0.003 [#]	0 (0/10)
PSE47A-2+PSE11-3	9	10+10	0.01 [#]	0 (0/10)
PSE53-1+PSE52-1	6	10+10	0.20*	0 (0/9)
PSE54+PSE10-1	8	10+10	0.0154	20 (2/10)
	10	10+10	0.0025	20 (2/10)
PSE54+PSE47A-2	10	10+10	0.55	10 (1/10)
PSE54+PSE11-3	10	10+10	0.31	10 (1/10)
PSE54+PSE52-1	10	10+10	0.0142	33 (3/9)
	11	10+10	0.96	0 (0/3)
PSE54+PSE53-1	10	10+10	0.0085	12.5 (1/8)
PSE54+ PSE53	13	10+10	0.61	22 (2/9)
PSE54+PSE21-5	7	10+10	0.0332	14 (1/7)
PSE54+PSE21	13	10+10	0.0213	40 (4/10)
	14	10+10	0.13*	70 (7/10)
PSE54+PSE27-1	10	10+10	0.20*	22 (2/9)

PSE54+ PSE27	13	10+10	0.16*	10 (1/10)
	14	10+10	0.052*	80 (8/10)
PSE54+PSE44-4	10	10+10	0.0076	40 (4/10)
	11	10+10	0.14*	57.1 (4/7)
PSE54+OprF-OprI	13	10+10	0.0403	50 (5/10)
PSE10-1+PSE25-1	8	10+10	0.44	0 (0/7)
PSE47A-2+PSE52-1+PSE53-1	9	10+10+10	0.06	0 (0/8)
PSE54+PSE44-4+PSE10-1	12	10+10+10	0.69	0 (0/10)
PSE54+PSE44-4+PSE21-5	12	10+10+10	0.34	0 (0/10)
PSE54+PSE44-4+PSE27	12	10+10+10	0.32	0 (0/10)
PSE54+PSE44-4+PSE52	12	10+10+10	0.61	0 (0/5)
PSE54+PSE44-4+PSE53-1	12	10+10+10	0.25	0 (0/7)
PSE54+PSE44-4+PSE47A-2	12	10+10+10	0.17*	16.6 (1/6)
PSE54+PSE44-4+PSE11	12	10+10+10	0.46	0 (0/10)
PSE54+PSE52-1+PSE53-1	12	10+10+10	0.68	0 (0/4)
PSE54+PSE27+OprF-OprI	13	10+10+10	0.0021	60 (6/10)
PSE54+ PSE53+OprF-OprI	13	10+10+10	0.17*	20 (2/10)
PSE54+PSE53+ PSE27	13	10+10+10	0.08*	20 (2/10)
PSE54+PSE53+ PSE27	13	6.7+6.7+6.7	0.17*	20 (2/10)
OprF-OprI	11	10	0.28	0 (0/8)
	12	10	0.43	10 (1/10)
	13	10	0.0446	20 (2/10)

Legend of table 2:

p value in Mantel-Cox test against negative control group: Survival curve of each protein was compared with the survival curve of the negative control of the same round in which the protein was tested. Survival was measured for 120 hrs at intervals of twelve hours and compared with un-vaccinated and PAO1 vaccinated control groups. Percentage of mice survival after the 36 hours was evidence of positive immunization results *in vivo*.

* t-test significant with increased animal number: antigen vs negative control.

t-test significant: antigen vs. negative control significant but mice immunized with the antigens dead before the negative controls. Nd: not done

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CLAIMS

1. An immunogenic composition comprising a PSE54 (PA5340) antigen and optionally one or more antigens selected from the list of: a PSE44-4 (PA3526) antigen; a PSE10-1 (PA1178) antigen; a PSE21-5 (PA5112) antigen; a PSE27-1 (PA0328) antigen; a PSE52-1 (PA4765) antigen; a PSE53-1 (PA5047) antigen; PSE11-3 (PA1248) antigen; a PSE41-5 (PA2407) antigen; a PSE47A-2 (PA4082); PSE5-1 (PA0595); PSE13-2 (PA1954); PSE17-1 (PA3692); PSE18-2 (PA4370); PSE20-1 (PA4735); PSE23-1 (PA3647); PSE24-1 (PA0126); PSE25-1 (PA0189); PSE26-1 (PA0274); PSE28-2 (PA0537); PSE31-2 (PA0737); PSE33-2 (PA1086); PSE42-1 (PA2793); PSE45-2 (PA3535); PSE50-1 (PA4578); PSE51-4 (PA4667); PSE19-1 (PA4710); PSE34-1 (PA1106); PSE36-3 (PA1324); PSE38-1 (PA1777).
2. The composition of claim 1, comprising a PSE54 (PA5340) antigen in combination with at least one other antigen selected from the list: a PSE44-4 (PA3526) antigen; a PSE10-1 (PA1178) antigen; a PSE21-5 (PA5112) antigen; a PSE27-1 (PA0328) antigen; a PSE52-1 (PA4765) antigen; a PSE53-1 (PA5047) antigen; PSE11-3 (PA1248) antigen; a PSE41 (PA2407) antigen; a PSE47A-2 (PA4082); PSE5-1 (PA0595); PSE13-2 (PA1954); PSE17-1 (PA3692); PSE18-2 (PA4370); PSE20-1 (PA4735); PSE23-1 (PA3647); PSE24-1 (PA0126); PSE25-1 (PA0189); PSE26-1 (PA0274); PSE28-2 (PA0537); PSE31-2 (PA0737); PSE33-2 (PA1086); PSE42-1 (PA2793); PSE45-2 (PA3535); PSE50-1 (PA4578); PSE51-4 (PA4667); PSE19-1 (PA4710); PSE34-1 (PA1106); PSE36-3 (PA1324); PSE38-1 (PA1777).
3. The composition according to claim 2, further comprising one or more antigens selected from the list: PilA, OprF-OprI, FliC, FliD, ExoA.
4. The composition of claim 2, comprising at least one antigen selected from: PSE10-1 (PA1178), PSE52-1 (PA4765), PSE53-1 (PA5047) and PSE21-5 (PA5112).
5. The composition of claim 2, comprising one or more antigens selected from the group consisting of: a PSE10-1 (PA1178) antigen; a PSE44-4 (PA3526) antigen; a PSE52-1 (PA4765) antigen; a PSE53-1 (PA5047) antigen; a PSE21-5 (PA5112) antigen; a PSE27-1 (PA0328) antigen; a PSE47A-2 (PA4082) antigen; or OprF-OprI.
6. The composition of any preceding claim, wherein one or more of said antigens is adsorbed to an aluminium hydroxide adjuvant, and optionally wherein the composition includes a histidine buffer.
7. The composition of any preceding claim, further comprising: one or more conjugates of (i) a *P. aeruginosa* exopolysaccharide and (ii) a carrier protein.
8. An immunogenic composition comprising the composition of any preceding claims, and further comprising one or more of:
 - (A) one or more conjugates of (i) a *S.aureus* exopolysaccharide;

- (B) one or more protein antigens of (i) a *S.aureus* or
 - (C) one or more pathogenic *E. coli* antigen/s
 - (D) one or more pathogenic *B. cenocepacia* antigen/s
9. An immunogenic composition comprising the composition of claim 1 and one or more of (i) a OprF-OprI antigen; (ii) a FliC antigen; (iii) a FliD antigen; and/or (iv) a PilA antigen.
 10. The composition according to claim 1 wherein the one or more antigens is a OprF-OprI antigen.
 11. The composition of any preceding claim, including an adjuvant.
 12. A pharmaceutical composition comprising:
 - (a) a polypeptide comprising an amino acid sequence having 80% or more identity to SEQ ID NO: 10 which can elicit an antibody that recognises SEQ ID NO: 10; or
 - (b) a polypeptide comprising amino acid sequence SEQ ID NO:45; and
 optionally further comprising an amino acid sequence having 80% or more identity to any one of SEQ ID NOs: 1-9, 11-35 or SEQ ID NOs: 36-65.
 13. The immunogenic composition of any one of claims 1-11, for use:
 - (i) in raising an immune response against *Pseudomonas aeruginosa* infections in a mammal; or
 - (ii) as a prophylactic or therapeutic vaccine against nosocomial infections caused by *Pseudomonas aeruginosa*.

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