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(54) Title: *ANTI-ACINETOBACTER BAUMANNII* IMMUNOGENS

(57) Abstract: The present invention relates generally to the field of immunology and, in particular, to agents and compositions suitable for administration to a host, thereby immunizing and generating protection of the host against infection and disease by *A. baumannii*. The agents of the invention are novel anti-*A. baumannii* immunogens, more specifically novel polypeptide fragments of the putative ferric siderophore and putative ferric hydroxamate siderophore receptors of *A. baumannii* capable of conferring protection against an infection caused by *A. baumannii*. The invention also provides specific examples of the agents of the invention, such as specific polypeptides having a defined amino acid sequence. These specific polypeptides prove the concept of the invention.



Anti-*Acinetobacter baumannii* immunogens

Field of the Invention

5 The present invention relates generally to the field of immunology and, in particular, to agents and compositions suitable for administration to a host, thereby immunizing and generating protection of the host against infection and disease by *Acinetobacter baumannii*.

Background art

The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention, and is not admitted to describe or constitute prior art to the present invention.

15 *Acinetobacter baumannii* is an aerobic gram-negative bacillus with increasing importance as a cause of nosocomial and community-acquired infections. The frequency of infections caused by *A. baumannii* has increased dramatically in the last three decades. At the same time, the prevalence of infections caused by *A. baumannii* strains with resistance to multiple antibiotic classes has increased. Due
20 to the increase of infections caused by *A. baumannii*, and the emergence of highly resistant strains, the development of novel treatment and prevention strategies for infection caused by this pathogen is required. *Acinetobacter baumannii* can cause different types of infection depending on the route of entry into the host. The infections caused by *A. baumannii* are mainly pneumonia, bacteremia, urinary
25 tract infections, surgical site infections and meningitis.

Respiratory infections caused by *A. baumannii* are the most common with an increased risk of death. A study conducted in 2003 in the United States reported that 6.9% of cases of pneumonia in patients admitted to the intensive care unit
30 (ICU) were caused by *A. baumannii*, which represented an increase of 72% compared to cases observed in 1986. In other countries cases of pneumonia caused by *A. baumannii* have been reported at a frequency even higher, of 9.6% in a study conducted in 12 Latin American countries, 27% in Turkey and 35% in the India. The mortality rate associated with cases of nosocomial pneumonia
35 caused by *Acinetobacter* is very high, with rates ranging between 35 and 70%.

Treatment of infections caused by *A. baumannii* has been complicated over the last two decades by the emergence of multiresistant and panresistant strains. In fact a study carried out in the U.S. between 1986 and 2003 showed that resistance to commonly used antibiotics has increased dramatically during this period, including an increase in the percentage of isolates that were resistant to ceftazidime (between 24% to 67%), amikacin (between 3% and 20%) and imipenem (between 0% and 20%). Furthermore a study conducted worldwide in 2005 analyzing the antibiotic resistance of *A. baumannii* isolates showed that 34% of the isolates were resistant to ceftazidime, 40% to ciprofloxacin, 30% to levofloxacin, 26% to gentamicin and 22% to cefepime. Thus different studies have demonstrated the ability of *A. baumannii* to rapidly acquire resistance to antibiotics causing serious nosocomial infections. This has resulted in a high prevalence of *A. baumannii* strains resistant to most of the antibiotics commonly used except colistin. However, in recent years it has been observed the emergence of resistant strains including resistance to colistin, so-called panresistant strains.

In summary, the frequency of severe infections caused by *A. baumannii* has increased significantly over the past two decades. In parallel, infections caused by multiresistant and panresistant strains of *A. baumannii* have also increased. Because of this trend, it is necessary to develop new strategies for treatment and prevention of infections caused by this organism. The vaccine described in this patent represents a novel therapeutic approach to reduce morbidity and mortality resulting from infections caused by *A. baumannii*.

Brief description of the Invention

The present invention relates to novel anti-*Acinetobacter baumannii* immunogens, more specifically to novel polypeptide fragments of the putative ferric siderophore and putative ferric hydroximate siderophore receptors of *A. baumannii* capable of conferring protection against an infection caused by *A. baumannii*. The inventors also provide specific examples as proof of this concept.

Therefore, a first aspect of the invention refers to a composition comprising polypeptide sequence SEQ ID No 28 (putative ferric hydroximate siderophore receptor (*A. baumannii* ATCC 17978; accession number YP_001084696)) or a

fragment thereof, wherein the fragments are biologically active fragments, preferably selected from the list consisting of SEQ ID No 12 to SEQ ID No 23 or sequences having at least 85% sequence identity with peptide sequences SEQ ID No 12 to SEQ ID No 23.

5

Preferably the composition of the first aspect of the invention further comprises polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor (*A. baumannii* ATCC 17978; accession number YP_001084684)) or a fragment thereof, wherein the fragments are biologically active fragments, preferably
10 selected from the list consisting of SEQ ID No 1 to SEQ ID No 11 or sequences having at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11.

15

More preferably the composition of the first aspect of the invention comprises polypeptide sequence SEQ ID No 28 (putative ferric hydroximate siderophore receptor) and polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor).

20

A second aspect of the invention, refers to a composition comprising polypeptide sequence SEQ ID No 28 (putative ferric hydroximate siderophore receptor (*A. baumannii* ATCC 17978; accession number YP_001084696)) or a fusion protein comprising at least 2, preferably at least three, or more preferably at least four, amino acid sequences selected from the following list: SEQ ID No 12 to SEQ ID No 23 or variant sequences having at least 85% sequence identity with peptide
25 sequences SEQ ID No 12 to SEQ ID No 23.

30

Preferably the composition of the second aspect of the invention further comprises polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor (*A. baumannii* ATCC 17978; accession number YP_001084684)) or a fusion protein comprising at least 2, preferably at least three, or more preferably at least four, amino acid sequences selected from the following list: SEQ ID No 1 to SEQ ID No 11 or variant sequences having at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11.

A third aspect of the invention refers to a fusion protein, wherein the fusion protein (from hereinafter fusion protein of the invention) comprises peptide sequences of the putative ferric siderophore receptor and of the putative ferric hydroximate siderophore receptor of *A. baumannii*, and wherein:

- 5 a. the peptide sequences of the putative ferric siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 1 to SEQ ID No 11 or variant sequences having at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11; and
- 10 b. the peptide sequences of the putative ferric hydroximate siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 12 to SEQ ID No 23 or variant sequences having at least 85% sequence identity with peptide sequences SEQ ID No 12 to SEQ ID No 23.

15 A further embodiment of the invention refers to the fusion protein of the third aspect of the invention, wherein:

- 20 a. the peptide sequences of the putative ferric siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 1 to SEQ ID No 11; and
- b. the peptide sequences of the putative ferric hydroximate siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 12 to SEQ ID No 23.

- 25 In another embodiment of the invention, the fusion protein of the third aspect of the invention comprises amino acid sequence SEQ ID No 24 or SEQ ID No 25.

30 A fourth aspect of the invention refers to a nucleic acid (from herein after nucleic acid of the invention) encoding the fusion protein of the invention, wherein preferably said nucleic acid is SEQ ID No 26.

A fifth aspect of the invention refers to an expression vector (from hereinafter expression vector of the invention) containing the nucleic acid of the invention.

A sixth aspect of the invention refers to a host cell comprising the expression vector of the invention.

5 A seventh aspect of the invention refers to a pharmaceutical composition (from hereinafter pharmaceutical composition of the invention) comprising the fusion protein of the invention or the composition of the first or second aspect of the invention and optionally a pharmaceutically acceptable carrier.

10 A further embodiment of the invention refers to the pharmaceutical composition of the invention further comprising an adjuvant.

15 A further aspect of the invention refers to the fusion protein of the invention or the composition of the first or second aspect of the invention or the pharmaceutical composition of the invention for its use as a medicament.

In a yet further aspect of the invention, the fusion protein of the invention or the composition of the first or second aspect of the invention or the pharmaceutical composition of the invention are used as a vaccine which preferably further comprises an adjuvant.

20 In a still further aspect of the invention, the fusion protein of the invention or the composition of the first or second aspect of the invention or the pharmaceutical composition of the invention are used for conferring protection against an infection caused by *A. baumannii* in a subject, wherein preferably said subject is a human subject.

25 The polypeptide or fusion protein of the invention or the composition of the first or second aspect of the invention or the pharmaceutical composition of the invention can be administered in one or several, such as two, three, four, five, six, seven, eight, nine, ten or more than ten doses. There are no particular constraints concerning the amount of active ingredient per dose.

A still further aspect of the present invention refers to a composition comprising an antibody or a fragment thereof capable of binding SEQ ID No 27 or SEQ ID No 28

or a fusion protein as defined in the second aspect or the fusion protein of the invention, wherein preferably said composition is a pharmaceutical composition, preferably a vaccine, and wherein said pharmaceutical composition is use in the treatment or prevention of an infection caused by *A. baumannii*.

5

Another aspect of the invention refers to an antibody or active fragment thereof obtained by immunization of a mammal with the composition of the first or second aspect of the invention or with the fusion protein of the invention. Preferably said antibody or active fragment is comprised in a composition wherein preferably said
10 composition is a pharmaceutical composition and wherein said pharmaceutical composition is used in therapy, particularly for the treatment or prevention of an infection caused by *A. baumannii*.

Brief description of the drawings

15 **Figure 1:** Amino acid (A) and nucleotide (B) sequences of the multi-peptide (fusion protein) vaccine antigen resulting from the concatenation of the 23 selected peptides. The start codon methionine (M) and the stop codon (*) added to the sequence have been highlighted.

20 **Figure 2:** Agarose gel. Digestion of the transformant bacteria after cloning the synthetic gene into the plasmid pET-15b (pET-VXD001). Notes: lanes 1-4: digestion of 4 transformants, lane M: molecular weight marker.

25 **Figure 3.** Western blot analysis using anti-His antibody to determine the expression of the recombinant protein in the *E. coli* BL21 (DE3) strain. Lanes: BL21: negative control *E. coli* strain untransformed, BL21 + pET-vxd001: *E. coli* strain transformed with plasmid pET-VXD001.

30 **Figure 4:** Purification of the vaccine. A. Coomassie staining. B. Western blot with an anti-6xHis antibody.

Figure 5: Antibodies levels against the recombinant protein in the serum of mice after two injections of the multi-peptide vaccine (fusion protein) (VXD001, n = 8 mice) or two injections of adjuvant only (adjuvant control, n = 8 mice). Antibody
35 levels in vaccinated mice were significantly higher than in unvaccinated mice (*p <

0.001, Mann-Whitney U test). The dashed line represents the limit of detection of the assay.

Figure 6: Survival of vaccinated and control mice after inoculation with *A. baumannii*.

Figure 7: Immunization with an inactivated whole cell vaccine produces antibodies against OmpA. Sera collected from mice on day 28 after immunization with 1×10^8 inactivated bacteria on days 0 and 21, or control sera, were used to probe Western blots containing 3 μg of ATCC 19606 whole bacteria lysates (WBL) or 0.5 μg of 6x His-tagged OmpA (A). Endpoint titers of OmpA-specific IgG in 28-day serum from inactivated whole cell-vaccinated and control mice ($n = 8$ mice/group). Boxes represent the first and third quartiles with the median indicated by a horizontal line, and whiskers indicating the range. * $P < 0.001$ compared to control mice; Mann-Whitney U test (B).

Figure 8. Antibody response to immunization with purified refolded OmpA. Sera were collected from vaccinated and control mice ($n = 8$ mice/group) before immunization and at 7 and 21 days after the first immunization with OmpA and levels of OmpA-specific total IgG were determined by ELISA. * $P < 0.001$ compared to the corresponding timepoint in control mice; # $P = 0.001$ compared to 7-day IgG titers in vaccinated mice; Mann-Whitney U test (A). Endpoint titers of OmpA-specific IgG1 and IgG2c in 21-day serum from vaccinated and control mice ($n = 8$ mice/group). * $P < 0.001$ compared to control mice; Mann-Whitney U test (B). For all panels the discontinuous lines represent the limit of detection of the assay.

Figure 9. Infection of immunized and control mice with *A. baumannii*. Spleen bacterial loads in vaccinated and control mice ($n = 8$ mice/group) 12 hours post-infection with 8.5×10^4 cfu ($12.9 \times \text{LD}_{50}$) of the ATCC 19606 strain. Data points represent bacterial loads from individual mice with horizontal lines indicating the median (A). Survival of vaccinated and control mice ($n = 8$ mice/group) after challenge with the ATCC 19606 and Ab-1 strains (B). Figure 4. Whole cell ELISAs using 19606 and Ab-1 cells. IgG titers against ATCC 19606 (A) and Ab-1 (B) cells in 3-week serum from mice vaccinated with OmpA, adjuvant control mice, and

mice immunized with a whole cell vaccine (n = 8/group). * P < 0.001 compared to mice immunized with the whole cell vaccine. For all panels the discontinuous lines represent the limit of detection of the assay.

5 **Abbreviations used**

Ab	antibody
nAb	neutralizing antibody
mAb	monoclonal antibody

10 **Detailed Disclosure of the Invention**

The following detailed description discloses specific and/or preferred variants of the individual features of the invention. The present invention also contemplates as particularly preferred embodiments those embodiments, which are generated
15 by combining two or more of the specific and/or preferred variants described for two or more of the features of the present invention. Unless expressly specified otherwise, the term "comprising" is used in the context of the present document to indicate that further members may optionally be present in addition to the members of the list introduced by "comprising".

20 The present invention relates to improved anti-*A. baumannii* immunogens, more specifically to polypeptide fragments of the putative ferric siderophore and putative ferric hydroxamate siderophore receptors of *A. baumannii* capable of conferring protection against an infection caused by *A. baumannii*.

25 To date, few protective subunit antigens have been identified for *A. baumannii*. In order to overcome this problem, the present inventors have dedicated numerous efforts trying to find a suitable protective subunit antigen. In this sense, they first found outer membrane protein A as an attractive choice as a vaccine antigen for
30 preventing *A. baumannii* disease in an experimental mouse model. Outer membrane protein A (OmpA) is one of the few virulence factors that have been characterized for *A. baumannii*. *A. baumannii* OmpA plays key roles in infection of the host including adherence to epithelial cells, induction of apoptosis in host cells, and differentiation of host immune cells. In addition, immunization with OmpA

homologues from other Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, has been shown to produce a protective immune response in animal models.

5 However, the present inventors come to the surprising conclusion that immunization with purified *A. baumannii* OmpA produces high titers of antigen-specific IgG, but does not protect against infection. In this sense, as illustrated in Figure 7A and Example 1, embodiment 2.3, there was no difference in bacterial load between vaccinated and control mice ($P = 0.674$; Mann-Whitney U test). In
10 addition, vaccinated mice demonstrated no survival benefit compared to control mice ($n = 8$ mice/ group) after infection with either 8.5×10^4 cfu ($12.9 \times \text{LD}_{50}$) of the ATCC 19606 strain or 1.0×10^3 cfu ($3.3 \times \text{LD}_{50}$) of the clinical isolate Ab-1 ($P = 0.858$ and $P = 0.535$, respectively; log-rank test), indicating that vaccination with OmpA does not protect against *A. baumannii* infection. Similar results were
15 obtained after vaccination with twice the dose of purified OmpA (dose = $20 \mu\text{g}/\text{immunization}$).

These findings, indicate that although immunization with OmpA from some Gram-negative pathogens can induce protective immunity, it is not the case for all
20 Gram-negative bacteria. Therefore, further studies were necessary in order to identify further antigens and determine if immunization with said antigens was capable of providing protective immunity to *A. baumannii*.

In order to identified these further immunogens, the inventors grew *A. baumannii* strain ATCC 19606 in 100 ml of Mueller-Hinton broth or 100 ml of human serum at
25 37°C with mixing at 180 rpm. Upon reaching the exponential phase of growth (optical density at 600 nm of 0.6) the bacteria were collected by centrifugation and the outer membrane proteins were isolated. The isolated proteins were separated by SDS-PAGE and stained with Coomassie. The levels of expression of the outer
30 membrane proteins were compared between the two samples using isobaric tag for relative and absolute quantitation (iTRAQ). Results from the iTRAQ experiment showed that a number of proteins were over expressed in serum vs. broth, among them two proteins were specifically chosen for the construction of

the vaccine. These were putative ferric siderophore receptor protein and the putative ferric hydroxamate siderophore receptor.

Protein	Fold over expression in serum vs. broth
putative ferric siderophore receptor protein	3.2
putative ferric hydroxamate siderophore receptor	4.6

- 5 Then the inventors identified peptide fragments of these two proteins that were later incorporated into the vaccine of the present invention, namely sequences SEQ ID No 1 to SEQ ID No 23.

10 To determine the immunogenicity and efficacy of the vaccine, a mouse model of sepsis in C57/B6 mice was employed. As further detailed in Example 2 herein below, for immunization, the concentration of the purified recombinant protein (SEQ ID No 25 containing all 23 sequences mentioned above) was adjusted to 100 µg/ml in phosphohate buffered saline and then mixed 1:1 (v:v) with an aluminum hydroxide adjuvant. Mice (n=8) received 200 µl (10 µg of the
15 recombinant protein) of vaccine by intramuscular injection into the quadriceps muscle. As a control, one group (n=8) received the same volume of a mixture of adjuvant with phosphate buffered saline (without the recombinant protein). Mice were immunized on the first day of the study (day 0) and on day 14. Serum was collected from mice on days 0, 7 and 21 of the study for analysis of antigen-
20 specific antibodies by enzyme-linked immunosorbant assay (ELISA). On day 21, mice were challenged with the *A. baumannii* strain ATCC 19606 by intraperitoneal injection of (1×10^5) colony forming units. Survival of mice was measured daily over the following 7 days.

- 25 Serum collected from mice on days 0, 7, and 21 were analyzed by ELISA in order to determine serum-levels of antibodies against the recombinant protein. As shown in Figure 5, on day 7 low levels of antigen-specific IgG were detected in mice immunized with the recombinant protein, and on day 21, antigen-specific IgG were detected in all immunized mice (median titer = 2.3×10^4). Control mice had
30 no detectable antigen-specific antibodies at any timepoint.

One week after the second immunization, mice were inoculated with the reference strain ATCC 19606 of *A. baumannii* by intraperitoneal injection. All vaccinated mice survived, while only 50% of control mice receiving the adjuvant only survived (Figure 6). The difference in survival was statistically significant ($p = 0.026$, log-rank test).

Thus, the inventors have herein successfully shown that the two chosen proteins, namely putative ferric siderophore receptor and putative ferric hydroxamate siderophore receptor or a fragment of any of these sequences thereof, can act as efficient protective subunit antigens for *A. baumannii*.

Therefore, a first aspect of the invention refers to a composition comprising polypeptide sequence SEQ ID No 28 (putative ferric hydroxamate siderophore receptor (*A. baumannii* ATCC 17978; accession number YP_001084696)) or a fragment thereof, wherein the fragments are biologically active fragments, preferably selected from the list consisting of SEQ ID No 12 to SEQ ID No 23 or sequences having at least 85% sequence identity with peptide sequences SEQ ID No 12 to SEQ ID No 23.

Preferably the composition of the first aspect of the invention further comprises polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor (*A. baumannii* ATCC 17978; accession number YP_001084684)) or a fragment thereof, wherein the fragments are biologically active fragments, preferably selected from the list consisting of SEQ ID No 1 to SEQ ID No 11 or sequences having at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11.

More preferably the composition of the first aspect of the invention comprises polypeptide sequence SEQ ID No 28 (putative ferric hydroxamate siderophore receptor) and polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor).

A further separate embodiment of the invention, refers to a composition (from hereinafter "the composition comprising fragments of SEQ ID No 27") comprising at least one, preferably at least two, preferably at least three, or more preferably at least four, amino acid sequences selected from the following list consisting of:
5 SEQ ID No 1 to SEQ ID No 11 or variant sequences having at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11.

A further embodiment of the invention refers to a nucleic acid encoding any of the amino acid sequences of the "composition comprising fragments of SEQ ID No
10 27".

Another embodiment of the invention refers to an expression vector containing the nucleic acid of the previous embodiment.

15 A still further embodiment of the invention refers to a host cell comprising the expression vector of the previous embodiment.

A still further embodiment of the invention refers to a pharmaceutical composition comprising the "composition comprising fragments of SEQ ID No 27" and
20 optionally a pharmaceutically acceptable carrier.

A further embodiment of the invention refers to the pharmaceutical composition of the previous embodiment further comprising an adjuvant. Preferably said pharmaceutical composition is use as medicament, more preferably as a vaccine.
25 Still more preferably said pharmaceutical composition is use for conferring protection against an infection caused by *A. baumannii* in a subject, wherein preferably said subject is a human subject.

In addition, antibodies or fragments thereof capable of binding any of the amino
30 acid fragments as defined in the "the composition comprising fragments of SEQ ID No 27" can also be used to confer protective immunity in a subject, preferably a human, to the infection caused by *A. baumannii*. These antibodies or fragments thereof can easily be obtained from antisera.

In the context of the present invention the term "*A. baumannii*" must be understood as a cellular organism of the superkindom *Bacteria*, phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Pseudomonadales*, family *Moraxellaceae*, genus *Acinetobacter*. The species *Acinetobacter baumannii* Bouvet & Grimont, 1986, (*Int. J. Syst. Bacteriol.*, 1986, 36, 228-240) refers to a species of Gram-negative bacteria belonging to the phylum *Proteobacteria*. *Acinetobacter* species are strictly aerobic nonfermentative,, oxidase-negative bacilli that occur in pairs under a microscope. Are widely distributed in nature, are important in the soil and contribute to mineralization. Type strain: strain ATCC 19606 = CCUG 19096 = CIP 70.34 = DSM 30007 = JCM 6841 = LMG 1041 = NCCB 85021 = NCTC 12156. GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of the type strain: X81660.

The fragments described above differ from the variant sequences by at least one amino acid. More preferred variants are the ones having 85 % or more, including 90 % or more, such as 93 % or more, and preferably 95 % or more, 96 % or more, 97 % or more, 98 % or more, 99 % or more sequence identity with any of the polypeptides shown as SEQ ID No 1 to SEQ ID No 23.

More preferably, the invention relates to a variant sequence characterized by at least one (at least two, at least three, at least four) mutation(s) relative to any of the polypeptides shown as SEQ ID No 1 to SEQ ID No 23. According to the invention as described in this specification, "mutation" can mean any one selected from insertion(s), deletion(s) and substitution(s). Substitution(s) may be preferred.

A second aspect of the invention, refers to a composition comprising polypeptide sequence SEQ ID No 28 (putative ferric hydroximate siderophore receptor (*A. baumannii* ATCC 17978; accession number YP_001084696)) or a fusion protein comprising at least 2, preferably at least three, or more preferably at least four, amino acid sequences selected from the following list: SEQ ID No 12 to SEQ ID No 23 or variant sequences having at least 85% sequence identity with peptide sequences SEQ ID No 12 to SEQ ID No 23.

Preferably the composition of the second aspect of the invention further comprises polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor (*A. baumannii* ATCC 17978; accession number YP_001084684)) or a fusion protein comprising at least 2, preferably at least three, or more preferably at least four, amino acid sequences selected from the following list: SEQ ID No 1 to SEQ ID No 11 or variant sequences having at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11.

The fusion protein of the second aspect of the invention as well as the fusion protein of the invention as detailed herein below, might comprise at least two, three or four of the polypeptide sequences or variant sequences described above. Additionally, said fusion protein might comprise further polypeptide sequences not particularly limited such as for example polypeptide sequences with immunostimulatory properties and/or polypeptide sequences which may be helpful for purification of the fusion protein of the invention may be preferred. Sequences helpful for purification are affinity tags, optionally separated from the polypeptide sequence of the above-described polypeptide by a protease cleavage site. A preferred affinity tag may be a polyhistidine tag (which stands for a plurality of subsequent histidine residues, e.g. HHHHHH). A preferred affinity tag is GGGGSHHHHHH.

After purification by the use of the affinity tag, the fusion protein can optionally be further purified using a size-exclusion chromatography step. Alternatively, the fusion protein can be purified using ion-exchange chromatography methods, as polishing purification step.

Further, the invention relates to a peptide conjugate, in which the fusion protein or polypeptide of the invention is (covalently or non-covalently) bound to a further moiety.

A third aspect of the invention refers to a fusion protein, wherein the fusion protein (from hereinafter the fusion protein of the invention) comprises peptide sequences of the putative ferric siderophore receptor and of the putative ferric hydroximate siderophore receptor of *A. baumannii*, and wherein:

a. the peptide sequences of the putative ferric siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 1 to SEQ ID No 11 or variant sequences having at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11; and

b. the peptide sequences of the putative ferric hydroximate siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 12 to SEQ ID No 23 or variant sequences having at least 85% sequence identity with peptide sequences SEQ ID No 12 to SEQ ID No 23.

A further embodiment of the invention refers to the fusion protein of the third aspect of the invention, wherein:

a. the peptide sequences of the putative ferric siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 1 to SEQ ID No 11; and

b. the peptide sequences of the putative ferric hydroximate siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 12 to SEQ ID No 23.

In another embodiment of the invention, the fusion protein of the third aspect of the invention comprises or consists of amino acid sequence SEQ ID No 24 or SEQ ID No 25.

A fourth aspect of the invention refers to a nucleic acid (from hereinafter nucleic acid of the invention) encoding the fusion protein of the invention or the fusion protein of the second aspect of the invention. Preferably said nucleic acid is SEQ ID No 26

A fifth aspect of the invention refers to an expression vector (from hereinafter expression vector of the invention) containing the nucleic acid of the invention.

A sixth aspect of the invention refers to a host cell comprising the expression vector of the invention.

The nucleic acid sequence of the invention can be easily calculated *in silico* by reverse translation. If expression in a particular host is desired, the codon usage frequency of said host may be considered. The invention also relates to a single-stranded nucleic acid which can hybridize under stringent conditions with the first described nucleic acid. The invention also relates to a double-stranded nucleic acid comprising the first-described single-stranded nucleic acid and its complementary – or essentially complementary strand. The invention also relates to an expression vector, such as a plasmid, containing the aforementioned nucleic acid.

The polypeptides, fragments or fusion proteins described in the present invention may be obtained by any method known in the art, such as by chemical peptide synthesis *in vitro*, or by expression of a gene encoding it, e.g. the above-described nucleic acid or vector, in a host cell. A method for synthesizing the peptide *in vitro* as a method for obtaining the polypeptide from a host cell and subsequently obtaining and optionally purifying it are therefore also comprised in the invention. The host cell is also comprised in the invention.

A seventh aspect of the invention refers to a pharmaceutical composition (from hereinafter pharmaceutical composition of the invention) comprising the fusion protein of the invention or the composition of the first or second aspect of the invention and optionally a pharmaceutically acceptable carrier.

A further embodiment of the invention refers to the pharmaceutical composition of the invention further comprising an adjuvant.

A further aspect of the invention refers to the fusion protein of the invention or the composition of the first or second aspect of the invention or the pharmaceutical composition of the invention for its use as a medicament.

In a yet further aspect of the invention, the fusion protein of the invention or the composition of the first or second aspect of the invention are used as a vaccine which preferably further comprises an adjuvant.

In a still further aspect of the invention, the fusion protein of the invention or the composition of the first or second aspect of the invention or the pharmaceutical composition of the invention are used for conferring protection against an infection
5 caused by *A. baumannii* in a subject, wherein preferably said subject is a human subject.

In one embodiment the pharmaceutical composition of the invention is in the format of a liposome formulation. Exemplary liposomes may comprise di-myristoyl-
10 phosphatidylcholine (DMPC), cholesterol, and di-myristoyl-phosphatidylglycerol (DMPG). In certain embodiments, the molar ratio of DMPC to cholesterol to DMPG in the composition is about 9:7:1. Also provided are methods for producing such liposomes. The liposomes typically comprise phospholipids, either as a homogenous preparation (e.g., a single type of phospholipid) or a mixture of
15 different phospholipids. For instance, phospholipids with different chain lengths (e.g., one or more of C14, C16, C18, C20, or natural phospholipids with mixed chain lengths) may be used. Mixtures of cholesterol(s) and lipid(s) at various ratios may also be used. In some embodiments, a phospholipid providing a 15 negative surface charge to the liposome may be used (e.g., DMPG, DMPA, DOTAP,
20 DOTMA). In certain embodiments, the liposomes are produced by combining a lipid with the polypeptide in the presence of octyl- β -D-glucopyranoside (β -OG), Tween 20 and / or other suitable detergents, which may be necessary to solubilize and stabilize the hydrophobic membrane proteins. In some embodiments, the liposomes within a composition are of substantially similar sizes (e.g., an z-average diameter of approximately any of 70 to 130, 70-80, 80-90, 90-100, 100-
25 110, 110-120, and 120-130 nm, as determined by dynamic light scattering). The liposomes may be prepared using methods described in, for example, U.S. Pat. No. 6,843,942.

30 The medicament or vaccine as described in the present invention can be administered to an animal (such as mammal) or human subject. Administration to a human subject is preferred. It can be administered in one or several, such as two, three, four, five, six, seven, eight, nine, ten or more than ten doses. There are no particular constraints concerning the amount of active ingredient per dose.

In addition, antibodies or fragments thereof capable of binding any of the polypeptides as defined in the compositions of the first aspect of the invention or capable of binding any of the fusion proteins as defined in the second or third
5 aspect of the invention can also be used to confer protective immunity in a subject, preferably a human, to the infection caused by *A. baumannii*. These antibodies or fragments thereof can easily be obtained from antisera.

Antisera to the polypeptides or fusion proteins described in the present invention
10 can be generated by standard techniques, for example, by injection of any of the peptides of the invention into an appropriate animal and collection and purification of antisera from animals. Antibodies or fragments thereof which bind SEQ ID No 1 to SEQ ID No 23 or SEQ ID No 24, 25, 27 or 28 or a variant sequence thereof in accordance with the invention can be identified by standard immunoassays. The
15 antibodies so obtained (from hereinafter antibodies of the invention) may be used to isolate or purify peptides for incorporation into the vaccine composition of the invention or to directly confer protective immunity in a subject to the infection caused by *A. baumannii*. Preferably the antibodies or fragments thereof are monoclonal antibodies. Thus, another aspect of the invention refers to an antibody
20 or active fragment thereof obtained by immunization of a mammal with the composition of the first aspect of the invention or with the fusion protein as defined in the second aspect of the invention or with the fusion protein of the invention. Preferably said antibody or active fragment is comprised in a composition wherein preferably said composition is a pharmaceutical composition and wherein said
25 pharmaceutical composition is use in therapy, particularly for the treatment or prevention of an infection caused by *A. baumannii*.

Thus, a still further aspect of the present invention refers to a composition comprising an antibody of the invention or a fragment thereof, wherein preferably
30 said composition is a pharmaceutical composition and wherein said pharmaceutical composition is use in the treatment or prevention of an infection caused by *A. baumannii*.

Antibodies molecules suitable for use in the present invention include:

"intact" antibodies which comprise an antigen-binding variable region as well as a light chain constant domain (CL) and heavy chain constant domains, CH1, CH2 and CH3,

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"Fab" fragments resulting from the papain digestion of an intact antibody and which comprise a single antigen-binding site and a CL and a CH1 region,

"F(ab')₂" fragments resulting from pepsin digestion of an intact antibody and which contain two antigen-binding sites,

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"Fab'" fragments contain the constant domain of the light chain and the first constant domain (CH1) of the heavy chain and has one antigen-binding site only. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH 1 domain including one or more cysteines from the antibody hinge region.

15

"Fv" is the minimum antibody fragment which contains a complete antigen-recognition and antigen-binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent-association. It is in this configuration that the three hypervariable regions (CDRs) of each variable domain interact to define an antigen-binding site on the surface of the VH - VL dimer. Collectively, the six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

20

25

Single-chain FV or "scFv" antibody fragments comprise the VL and VH, domains of antibody, wherein these domains are present in a single polypeptide chain. Preferably, the VL and VH regions are connected by a polypeptide linker which enables the scFv to form the desired structure for antigen binding.

30

"Diabodies" comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) on the same polypeptide chain (VH-VL) connected by a peptide linker that is too short to allow pairing between the two domains on the same chain. This forces pairing with the complementary domains of another chain and promotes the assembly of a dimeric molecule with two functional antigen binding sites.

"Bispecific antibodies" (BAbs) are single, divalent antibodies (or immunotherapeutically effective fragments thereof) which have two differently specific antigen binding sites. The two antigen sites may be coupled together chemically or by genetic engineering methods known in the art.

All these antibody fragments can be further modified using conventional techniques known in the art, for example, by using amino acid deletion(s), insertion(s), substitution(s), addition(s), and/or recombination (and/or any other modification(s) (e.g. posttranslational and chemical modifications, such as glycosylation and phosphorylation) known in the art either alone or in combination. Methods for introducing such modifications in the DNA sequence underlying the amino acid sequence of an immunoglobulin chain are well known to the person skilled in the art; see, e.g., Sambrook et al.; Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory Press, 2nd edition 1989 and 3rd edition 2001 .

Examples

Example 1. Immunogenicity and efficacy of the OmpA vaccine in mice

1. Materials and Methods

1.1 Bacterial strains

A. baumannii ATCC 19606 is an antibiotic sensitive reference strain, and Ab-1 is a previously characterized multidrug resistant clinical isolate.

1.2 Purification of *A. baumannii* OmpA

A. baumannii OmpA from the ATCC 19606 strain was purified from *E. coli* under denaturing conditions and refolded into its native conformation as described previously. This protocol involves the removal of endotoxin, resulting in endotoxin levels < 1.3 EU/mg OmpA. After refolding, the purified protein was extensively
5 dialyzed into PBS.

1.3 Mice and immunization

Experiments involving mice were approved by the Institutional Committee on Ethics and Experimentation. For immunization, purified OmpA was diluted to a
10 concentration of 100 µg/ml in PBS and combined 1:1 (v:v) with the aluminium adjuvant, Adjuphos (Brenntag, Frederikssund, Denmark). Six to eight-week, female C57BL/6 mice were immunized by intramuscular injection with 100 µl of the mixture into each quadriceps muscle (dose = 10 µg OmpA/mouse) on days 0 and 14. Control mice were immunized similarly with PBS and adjuvant.

1.4 ELISAs and Western blots

Blood was collected from the retro-orbital plexus before immunization, 7 days after the first immunization (day 7), and 7 days after the second immunization (day 21). Sera from mice immunized with an *A. baumannii* inactivated whole cell vaccine
20 were obtained from a previous study. For Western blots, 3 µg of whole bacterial lysates from the 94 ATCC 19606 strain and 0.5 µg of purified OmpA were resolved on a 12% polyacrylamide gel and transferred to nitrocellulose. The membrane was probed with a 1:2,000 dilution of serum from mice immunized with an *A. baumannii* whole cell vaccine or control serum before developing using chemiluminescence.
25 For OmpA ELISAs, 96-well plates were coated with 0.1 µg of OmpA per well, and ELISAs were performed as described previously [10]. Whole cell ELISAs were performed identically except that 1×10^7 cells of the indicated *A. baumannii* strain in 100 µl PBS were placed into each well and allowed to dry overnight at 37 °C.

1.5 Infection with *A. baumannii*

A mouse model of disseminated sepsis was used. *A. baumannii* ATCC 19606 and Ab-1 strains were grown for 18 hours at 37 °C in Mueller-Hinton broth, and the concentration of the resulting cultures adjusted using physiologic saline. The
30

bacterial suspension was mixed 1:1 with porcine mucin, 0.5 ml was injected intraperitoneally, and mice were monitored for survival for 7 days. As described previously by our group, porcine mucin is used since it allows for a smaller inoculum to be used in order to achieve mortality. Bacterial concentrations in the inocula were determined by plating on blood agar. LD50 values for these strains are ATCC 19606, 6.6×10^3 cfu and Ab-1, 3.0×10^2 . Bacterial loads were determined 12 hours post-infection by aseptically removing spleens and plating 10-fold dilutions of tissue homogenates on blood agar.

1.6 Statistical Analysis

Antibody titers and bacterial loads were compared using the Mann-Whitney U test. Survival data were compared using the log-rank test.

2. Immunogenicity and efficacy of the OmpA vaccine in mice

2.1 Immunization with a whole cell vaccine produces antibodies against OmpA

Previously to the present study the inventors demonstrated that immunization with a whole cell vaccine provides protection against infection with *A. baumannii*. Based on these results, the inventors sought to determine if serum from mice immunized with the whole cell vaccine contained significant levels of antibodies against OmpA. Sera collected on day 28 after immunization with 1×10^8 inactivated bacterial cells on days 0 and 21 were used in Western blots to probe both *A. baumannii* whole bacterial lysates and purified OmpA. Vaccine serum reacted with multiple *A. baumannii* proteins present in whole bacterial lysates and, importantly, the serum also reacted strongly with purified OmpA (Figure 7A). ELISAs demonstrated that immunization with the whole cell vaccine produced significant titers of antibodies against OmpA, whereas control mice had no detectable antibodies (Figure 7B). Based on these results the inventors sought to determine if immunization with purified OmpA could produce a protective immune response.

2.2 Antibody response to immunization with OmpA

Mice were vaccinated with OmpA on days 0 and 14 and serum was collected before immunization on days 7 and 21 for analysis. As shown in Figure 8A, sera collected 7 days (n = 8 mice/group) had detectable levels of total IgG against

OmpA ($P < 0.001$ compared to control mice). Total IgG levels were significantly higher in 21-day serum compared to 7-day serum ($P = 0.001$), indicating that a second immunization increased antibody levels. Levels of the IgG subclasses IgG1, IgG2b, IgG2c, and IgG3 were determined in 21-day serum from vaccinated and control mice. All subtypes were present in 21-day serum.

2.3 Infection of vaccinated mice with *A. baumannii*

The inventors next sought to determine if the immune response produced by immunization with purified refolded OmpA could protect against infection with *A. baumannii*. Seven days after the second immunization (day 21) mice were challenged with 8.5×10^4 cfu ($12.9 \times \text{LD}_{50}$) of the ATCC 19606 strain ($n = 8$ mice/group) and spleens were harvested 12 hours post-infection to determine bacterial loads. As shown in Figure 9A, there was no difference in bacterial load between vaccinated and control mice ($P = 0.674$; Mann-Whitney U test). In addition, vaccinated mice demonstrated no survival benefit compared to control mice ($n = 8$ mice/group) after infection with either 8.5×10^4 cfu ($12.9 \times \text{LD}_{50}$) of the ATCC 19606 strain or 1.0×10^3 cfu ($3.3 \times \text{LD}_{50}$) of the clinical isolate Ab-1 ($P = 0.858$ and $P = 0.535$, respectively; log-rank test), indicating that vaccination with OmpA does not protect against *A. baumannii* infection. Similar results were obtained after vaccination with twice the dose of purified OmpA (dose = 20 μg /immunization; data not shown).

Example 2. Immunogenicity and efficacy of the fusion protein vaccine in mice

1.1 Expression and purification of the multi-peptide vaccine

In order to express and purify the fusion protein or multi-peptide vaccine, a gene corresponding to the DNA sequence of the concatenated proteins was synthesized (DNA sequence SEQ ID No 26). The synthesized gene encoding the multi-peptide vaccine (amino acid sequence SEQ ID No 25) was cloned into the pET-15b plasmid that contains an IPTG inducible promoter, and incorporates a tail of six histidines for purification using a nickel matrix. The synthetic gene and the pET-15b plasmid were digested using the NdeI and BamHI endonucleases at 37 °C during 2 h, to generate cohesive ends. The digested plasmid and insert were purified and mixed in a 15 μl ligation reaction containing the T4 ligase enzyme for 2h at room temperature.

Seven μ l of the ligation mixture were used to transform 50 μ l of *E.coli* TOP10 competent cells and the mixture was incubated 30 minutes on ice, 30 seconds at 42 °C for a heat shock and 2 minutes on ice to recover. Then, 500 μ l of SOC solution previously preheated to 37 °C was added to the mixture and the mixture was incubated for 1h at 37 °C. To select positive transformants 100 μ l of the bacterial culture was plated in a LB plate supplemented with ampicillin (50 μ g/ml). In order to confirm correct insertion of the synthetic gene into the pET-15b plasmid 4 of the transformant colonies were selected, inoculated in 4 ml of LB containing 50 μ g/ml of ampicillin and grown for 18 hours. The isolation of plasmid DNA was carried out using the miniprep kit Wizard Plus SV Minipreps DNA Purification System (Promega). The resulting DNA was digested with the enzymes NdeI and BamHI at 37 °C for 2 hours to cleave the insert from the plasmid and the digested fragments were separated by electrophoresis in a 1% agarose gel. As shown in Figure 3, three of the four transformants selected were positive yielding after digestion a 2.4 kb band corresponding to the plasmid and a 1.8kb band corresponding to the insert. The pET15b construct containing the concatenated DNA was designated as pET-VXD001. Plasmid number 2 (pET-VXD001) was selected for transforming the *E. coli* BL21 (DE3) strain designed for expression of recombinant proteins.

With the strain BL21 (DE3) transformed with the pET-VXD001 plasmid coding for the multi-peptide vaccine, the protocol for purification of the recombinant protein was optimized on a small scale. The inventors first tested whether the protein was expressed in the *E.coli* BL21 (DE) transformed strain. To do this broth 50 ml of LB cultures were inoculated with the BL21-pET-VXD001 bacteria, and the expression of the recombinant protein was induced by adding 1 mM IPTG to the culture and incubated at 37 °C for 4 hours with stirring. As a negative control, a 50 ml culture was inoculated in parallel with untransformed BL21 bacteria. After 4 hours of incubation, cells were centrifuged and lysed with hypotonic buffer (20 mM NaPO₄, pH 7.5, 0.5% NP-40) and sonicated.

The protein mixture was separated by electrophoresis in a 8% acrylamide gel and transferred to a nitrocellulose membrane and the protein was detected by Western

blot using an anti-6x histidine antibody (GE Healthcare). As shown in Figure 3, the recombinant protein was expressed and recognized by the anti-His antibody.

5 After verifying protein expression, the solubility of the recombinant protein was determined by separating the soluble fraction from the insoluble of the protein lysate by centrifugation. After centrifugation, the pellet containing the insoluble fraction was solubilized using an 8M urea solution.

10 Both fractions, soluble and insoluble, were separated by electrophoresis using in a 10% acrylamide gel, transferred to a nitrocellulose membrane after which a Western blot was carried out using an anti-6x histidine antibody (GE Healthcare). The results showed that the protein was located in the insoluble fraction.

15 With these results, purification was performed on a larger scale and 1 liter of LB broth was inoculated with the BL21-pET-VXD001 transformant and grown to an optical density of 0.6 at 600 nm. The expression of the protein was induced by adding 1mM IPTG to the culture and incubated for 4 hours. For protein purification through the nickel matrix 8M urea denaturing conditions were used. The purified recombinant protein was separated by electrophoresis in an acrylamide gel and
20 visualized by Coomassie staining, Western blotting (Figure 5) and spectrometry. Results confirmed a purity of the recombinant protein of 95%.

1.2 Immunogenicity and efficacy of the fusion protein or multi-peptide vaccine in mice.

25 To determine the immunogenicity and efficacy of the vaccine, a mouse model of sepsis in C57/B6 mice was employed. For immunization, the concentration of the purified recombinant protein was adjusted to 100 µg/ml in phosphate buffered saline and then mixed 1:1 (v:v) with an aluminum hydroxide adjuvant. Mice (n=8) received 200 µl (10 µg of the recombinant protein) of vaccine by intramuscular
30 injection into the quadriceps muscle. As a control, one group (n=8) received the same volume of a mixture of adjuvant with phosphate buffered saline (without the recombinant protein). Mice were immunized on the first day of the study (day 0) and on day 14. Serum was collected from mice on days 0, 7 and 21 of the study

for analysis of antigen-specific antibodies by enzyme-linked immunosorbant assay (ELISA).

5 On day 21, mice were challenged with the *A. baumannii* strain ATCC 19606 by intraperitoneal injection of (1×10^5) colony forming units. Survival of mice was measured daily over the following 7 days.

10 Indirect enzyme-linked immunosorbent assays (ELISAs) were used to quantify the antigen-specific antibody response in vaccinated and control mice. Ninety-six well plates were coated with 0.1 μ g of the recombinant protein/well. The wells were washed twice with 0.1% Tween 20 in PBS (PBST) and blocked with 5% dry milk in PBST (PBSTM) for 30 min at room temperature. After the wells were washed twice with PBST, serial two-fold dilutions of serum in PBSTM were added to the wells and incubated for 90 min at 37°C. The plates were washed three times with
15 PBST, and 100 μ l of horseradish peroxidase-conjugated anti-immunoglobulin G (IgG) diluted in PBSTM (1:10,000) were added to each well and incubated at room temperature for 1 h. After the wells were washed four times with PBST, 100 μ l of horseradish peroxidase substrate was added to each well and developed for 20 min at room temperature. The reaction was stopped by the addition of 50 μ l 1M
20 HCl, and the absorbance was read at 450 nm. The endpoint titer was defined as the highest dilution at which the optical density at 450 nm was at least 0.1 above that of the background wells (wells receiving no serum).

1.3 Immunogenicity of the fusion protein multi-peptide vaccine

25 Serum collected from mice on days 0, 7, and 21 were analyzed by ELISA in order to determine serum-levels of antibodies against the recombinant protein. As shown in Figure 5, on day 7 low levels of antigen-specific IgG were detected in mice immunized with the recombinant protein, and on day 21, antigen-specific IgG were detected in all immunized mice (median titer = 2.3×10^4). Control mice had
30 no detectable antigen-specific antibodies at any timepoint.

1.4 Protective Efficacy of the fusion protein multi-peptide vaccine

One week after the second immunization, mice were inoculated with the reference strain ATCC 19606 of *A. baumannii* by intraperitoneal injection. All vaccinated

mice survived, while only 50% of control mice receiving the adjuvant only survived (Figure 6). The difference in survival was statistically significant ($p = 0.026$, log-rank test).

5 **Example 3. Immunization with the putative ferric hydroximate siderophore receptor (SEQ ID No 28)**

1.1. Gene cloning and protein purification

10 The open reading frame of the putative ferric hydroximate siderophore receptor is amplified by polymerase chain reaction from genomic DNA of the *A. baumannii* strain ATCC 19606 using sequence specific primers. The amplified gene is subsequently cloned into the plasmid pET15-b via corresponding restriction sites such that it is fused in-frame to the 6-histidine tag that is encoded by the plasmid.

15 The plasmid is then transformed into the *Escherichia coli* strain BL21(DE3) in order to facilitate expression of the tagged recombinant protein. A one litre culture containing *E. coli* BL21(DE3) transformed with the pET-15b- putative ferric hydroximate siderophore receptor gene is grown to the mid exponential phase and induced by the addition of IPTG to a final concentration of 1mM. The culture is

20 incubated at 37°C for four hours with aeration in order to allow for expression of the recombinant protein. After four hours, the bacterial cells are collected, lysed, and the recombinant protein is purified via a nickel column.

25 1.2. Immunization and characterization of the immune response

For immunization studies, the recombinant protein is combined with an adjuvant (in this case aluminum phosphate) and administered via intramuscular injection into C57BL/6 mice at a final dose of 10 micrograms of the recombinant protein.

30 Two weeks after the initial immunization, mice are immunized with the same vaccine dose and formulation. Serum samples are collected from the mice before immunization, one week after the first immunization, and one week after the second immunization. Antigen-specific antibody levels are determined in the

serum samples by enzyme-linked immunosorbant assay (ELISA) using the purified recombinant protein as a capture antigen.

1.3. Survival studies

5

One week after the second immunization, mice are infected via intraperitoneal infection with a lethal dose of the *A. baumannii* strain ATCC 19606 and survival is monitored over the following 7 days. Mice immunized in the same way with only the adjuvant are used as negative controls and survival is compared between the

10

Overview of sequences mentioned:

	SEQ ID NO: 1:	TSSDTFRSDGLA
15	SEQ ID NO: 2:	TSRGSQFDGNGDRISLSPWQGSTMDDT
	SEQ ID NO: 3:	YKKQD TDYGP DYSYLP TTSKSN DATTPT YKAIKGLKLSNPL
	SEQ ID NO: 4:	RNEKSRFFPYGLSNKSVTSVNQSQSEIEV
	SEQ ID NO: 5:	EKDKQFVDILATQYPYLVYPTGQRKGYGPNT EIQN
	SEQ ID NO: 6:	IQADTDAYIPSRETTMVPAGSTHDDKPL
20	SEQ ID NO: 7:	PDVQRMLRDVSTYTVSTANLQPITVNS
	SEQ ID NO: 8:	NTSDKTVQFNNRAAKVVD TDQRV
	SEQ ID NO: 9:	TRGQYKDVANKWHELNSFTVAP
	SEQ ID NO: 10:	IKGTNKAYKDDRELA AFATTQDEAFQNAVKNDANSA
	SEQ ID NO: 11:	YNVWNRQYRTVFAQQAAVSANPLLAIPAEGR T
25	SEQ ID NO: 12:	VKGADSLTNAFGDPSATINNIRKRPTQE
	SEQ ID NO: 13:	SGKVRGRIMGYEQTGDSYLD RYSAEKNG
	SEQ ID NO: 14:	SSEQNKPNANN
	SEQ ID NO: 15:	NPNPDWAHWDNETQN
	SEQ ID NO: 16:	LDTKHNSRLLYYYGYPKADGSGVSLTPWGGQE HQEK
30	SEQ ID NO: 17:	VRNHQQDKQSTGTINDSNVIKSTTTD WASWTPQSIT
		WSDFTEAANYKQNI
	SEQ ID NO: 18:	VQAESKGESYSSPMSYSESK
	SEQ ID NO: 19:	RPQTGIDKDTNQALKPIEGKS
	SEQ ID NO: 20:	TEQNNYPLRNSDGNPLNRKVPTSDLESQG

SEQ ID NO: 21: AQFSIKDTKNGGEARTYNPN
SEQ ID NO: 22: QDGIKLYDSNVNGTIKQDAY
SEQ ID NO: 23: DKKYLNSFPDGGQAFYGAPAN

5 **SEQ ID NO: 24:**

VKGADSLTNAFGDPSATINNIRKRPTQESQEQNKPNANNNPNPDWAHWDNETQ
NLDTKHNSRLLYYYGYPKADGSGVSLTPWGGQEHQEKVRNHQQDKQSTGTIND
SNVIKSTTTDWASWTPQSITWSDFTEAANYKQNVQAESKGESYSSPMSYSESK
RPQTGIDKDTNQALKPIEGKSTEQNNYPLRNSDGNPLNRKVPTSDLESQGAQFSI
10 KDTKNGGEARTYNPNQDGIKLYDSNVNGTIKQDAYDKKYLNSFPDGGQAFYGAPA
NTSSDTRSDGLATSRGSQFDGNGDRISLSPWQGSTMDTDYKDKQDQTDYGPD
YSYLPPTSSKSNDAATTPTYKAIKGLKLSNPLRNEKSRFFPYGLSNKSVTSVNQSQS
EIEVEKDKQFVDILATQYPYLVYTPTGQRKGYGPNTEIQNIQADTDAYIPSRETTM
VPAGSTHDDKPLPDVQRMRLRDVSTYTVSTANLQPITVNSNTSDKTVQFNNRAAK
15 VVDTDQRVTRGQYKDVANKWHELNSFTVAPIKGNTKAYKDDRELAFAFATTQDEA
FQNAVKNDAANSAYNVWNRQYRTVFAQQAASVSNANPLLAIPAEGRT

SEQ ID NO: 25:

MSGKVRGRIMGYEQTGDSYLDRYSAEKNVKGADSLTNAFGDPSATINNIRKR
20 TQESQEQNKPNANNNPNPDWAHWDNETQNLDTKHNSRLLYYYGYPKADGSGV
SLTPWGGQEHQEKVRNHQQDKQSTGTINDSNVIKSTTTDWASWTPQSITWSDF
TEAANYKQNVQAESKGESYSSPMSYSESKRPQTGIDKDTNQALKPIEGKSTEQ
NNYPLRNSDGNPLNRKVPTSDLESQGAQFSIKDTKNGGEARTYNPNQDGIKLYD
SNVNGTIKQDAYDKKYLNSFPDGGQAFYGAPANTSSDTRSDGLATSRGSQFDG
25 NGDRISLSPWQGSTMDTDYKDKQDQTDYGPDYSYLPPTSSKSNDAATTPTYKAIK
LKLSNPLRNEKSRFFPYGLSNKSVTSVNQSQSEIEVEKDKQFVDILATQYPYLVYT
PTGQRKGYGPNTEIQNIQADTDAYIPSRETTMVPAGSTHDDKPLPDVQRMRLRDV
STYTVSTANLQPITVNSNTSDKTVQFNNRAAKVVDTDQRVTRGQYKDVANKWH
ELNSFTVAPIKGNTKAYKDDRELAFAFATTQDEAFQNAVKNDAANSAYNVWNRQYR
30 TVFAQQAASVSNANPLLAIPAEGRT

SEQ ID NO: 26:

ATGTCTGGTAAAGTTCGTGGTCGTATCATGGGTACGAACAGACCGGTGA
CTCTTACCTGGACCGTTACTCTGCTGAAAAAACGGTGTTAAAGGTGCTGACT

CTCTGACCAACGCTTTCGGTGACCCGTCTGCTACCATCAACAACATCCGTAAA
CGTCCGACCCAGGAATCTCAGGAACAGAACAAACCGAACGCTAACAACAACC
CGAACCCGGACTGGGCTCACTGGGACAACGAAACCCAGAACCTGGACACCA
AACACAACCTCTCGTCTGCTGTACTACTACGGTTACCCGAAAGCTGACGGTTCT
5 GGTGTTTCTCTGACCCCGTGGGGTGGTCAGGAACACCAGGAAAAAGTTCGTA
ACCACCAGCAGGACAAACAGTCTACCGGTACCATCA ACGACTCTAACGTTAT
CAAATCTACCACCACCGACTGGGCTTCTTGGACCCCGCAGTCTATCACCTGG
TCTGACTTCACCGAAGCTGCTAACTACAAACAGAACATCGTTCAGGCTGAATC
TAAAGGTGAATCTTACTCTTCTCCGATGTCTTACTCTGAATCTAAACGTCCGC
10 AGACCGGTATCGACAAAGACACCAACCAGGCTCTGAAACCGATCGAAGGTAA
ATCTACCGAACAGAACAACTACCCGCTGCGTAACTCTGACGGTAACCCGCTG
AACCGTAAAGTTCCGACCTCTGACCTGGAATCTCAGGGTGCTCAGTTCTCTAT
CAAAGACACCAAAAACGGTGGTGAAGCTCGTACCTACAACCCGAACCAGGAC
GGTATCAAACGTACGACTCTAACGTTAACGGTACCATCAAACAGGACGCTTA
15 CGACAAAAAATACCTGAACTCTTTCCTCCGGACGGTCAGGCTTTCTACGGTGCT
CCGGCTAACACCTCTTCTGACACCTTCCGTTCTGACGGTCTGGCTACCTCTC
GTGGTTCTCAGTTCGACGGTAACGGTGACCGTATCTCTCTGTCTCCGTGGCA
GGGTTCTACCATGGACACCGACACCTACAAAGACAAACAGGACACCGACTAC
GGTCCGGACTACTCTTACCTGCCGACCACCTCTAAATCTAACGACGCTACCA
20 CCCCAGCTACAAAGCTATCAAAGGTCTGAAACTGTCTAACCCGCTGCGTAA
CGAAAAATCTCGTTTCTTCCCGTACGGTCTGTCTAACAAATCTGTTACCTCT
GTTAACAGTCTCAGTCTGAAATCGAAGTTGAAAAAGACAAACAGTTCGTTGA
CATCCTGGCTACCCAGTACCCGTACCTGGTTTACACCCCGACCGGTCAGCGT
AAAGGTTACGGTCCGAACACCGAAATCCAGAACATCCAGGCTGACACCGACG
25 CTTACATCCCGTCTCGTGAAACCACCATGGTTCCGGCTGGTTCTACCCACGA
CGACAAACCGCTGCCGGACGTTACGCGTATGCTGCGTGACGTTTCTACCTAC
ACCGTTTCTACCGCTAACCTGCAGCCGATCACCGTTAACTCTAACACCTCTGA
CAAAACCGTTACGTTCAACAACCGTGCTGCTAAAGTTGTTGACACCGACCG
CGTGTTACCCGTGGTCAGTACAAAGACGTTGCTAACAAATGGCACGAACTGA
30 ACTCTTTCACCGTTGCTCCGATCAAAGGTACC

SEQ ID No 27: This sequence corresponds to the amino acid sequence of putative ferric siderophore receptor protein (*Acinetobacter baumannii* ATCC 17978; accession number YP_001084684).

SEQ ID No 28: This sequence corresponds to the amino acid sequence of putative ferric hydroxamate siderophore receptor (*Acinetobacter baumannii* ATCC 17978; accession number YP_001084696).

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CLAIMS

1. A composition comprising polypeptide sequence SEQ ID No 28 (putative ferric hydroximate siderophore receptor) or a fragment thereof, wherein the
5 fragment is selected from the list consisting of any of the following sequences: SEQ ID No 12 to SEQ ID No 23 or sequences having at least 85% sequence identity with peptide sequences SEQ ID No 12 to SEQ ID No 23.
- 10 2. The composition of claim 1 which further comprises polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor) or a fragment thereof, wherein the fragment is selected from the list consisting of any of the following sequences: SEQ ID No 1 to SEQ ID No 11 or sequences having
15 at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11.
3. A composition comprising polypeptide sequence SEQ ID No 28 (putative ferric hydroximate siderophore receptor) or a fragment thereof, wherein the
20 fragment is selected from the list consisting of any of the following sequences: SEQ ID No 12 to SEQ ID No 23.
4. The composition of claim 3 which further comprises polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor) or a fragment thereof, wherein the fragment is selected from the list consisting of any of the
25 following sequences: SEQ ID No 1 to SEQ ID No 11.
5. A composition comprising polypeptide sequence SEQ ID No 28 (putative ferric hydroximate siderophore receptor) and polypeptide sequence SEQ ID No 27 (putative ferric siderophore receptor).
- 30 6. A fusion protein comprising peptide sequences of the putative ferric siderophore receptor and of the putative ferric hydroximate siderophore receptor of acinetobacter baumannii, wherein:

- 5 a. the peptide sequences of the putative ferric siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 1 to SEQ ID No 11 or sequences having at least 85% sequence identity with peptide sequences SEQ ID No 1 to SEQ ID No 11; and
- 10 b. the peptide sequences of the putative ferric hydroximate siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 12 to SEQ ID No 23 or sequences having at least 85% sequence identity with peptide sequences SEQ ID No 12 to SEQ ID No 23.
7. The fusion protein of claim 6, wherein:
- 15 a. the peptide sequences of the putative ferric siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 1 to SEQ ID No 11; and
- b. the peptide sequences of the putative ferric hydroximate siderophore receptor include all of the peptides selected from a list of amino acid sequences consisting of sequences SEQ ID No 12 to SEQ ID No 23.
- 20 8. The fusion protein of any of claims 6 or 7, wherein the fusion protein comprises the amino acid sequence SEQ ID No 24.
9. The fusion protein of any of claims 6 to 8, wherein the fusion protein consists of SEQ ID No 25.
10. A nucleic acid encoding the fusion protein of any one of claims 6 to 9
- 25 11. The nucleic acid of claim 10, wherein the nucleic acid is SEQ ID No 26.
12. An expression vector containing the nucleic acid according to any of claims 10 or 11.
13. A host cell comprising the expression vector of claim 12.
14. A pharmaceutical composition comprising the fusion protein according to claims 6-9 or the composition of claims 1 to 5 and optionally a
- 30 pharmaceutically acceptable carrier.
15. The pharmaceutical composition according to claim 14 further comprising an adjuvant.
16. The pharmaceutical composition of any one of claims 14 or 15, wherein said composition is a vaccine.

17. The fusion protein according to any one of claims 6 to 9 or the composition according to any one of claims 1 to 5 for use as a medicament.
18. The fusion protein according to any one of claims 6 to 9 or the composition according to any one of claims 1 to 5 for its use in conferring protection against an infection caused by *A.baumannii* in a subject.
19. The pharmaceutical composition of any one of claims 14 or 15, or the vaccine of claim 16 for its use in conferring protection against an infection caused by *A.baumannii* in a subject.
20. Antibody or active fragment thereof obtained by immunization of a mammal with the composition of any of claims 1-5 or with the fusion protein of any of claims 6 to 9.
21. Antibody or active fragment thereof capable of binding any of the polypeptides as defined in the composition of any of claims 1-5 or capable of binding the fusion protein of any of claims 6 to 9.
22. The antibody or active fragment of claim 20 or 21, for its use as a medicament.
23. The antibody or active fragment of claim 20 or 21, for its use in conferring protection against an infection caused by *A.baumannii* in a subject.
24. A pharmaceutical composition comprising the antibody or active fragment of claim 20 or 21 and optionally a pharmaceutically acceptable carrier.
25. The pharmaceutical composition according to claim 24 further comprising an adjuvant.
26. The pharmaceutical composition of any one of claims 24 or 25, wherein said composition is a vaccine.

1/10

Figures

Figure 1A:

Peptide sequences	Number of amino acids
Putative ferric siderophore receptor (YP_001084684)	
TSSDTFRSDGLA	12
TSRGSQFDGNGDRISLSPWQGSTMDDT	28
YKDKQDTDYGPDYSYLPPTTSKSNDAATTPTYKAIGLKLNSPL	42
RNEKSRFFPYGLSNKSVTSVNQSQSEIEV	29
EKDKQFVDILATQYPYLVYTPTGQRKGYGPNTTEIQN	36
IQADTDAYIPSRETTMVPAGSTHDDKPL	28
PDVQRMLRDVSTYTVSTANLQPITVNS	27
NTSDKTVQFNNRAAKVVDTDQRV	23
TRGQYKDVANKWHELNSFTVAP	22
IKGTNKAYKDDRELAFAFATTQDEAFQNAVKNDA	36
YNVWNRQYRTVFAQQAASVSNANPLLAIPAEGR	33
Putative ferric hydroxamate siderophore receptor (YP_001084696)	
VKGADSLTNAFGDPSATINNIRKRPTQE	28
SGKVRGRIMGYEQTGDSYLDRYSAEKNG	28
SQEQNKPNANN	11
NPNPDWAHWDNETQN	15
LDTKHNSRLLYYYGYPKADGSGVSLTPWGGQEHQEK	36
VRNHQQDKQSTGTINDSNVIKSTTTDWASWTPQSITWSDFTEAANYKQNI	50
VQAESKGESYSSPMSYSESK	20
RPQTGIDKDTNQALKPIEGKS	21
TEQNNYPLRNSDGNPLNRKVPTSDLESQG	29
AQFSIKDTKNGGEARTYNPN	20
QDGIKLYDSNVNGTIKQDAY	20
DKKYLNSFPDGGQAFYGAPAN	20
Total number of peptides	23
Total number of amino acids	614

Figure 1B:

SEQ ID No 25:

MSGKVRGRIMGYEQTGDSYLDRYSAEKNGVKGADSLTNAFGDPSATINNIRKRPT
 QESQEQNKPNANNPNPDWAHWDNETQNLDTKHNSRLLYYYGYPKADGSGV
 SLTPWGGQEHQEKVRNHQQDKQSTGTINDSNVIKSTTTDWASWTPQSITWSDF
 TEAANYKQNIQVQAESKGESYSSPMSYSESKRPQTGIDKDTNQALKPIEGKSTEQ
 NNYPLRNSDGNPLNRKVPTSDLESQGAQFSIKDTKNGGEARTYNPNQDGIKLYD
 SNVNGTIKQDAYDKKYLNSFPDGGQAFYGAPAN

2/10

NGDRISLSPWQGSTMDTDYKDKQDTDYGPDYSYLPTTSKSNDAATTPTYKAIKG
LKLSNPLRNEKSRFFPYGLSNKSVTSVNQSQSEIEVEKDKQFVDILATQYPYLVYT
PTGQRKGYGPNTEIQNIQADTDAYIPSRETTMVPAGSTHDDKPLPDVQRMRLRDV
STYTVSTANLQPITVNSNTSDKTVQFNNRAAKVVDTDQRVTRGQYKDVANKWH
ELNSFTVAPIKGTNKAYKDDRELAAFATTQDEAFQNAVKNDAANSAYNVWNRQYR
TVFAQQAASVSNANPLLAIPAEGRT*

SEQ ID NO: 26:

ATGTCTGGTAAAGTTCGTGGTCGTATCATGGGTACGAACAGACCGGTGACC
TTACCTGGACCGTTACTCTGCTGAAAAAACGGTGTTAAAGGTGCTGACTCTC
TGACCAACGCTTTCGGTGACCCGTCTGCTACCATCAACAACATCCGTAAACG
TCCGACCCAGGAATCTCAGGAACAGAACAAACCGAACGCTAACAACAACCCG
AACCCGGACTGGGCTCACTGGGACAACGAAACCCAGAACCTGGACACCAAA
CACAACCTCTCGTCTGCTGTACTACTACGGTTACCCGAAAGCTGACGGTTCTG
GTGTTTCTCTGACCCCGTGGGGTGGTCAGGAACACCAGGAAAAAGTTCGTAA
CCACCAGCAGGACAAACAGTCTACCGGTACCATCAACGACTCTAACGTTATC
AAATCTACCACCACCGACTGGGCTTCTTGACCCCGCAGTCTATCACCTGGT
CTGACTTCACCGAAGCTGCTAACTACAAACAGAACATCGTTCAGGCTGAATCT
AAAGGTGAATCTTACTCTTCTCCGATGTCTTACTCTGAATCTAAACGTCCGCA
GACCGGTATCGACAAAGACACCAACCAGGCTCTGAAACCGATCGAAGGTAAA
TCTACCGAACAGAACAACTACCCGCTGCGTAACCTCTGACGGTAACCCGCTGA
ACCGTAAAGTTCCGACCTCTGACCTGGAATCTCAGGGTGCTCAGTTCTCTATC
AAAGACACCAAAAACGGTGGTGAAGCTCGTACCTACAACCCGAACCAGGACG
GTATCAAACCTGTACGACTCTAACGTTAACGGTACCATCAAACAGGACGCTTAC
GACAAAAAATACCTGAACTCTTCCCGGACGGTCAGGCTTTCTACGGTGCTC
CGGCTAACACCTCTTCTGACACCTTCCGTTCTGACGGTCTGGCTACCTCTCG
TGGTTCTCAGTTCGACGGTAACGGTGACCGTATCTCTCTGTCTCCGTGGCAG
GGTTCTACCATGGACACCGACACCTACAAAGACAAACAGGACACCGACTACG
GTCCGGACTACTCTTACCTGCCGACCACCTCTAAATCTAACGACGCTACCAC
CCCGACCTACAAAGCTATCAAAGGTCTGAAACTGTCTAACCCGCTGCGTAAC
GAAAAATCTCGTTTCTTCCCGTACGGTCTGTCTAACAAATCTGTTACCTCTGTT
AACCAGTCTCAGTCTGAAATCGAAGTTGAAAAAGACAAACAGTTCGTTGACAT
CCTGGCTACCCAGTACCCGTACCTGGTTTACACCCCGACCGGTCAGCGTAAA
GGTTACGGTCCGAACACCGAAATCCAGAACATCCAGGCTGACACCGACGCTT
ACATCCCGTCTCGTGAAACCACCATGGTTCCGGCTGGTTCTACCCACGACGA
CAAACCGCTGCCGGACGTTACGCGTATGCTGCGTGACGTTTCTACCTACACC
GTTTCTACCGCTAACCTGCAGCCGATCACCGTTAACTCTAACACCTCTGACAA
AACCGTTCAGTTCAACAACCGTGCTGCTAAAGTTGTTGACACCGACCGAGCGT

GTTACCCGTGGTCAGTACAAAGACGTTGCTAACAAATGGCACGAACTGAACT
CTTTCACCGTTGCTCCGATCAAAGGTACC

Figure 2

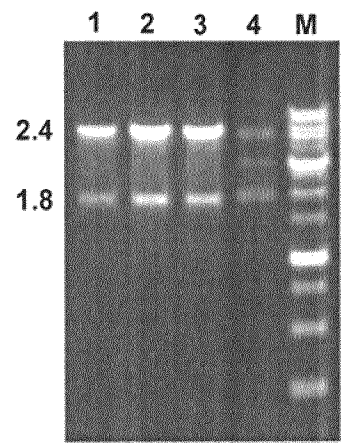


Figure 3

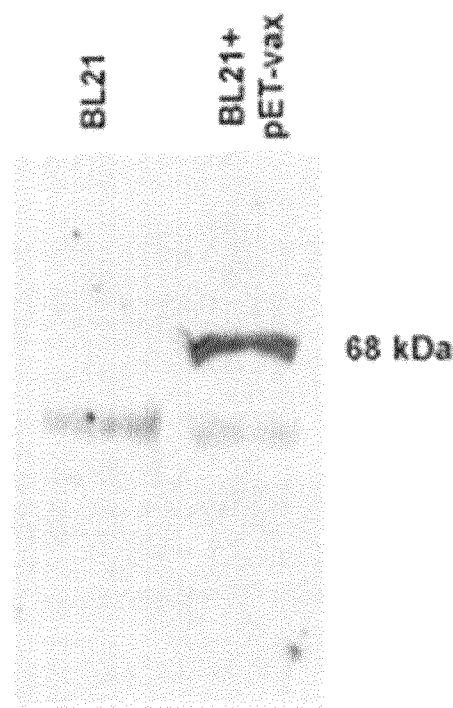
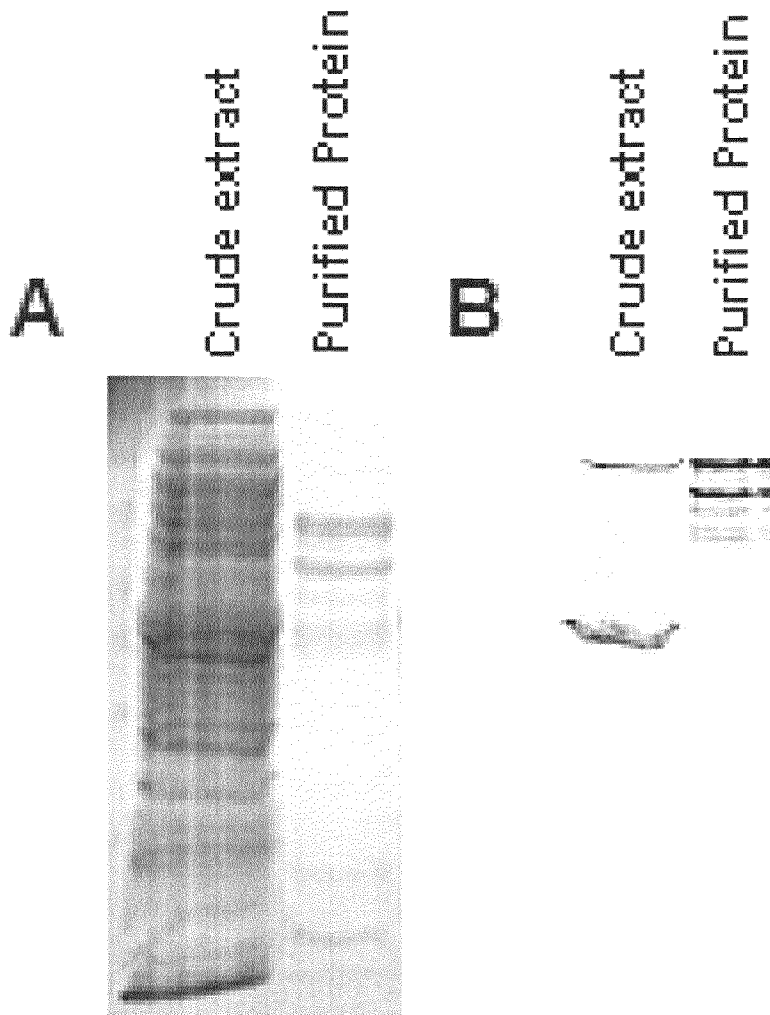
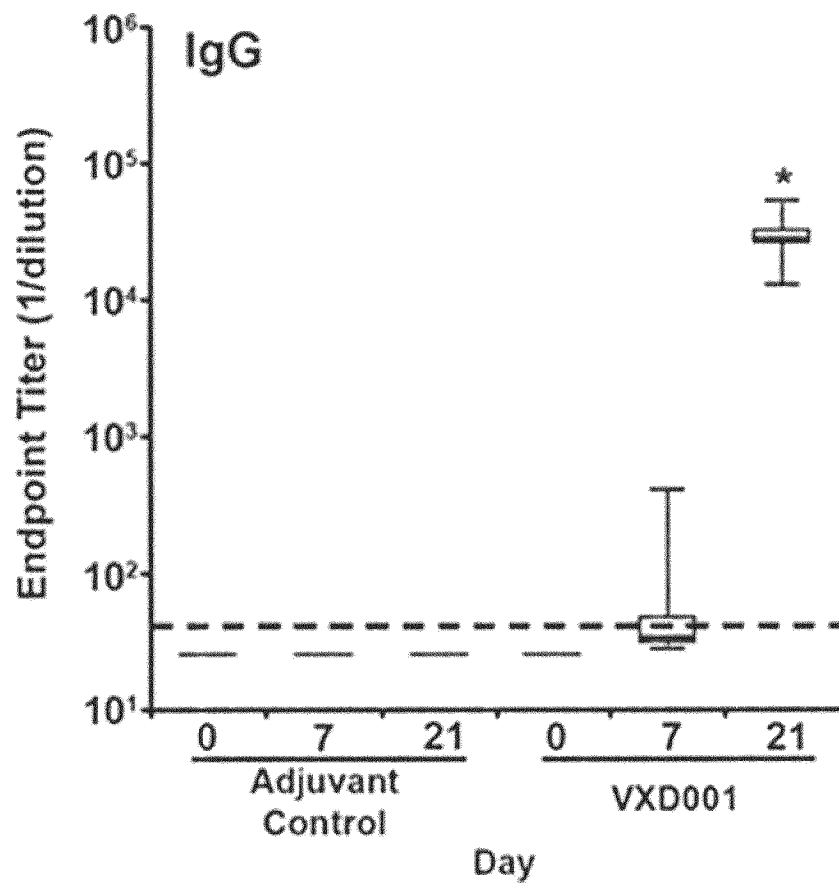


Figure 4



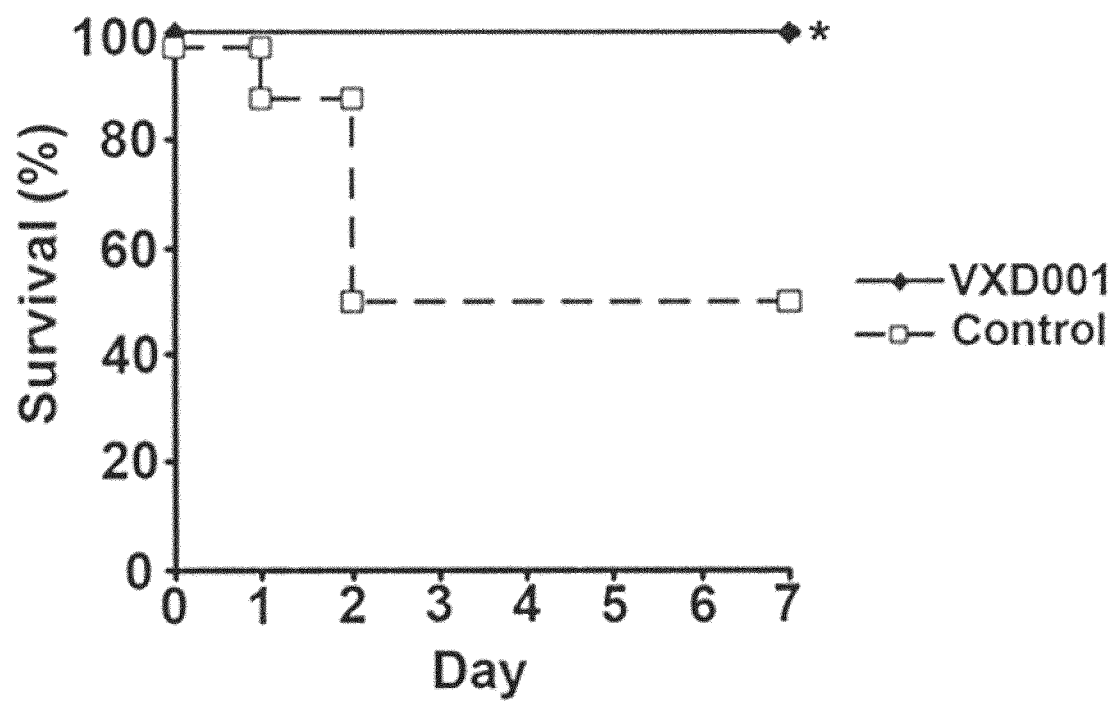
6/10

Figure 5



7/10

Figure 6



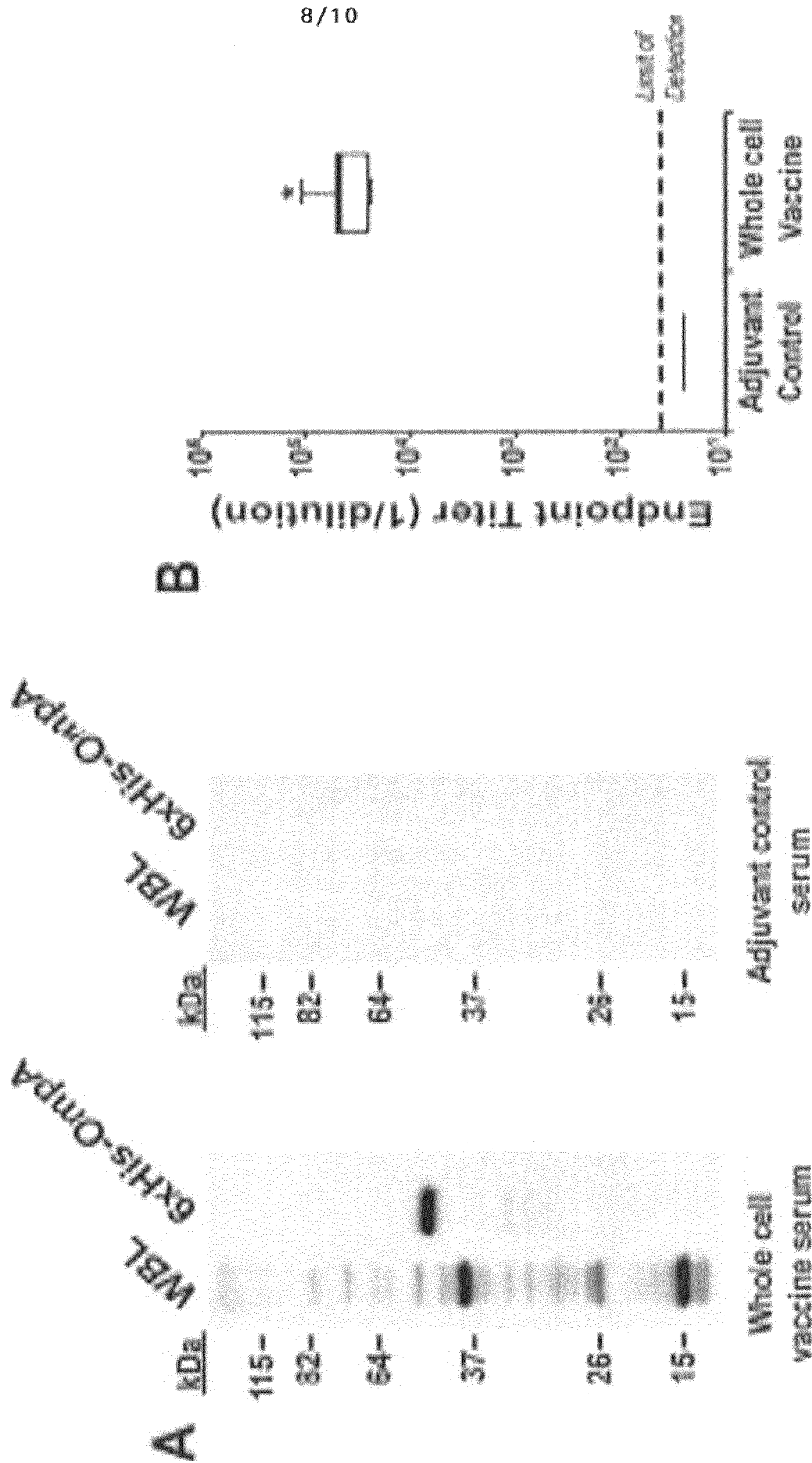
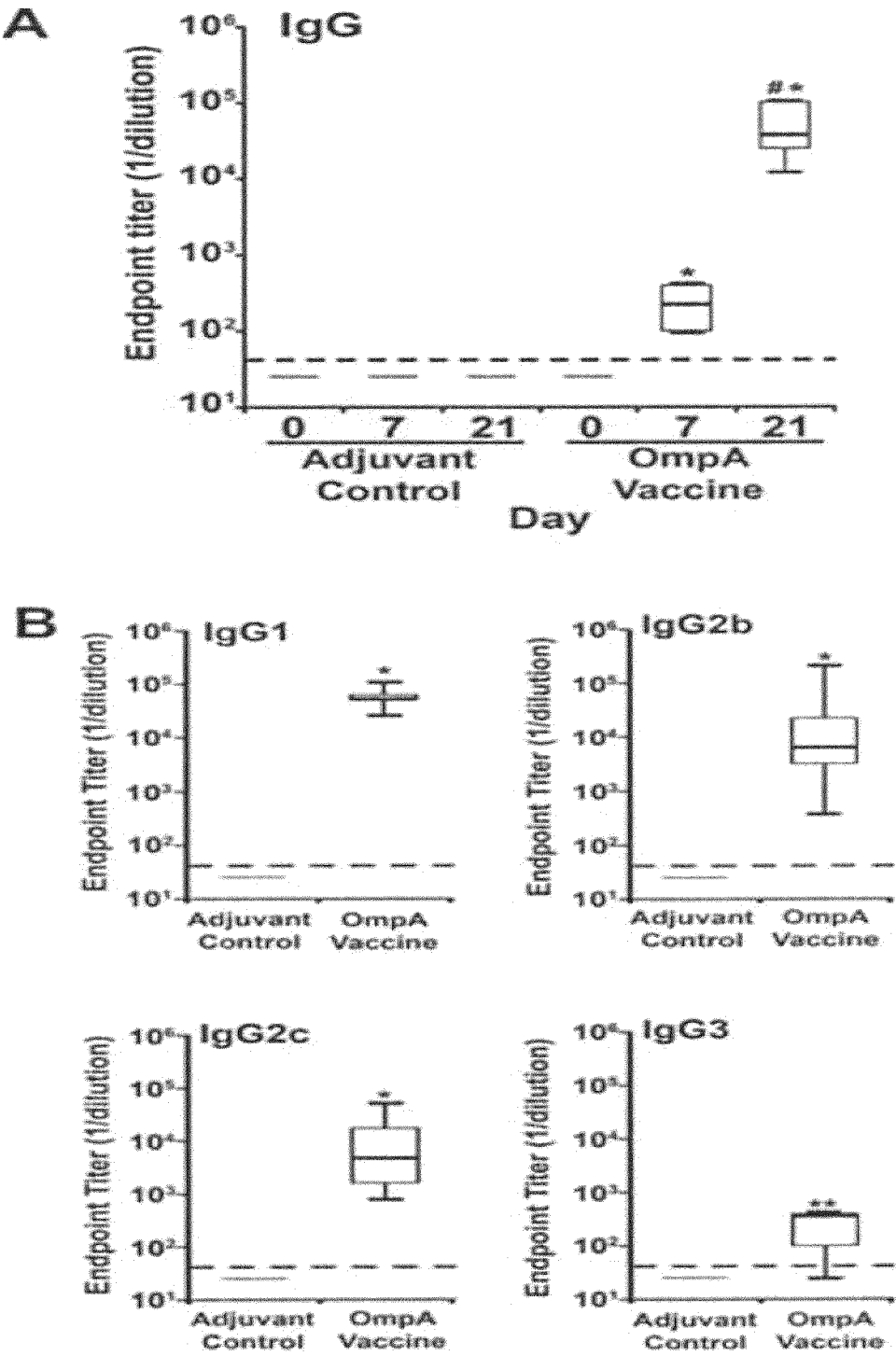


Figure 7

Figure 8



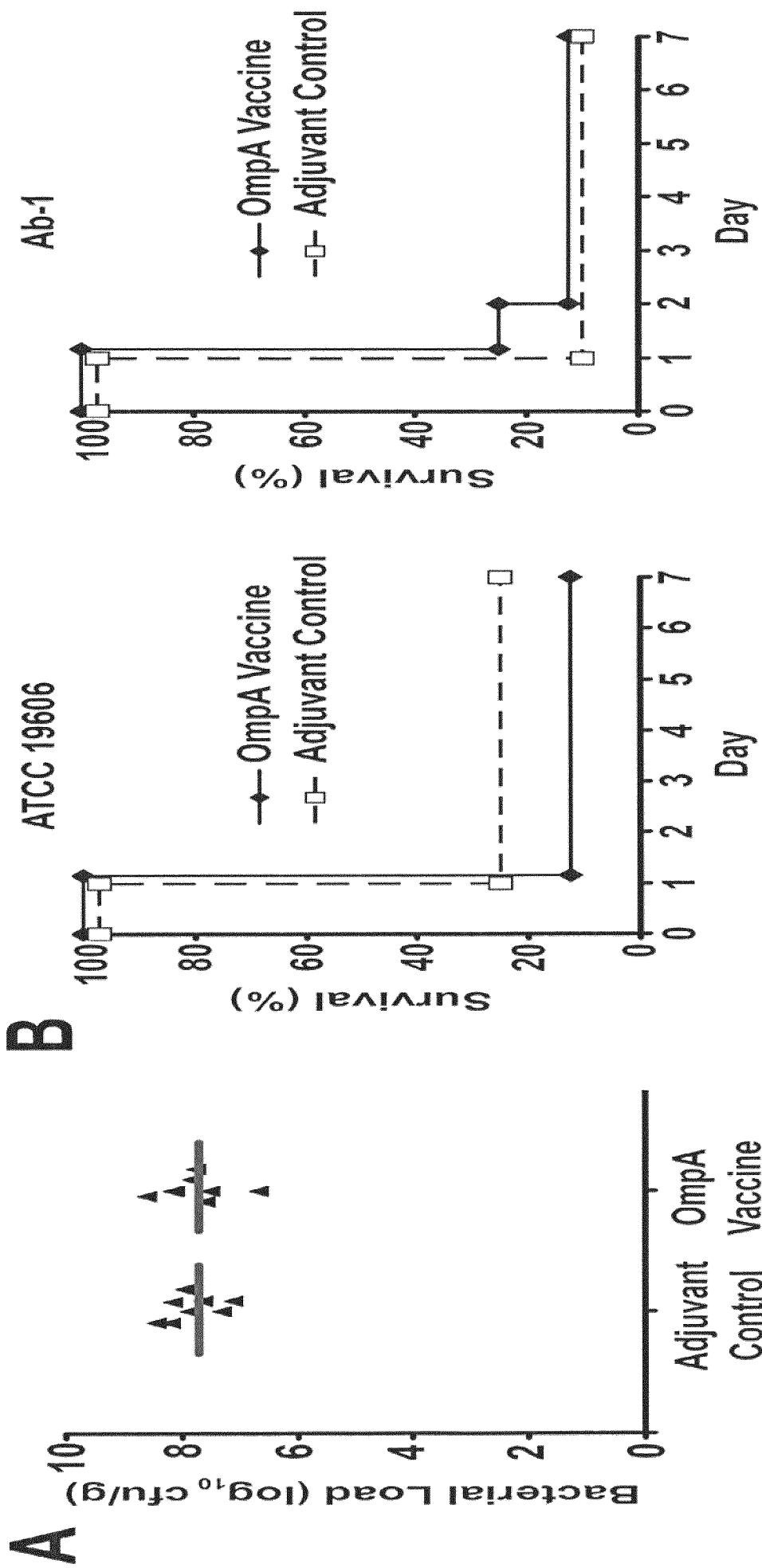


Figure 9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/069945A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/16
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, Sequence Search, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	-& DATABASE Geneseq [Online] 22 December 2011 (2011-12-22), "A. baumannii vaccine-related outer membrane protein, SEQ ID:19.", XP002693286, retrieved from EBI accession no. GSP:AZP28245 Database accession no. AZP28245 the whole document ----- -/-	1-26



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

12 December 2013

Date of mailing of the international search report

14/01/2014

Name and mailing address of the ISA/

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Merlos, Ana Maria

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/069945

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International application No
PCT/EP2013/069945

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2013/069945

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US 6562958	B1	13-05-2003	NONE

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