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(54) Title: *S. PNUEMONIAE* TRANSCYTOSIS PROTEIN

(57) Abstract: The present invention relates to a vaccine formulation providing protection against infection of a subject by a meningeal pathogen, said formulation comprising an effective amount of the protein MgtA or an immunogenic part thereof together with at least one of a pharmaceutically acceptable diluent, carrier, excipient or adjuvant therefore.

Title: *S. pneumoniae* transcytosis protein

FIELD OF THE INVENTION

The present invention is in the field of prophylactic or therapeutic treatment of an infectious diseases, in particular of infections by a meningeal pathogen. The invention further relates to vaccine formulations and pharmaceutical compositions for use in such methods as described in more defined herein below.

BACKGROUND OF THE INVENTION

Infection with the Gram-positive bacterium *Streptococcus pneumoniae* is a common cause of respiratory disease and serious invasive diseases such as pneumonia, sepsis and meningitis. Especially infants, the elderly and immuno-compromised individuals are at risk. Annually, over 1 million children die of pneumonia and meningitis caused by *Haemophilus influenza* (Gram negative), *Neisseria meningitides* (Gram negative) or *S. pneumoniae (Gram positive)*, and in the US alone 40.000 of these are caused by pneumonia or meningitis caused by *S. pneumoniae*. In addition, this pneumonococcal meningitis has a high mortality and morbidity rate compared to other pathogenic bacteria.

The pathway of infection for any etiological agent of meningitis begins with the establishment of localized infection in the host. Most meningeal pathogens are transmitted via the respiratory route. The pathogen must then evade host immune responses and enter the submucosa. Finally, the infectious agent must cross the blood-brain barrier to cause meningitis and access the cerebrospinal fluid (CSF). This is most often accomplished by invasion of the bloodstream and subsequent seeding of the central nervous system (CNS).

The integrity of body compartments is maintained in part by boundaries of epithelial and endothelial cells. For instance, microvascular endothelial cells form the major substrate of the blood brain barrier and *S. pneumoniae* can only cause meningitis if it traverses the microvascular endothelial cells. Thus, in order to become invasive, *S. pneumoniae* has to cross epithelial or endothelial cell layers, which occurs through a specific and receptor mediated processes called transcytosis (See below).

The specific receptor for *S. pneumoniae* on epithelial cells is the polymeric immunoglobulin receptor (pIgR) and on endothelial cells it is the platelet activating factor receptor (PAFR). Invasion takes place after binding to the receptors, when the bacteria are engulfed by either cell type and end up in a vesicle inside the eukaryotic cells. Generally, as soon as the vesicles are formed, conditions within change rapidly as divalent cations and other nutrients are pumped out. The vesicles are either recycled back to the cell surface or fuse with the lysosome, after which noxious substances are released that destroy the contents. However, not all vesicles containing *S. pneumoniae* fuse with the lysosome but most are circulated for some time within the cell and eventually transported to the other side of the cells where the contents are released. In this way the bacteria are transcytosed and have become invasive.

Adhesion to eukaryotic cells and interaction with the receptors is mediated primarily by phosphorylcholine and the choline binding protein A (CbpA). However, virtually nothing is known about which genes of *S. pneumoniae* play a role in the invasion, let alone during transcytosis. Without wishing to be bound by any theory, it is believed that - although the bacteria do not replicate within the eukaryotic cells - they have an active response to ensure that they survive the conditions within the vesicles and are transcytosed. *S. pneumoniae* strains that are more likely to cause invasive disease differ from others, which strongly suggests that there are specific genetic determinants that predispose to transcytosis. In addition, there are indications that invasion of eukaryotic cells by related streptococcal species

requires an active response. Altogether, this strongly suggests that *S. pneumoniae* has an active response to the translocation of endothelial cells and that its disruption will cripple the ability of *S. pneumoniae* to cause serious invasive disease.

A significant stress encountered by *S. pneumoniae* within the endothelial cell vesicles is probably Mg^{2+} limitation. Conditions within eukaryotic vesicles are not precisely known, but several bacteria experience Mg^{2+} limitation inside eukaryotic cells and induce the expression of Mg^{2+} transporters. More importantly, mutants in Mg^{2+} homeostasis genes in a wide variety of bacteria are less virulent and impaired in their ability to survive within eukaryotic cells. Several transporters are involved in Mg^{2+} homeostasis and their induction upon Mg^{2+} limitation is under complex regulatory control. During transcytosis, *S. pneumoniae* has to survive inside the endosomal vesicles, which qualifies as a low Mg^{2+} environment. Therefore it is conceivable that Mg^{2+} uptake is essential for this process. The response to Mg^{2+} limitation is important for virulence as mutation of the involved genes almost invariably leads to virulence defects and specifically to an inability to survive in eukaryotic cells. In Salmonella and other bacteria, several transporters are involved in Mg2+ homeostasis. One is the primary transporter CorA, which is thought to be a constitutively active pore in the membrane and whose expression is not regulated. In contrast, the ABC transporters MgtA and MgtB are induced upon encountering Mg^{2+} limiting conditions and import Mg^{2+} against the concentration gradient. Of particular interest is MgtC, a small membrane protein, whose function is unknown but is involved in Mg^{2+} homeostasis and has an essential role in virulence and intracellular survival in Salmonella and other bacteria. The induction upon Mg^{2+} limitation of *mgtA*, *mgtB* and *mgtC* genes is under complex regulatory control of several transcription factors that respond to the host environment. Analysis of the published *S. pneumoniae* genomes revealed that they contain homologues of these transporters. In this study we examined the role of one of these

transporters in *S. pneumoniae* Mg^{2+} homeostasis and in the interaction of the bacteria and Human Brain Microvascular Endothelial Cells (HBMEC).

SUMMARY OF THE INVENTION

The present inventors have now found that the *mgtA* gene encodes the main Mg^{2+} uptake mechanism in *S. pneumoniae,* have shown that this gene product plays a role in adhesion and invasion, and further have shown that expression of this gene is essential for normal growth and transcytosis of the cells, i.e. for the traversal of the endothelial cell layer. Predictive analysis shows that the *mgtA* gene product, the MgtA protein, is a membrane protein. This means that MgtA is immunogenic and accessible for antibodies and thus suitable for use as a vaccine. Also, it has been discovered that antibodies against MgtA recognize the bacteria and will protect the host against infection, either directly through immune clearance, or indirectly by blocking the activity of the protein, thereby inhibiting the growth of the bacteria. As a secondary effect, blocking the function of the protein reduces the adhesion abilities of the cells which reduces colonisation and invasion. Further, blocking of the activity will inhibit the ability of the bacteria to transcytosis and thus to cause invasive disease. The invention thus relates to MgtA-based vaccines as well as to anti-MgtA antibodies, both of which may be used in prophylactic or therapeutic treatment of an infectious diseases, in particular of infections by a meningeal pathogen, wherein the MgtA is derived from *S. pneumoniae* or homologs thereof.

In a first aspect, the present invention relates to a vaccine formulation providing protection against infection of a subject by a meningeal pathogen, said formulation comprising an effective amount of the protein MgtA or an immunogenic part thereof together with at least one of a pharmaceutically acceptable diluent, carrier, excipient or adjuvant therefore.

In a preferred embodiment, the immunogenic part is an antigenic determinant of the pathogen.

In another preferred embodiment, the formulation comprises an essentially cell-free preparation of said pathogen comprising said protein or said part thereof.

In another preferred embodiment, the immunogenic part of the protein MgtA comprises a part that is exposed on the membrane of said pathogen.

In still another preferred embodiment, the vaccine provides protections against meningitis, pneumonia, and/or sepsis caused by a meningeal pathogen. In a particularly preferred embodiment, the vaccine provides protections against meningeal streptococci, preferably selected form the group consisting of β-haemolytic *Streptococcus* spp. belonging to Lancefield group C, *S. acidominimus, S agalactiae, S. bovis, S. constellatus, S. equinus, S. equisimilis, S. mitis, S. oralis, S. pneumoniae, S. pyogenes, S. salivarius, S. sanguis, S. suis* and *S. uberis*, more preferably selected form the group consisting of *S agalactiae, S. pneumoniae, S. pyogenes, S. suis* and *S. uberis*.

In another aspect, the present invention relates to the protein MgtA or an immunogenic part thereof, for use as a vaccine.

In yet another aspect, the present invention relates to an anti-MgtA antibody or fragment thereof, preferably a humanized anti-MgtA antibody or fragment thereof.

In still another aspect, the present invention relates to an anti-MgtA antibody or fragment thereof, preferably a humanized anti-MgtA antibody or fragment thereof, for use as a medicament for the prophylactic or therapeutic treatment of an infection by a meningeal pathogen in a subject.

In still another aspect, the present invention relates to the use of anti-MgtA antibody or fragment thereof, preferably a humanized anti-MgtA antibody or fragment thereof, for the manufacture of a medicament for the prophylactic or therapeutic treatment of an infection by a meningeal pathogen in a subject.

In a further aspect, the present invention provides a pharmaceutical composition comprising an anti-MgtA antibody or fragment thereof, preferably

a humanized anti-MgtA antibody or fragment thereof, and a pharmaceutically acceptable carrier.

In still a further aspect, the present invention provides a method for prophylactic or therapeutic treatment of an infection by a meningeal pathogen in a subject comprising administering to a subject in need of such treatment an effective amount of a vaccine formulation according to the invention and/or an effective amount of a pharmaceutical composition as defined herein above.

In still a further aspect, the present invention provides a method for preparing an MgtA-based vaccine formulation, the said method comprising bringing into association, an effective amount of the protein MgtA or an immunogenic part thereof and at least one of a pharmaceutically acceptable diluent, carrier, excipient or adjuvant therefore.

In still a further aspect, the present invention provides a method for preparing a pharmaceutical composition as defined herein above, the said method comprising bringing into association, an effective amount of an anti-MgtA antibody, preferably a humanized anti-MgtA antibody, or fragment thereof, and a pharmaceutically acceptable carrier.

DESCRIPTION OF THE DRAWINGS

Figure 1 exemplifies the transcytosis process. A: Transcytosis is a specific and receptor mediated process. B: The transcytosis system. Endothelial cells (blue rectangles) are grown to a confluent monolayer on a membrane (Transwell® permeable supports, Corning Incorporated, Corning New York). Only bacteria that are able to transcytose are able to reach the lower compartment. Subsequently, the membrane with the cells and non-transcytosed bacteria will be removed and the amount of translocated bacteria can be analyzed.

Figure 2 displays the chromosomal organization of D39 and D39Δ*cps*. A: Chromosomal organization of D39 before integration of kanR in the capsule

locus. B: Chromosomal organization of D39 after integration of kanR in the capsule locus. kanR: kanamycin resistance gene.

Figure 3 displays the chromosomal organisation of D39Δ*cps* and D39Δ*cps*Δ*mgtA*. A: Chromosomal organisation of D39Δ*cps*, before transformation and integration of the PCR product containing the *mgtA* knockout. B: Chromosomal organisation of D39Δ*cps*, after transformation and integration of the PCR product containing the replacement of *mgtA* by the erythromycin resistance gene. eryR: erythromycin resistance gene.

Figure 4 shows the map of the plasmid pNZ8048::*mgtA* containing *mgtA* under control of PnisA. The complete *mgtA* gene was ligated into the plasmid by making use of NcoI and XbaI restriction sites.

Figure 5: *MgtA* is the main Mg^{2+} importer for *S. pneumoniae* under normal growth conditions. Growth of the wildtype (black squares) in GM17 compared to growth of the mutant in GM17 (black circles), GM17 containing 5 mM $MgCl_2$ (white circles) and 50 mM $MgCl_2$ (grey circles). Results are the mean of a duplicate experiment + the standard deviation and are representative of at leas three experiments performed in duplicate.

Figure 6: *MgtA* specifically transports Mg^{2+} in *S. pneumoniae* under normal growth conditions. Growth of the wildtype (black squares) in GM17 compared to growth of the mutant in GM17 (black circles), GM17 containing 5 mM $MgCl_2$ (white circles), GM17 containing 5 mM $CaCl_2$ (black triangles), GM17 containing 5 mM $MnCl_2$ (white diamonds), GM17 containing 50 μM $CuCl_2$ (white triangles) and GM17 containing 50 μM $ZnCl_2$ (black diamonds). Results are the mean of a duplicate experiment plus the standard deviation and are representative of at least three experiments performed in duplicate.

Figure 7: *MgtA* is the main Mg^{2+} importer for strain D39Δ*cps* under normal growth conditions and heterologous expression of the *mgtA* gene fully restores growth in GM17 in D39Δ*cps*. Growth of the wildtype (black squares) in GM17 compared to growth of the mutant in GM17 (black circles), GM17 containing 5 mM $MgCl_2$ (white circles), compared to the growth of

D39NisRKΔ*cps*Δ*mgtA* pNZ8048 (black triangles) and D39NisRKΔ*cps*Δ*mgtA* pNZ*mgtA* (white triangles).

Figure 8: Magnesium homeostasis of *S. pneumoniae* is essential for transcytosis. **A**: The Δ*mgtA* mutant has a similar adhesion to, invasion and survival in HBMEC as the parent strain. Adhesion, invasion and survival after 5 hrs of strains D39Δ*cps* (black bars) and D39Δ*cps*Δ*mgtA* (white bars). Results are the mean of three independent experiments, vertical bars represent the standard deviations. **B**: Appearance over 5 hours of strains strains D39Δ*cps* (black bars) and D39Δ*cps*Δ*mgtA* (white bars) in the lower compartment. Results are the mean of two independent experiments, vertical bars represent the standard deviations **C**: Appearance in the lower compartment of D39Δ*cps* *bgaA*::NisRK (black bars), D39Δ*cps* *bgaA*::NisRK Δ*mgtA* pNG8048E (white bars), D39Δ*cps* *bgaA*::NisRK Δ*mgtA* p*mgtA*. **D**: Appearance of D39D*cps* while 10 mM MgCl2 is added to the tissue culture medium (black bars), D39Δ*cps*Δ*mgtA* when no extra $MgCl_2$ is added to the medium (white bars) and the same strain when 10 mM $MgCl_2$ is present in the tissue culture medium during the assay (grey bars).

Figure 9: Appearance in the lower compartment over time of the total amount of bacteria (left part) and the *mgtA* mutant (right part) over time.

Fig. 10 shows two *S. pneumoniae* amino acid sequences coding for MgtA. Shown are the protein of S. *pneumoniae* TIGR4, indicated as SP1551 (TIGR code) and the protein of *S. pneumoniae* D39, indicated as SPD 1383

Fig 11 shows the corresponding nucleic acid sequence encoding the protein sequences displayed in Fig. 10.

Fig. 12 provides the Clustal-W alignment of SP1551 with highly homologous proteins.

M5005_Spy0516 : *Streptococcus pyogenes MGAS5005* Primary Locus: M5005_Spy0516 | TIGR Locus: NT10SP0571 | SWISS-PROT/TrEMBL AC: Q9A0T9 | GenBank ID: AAZ51134.1

SPy0623 : *Streptococcus pyogenes SF370 serotype M1* Primary Locus: SPy0623 | TIGR Locus: NT01SP0493 | SWISS-PROT/TrEMBL AC: Q9A0T9 | GenBank ID: AAK33594.1

spyM18_0689 : *Streptococcus pyogenes MGAS8232* Primary Locus: spyM18_0689 | TIGR Locus: NT03SP0621 | SWISS-PROT/TrEMBL AC: Q8P1V8 | GenBank ID: AAL97362.1

MGAS2096_Spy0528: *Streptococcus pyogenes MGAS2096*: Primary Locus: MGAS2096_Spy0528 | TIGR Locus: NT18SP0572 | SWISS-PROT/TrEMBL AC: Q1JCS8 | GenBank ID: ABF35580.1

MGAS9429_Spy0507 : *Streptococcus pyogenes MGAS9429* Primary Locus: MGAS9429_Spy0507 | TIGR Locus: NT19SP0543 | SWISS-PROT/TrEMBL AC: Q1JMQ4 | GenBank ID: ABF31695.1

M28_Spy0495 : *Streptococcus pyogenes MGAS6180* Primary Locus: M28_Spy0495 | TIGR Locus: NT13SP0547 | SWISS-PROT/TrEMBL AC: Q48UJ7 | GenBank ID: AAX71609.1

MGAS10750_Spy0535 : *Streptococcus pyogenes MGAS6180* Primary Locus: M28_Spy0495 | TIGR Locus: NT13SP0547 | SWISS-PROT/TrEMBL AC: Q48UJ7 | GenBank ID: AAX71609.1

MGAS10270_Spy0510 : *Streptococcus pyogenes MGAS10270* MGAS10270_Spy0510 | TIGR Locus: NT16SP0551 | SWISS-PROT/TrEMBL AC: Q1JHU8 | GenBank ID: ABF33575.1

M6_Spy0537: *Streptococcus pyogenes MGAS10394* Primary Locus: M6_Spy0537 | TIGR Locus: NT11SP0590 | SWISS-PROT/TrEMBL AC: Q5XD41 | GenBank ID: AAT86672.1

SpyM3_0440 *Streptococcus pyogenes MGAS315* Primary Locus: SpyM3_0440 | TIGR Locus: NT04SP0525 | SWISS-PROT/TrEMBL AC: Q8K867 | GenBank ID: AAM79047.1

SAJ_0627 *Streptococcus agalactiae 18RS21* Primary Locus: None | TIGR Locus: SAJ_0627 | SWISS-PROT/TrEMBL AC: None | GenBank ID: None

SAK_0664 *Streptococcus agalactiae A909* Primary Locus: None | TIGR Locus: SAK_0664 | SWISS-PROT/TrEMBL AC: Q3K2F7 | GenBank ID: ABA45439.1

SAM_0540 *Streptococcus agalactiae CJB111* Primary Locus: None | TIGR Locus: SAM_0540 | SWISS-PROT/TrEMBL AC: None | GenBank ID: None

SAN_061 *Streptococcus agalactiae COH1* Primary Locus: None | TIGR Locus: SAN_0617 | SWISS-PROT/TrEMBL AC: None | GenBank ID: None

SAG_0514 *Streptococcus agalactiae 2603V/R* Primary Locus: None | TIGR Locus: SAG_0514 | SWISS-PROT/TrEMBL AC: Q8E148 | GenBank ID: AAM99416.1

SP_1551 *Streptococcus pneumoniae TIGR4* Primary Locus: None | TIGR Locus: SP_1551 | SWISS-PROT/TrEMBL AC: Q97PQ2 | GenBank ID: AAK75638.1

SPN05340: *Streptococcus pneumoniae G54* Primary Locus: SPN05340 | TIGR Locus: NT05SP1364 | SWISS-PROT/TrEMBL AC: None | GenBank ID: None.

spr1410 *Streptococcus pneumoniae R6* Primary Locus: spr1410 | TIGR Locus: NT02SP1528 | SWISS-PROT/TrEMBL AC: Q8DP20 | GenBank ID: AAL00214.1

SMT0794: *Streptococcus mitis NCTC 12261* Primary Locus: None | TIGR Locus: SMT0794 | SWISS-PROT/TrEMBL AC: None | GenBank ID: None

Fig 13 shows the production of 6His-tagged MgtA in *L. lactis* (13A) and purified protein (13B).

Fig. 14 shows the presence of antibodies in the serum of vaccinated sheep.

Fig. 15 shows the presence of purified antibody.

Fig. 16 shows the antibody reactivity with several strains of *S. pneumoniae.*

Fig. 17 shows the antibody reactivity with other bacterial species (*L. lactis, S. epidermidis* and *S. aureus*).

Fig. 18 shows the antibody reactivity with other bacterial species (*S. pyogenes*).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The term "MgtA" refers to a gene product necessary for the uptake of Mg^{2+} and translocation of endothelial cell layers by *S. pneumoniae.* The amino acid sequence is depicted in Figure 10. Functional homologues of the protein are present in other meningeal pathogens.

The term "meningeal pathogen" refers to any bacterial species capable of causing meningitis in a mammal. In addition thereto, or alternatively, the term refers to any bacterial species of which the *mgtA* gene product, or a functional homologue thereof, is a virulence factor. Examples of meningeal pathogens include, but are not limited to *Streptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Neisseria gonorrhoea, Escherichia coli* (in particular strain K1), *Listeria monocytogenes, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella* spp., *Shigella* spp., *Clostridium* spp. and *Acinetobacter calcoaceticus.* These species are known to be associated with meningitis in mammals. Also, many of these bacteria comprise proteins that are functional MgtA homologues (i.e. that have homologous amino acid sequences; see below). The term is thus not restricted to bacteria causing meningitis, but also includes reference to bacteria in which

an *mgtA* gene product is expressed which is known to play a role in the process of Mg^{2+} uptake and /or transcytosis.

A "virulence factor" is referred to herein as a property of a pathogen that allows it to cause disease. Virulence factors may distinguish a pathogenic microorganism from otherwise identical non-pathogenic microorganisms by allowing pathogens to invade, adhere to, and colonize a host, and then harm the host, as for an organism to be pathogenic it must be able to invade a host, multiply in the host, evade host defences, and harm the host in some way. As used herein *mgtA* gene product or a functional homologue thereof, is a virulence factor.

The terms "invade" and "invasion" refer to the growing of infections into tissues, i.e., through and then beneath epithelial tissues in particular it encompasses the process of transcytosis.

The term "transcytosis" is used herein in its art-recognized meaning (see e.g. also e.g. Ring A et al. *J. Clin. Invest* **102**, 347 (1998); Zhang JR et al., *Cell* **102**, 827 (2000)) and refers to the transport of macromolecules and bacterial cells from one side of a cell to the other within a membrane-bounded carrier(s). Transcytosis is used by multicellular organisms to selectively move material between two different environments while maintaining the distinct compositions and integrity of those environments (see Figure 1A).

The terms "meningeal streptococcus" and its plural "meningeal streptococci" are used to indicate *Streptococcus* spp. that are capable of causing meningitis, i.e. that are capable of transcytosis. Examples of meningeal streptococci are β-haemolytic streptococci belonging to Lancefield Group C, *S. acidominimus*, *S agalactiae* (a.k.a. β-haemolytic streptococci belonging to Lancefield Group B), *S. bovis*, *S. constellatus*, *S. equinus*, *S. equisimilis*, *S. mitis*, *S. oralis*, *S. pneumoniae*, *S. pyogenes* (a.k.a. β-haemolytic streptococci belonging to Lancefield Group A), *S. salivarius*, *S. sanguis*, *S. suis* and *S. uberis*. In preferred embodiments of aspects of the present invention, the meningeal streptococcus is selected from the group consisting of *S agalactiae*,

S. pneumoniae, S. pyogenes, S. suis and *S. uberis*. Most preferably, it is *S. pneumoniae*.

The term "functional homologue" refers to a protein which retains the activity of the functional MgtA protein and preferably has a sequence homology of at least 60%. The homology is preferably determined using BLAST analysis, well known in the art. It is further preferred that the homologue has at least 70%, more preferably at least 80%, still more preferably at least 90% and most preferably 95% sequence identity to the sequence as displayed in Fig. 10. Further comprised in functional homologues are sequences having the activity of a functional MgtA protein (i.e. a bacterial Mg^{2+} ATP-ase) and having the so-called mgtA domain

D K T G T [LIVM] [TI]

Polypeptides have "homologous" sequences if the sequence of amino acids in the two sequences is the same when aligned for maximum correspondence as described herein. Sequence comparison between two or more polypeptides is generally performed by comparing portions of the two sequences over a comparison window to identify and compare local regions of sequence similarity. The comparison window is generally from about 7 to 1000 contiguous amino acids. The "percentage of sequence homology" for polypeptides, such as 50, 60, 70, 80, 90, 95, 98, 99 or 100 percent sequence homology may be determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the amino acid sequence in the comparison window may include additions or deletions (i.e. gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by: (a) determining the number of positions at which the identical amino acid occurs in both sequences to yield the number of matched positions; (b) dividing the number of matched positions by the total number of positions in the window of comparison; and (c) multiplying the result by 100 to yield the percentage of sequence homology. Optimal alignment of sequences for

comparison may be conducted by computerized implementations of known algorithms, or by inspection. Fully analogous to amino acid sequence comparison, nucleic acid sequence comparison may occur to determine sequence homology between two or more polynucleotides, for instance the polynucleotides encoding the MgtA protein. Readily available sequence comparison and multiple sequence alignment algorithms are, respectively, the Basic Local Alignment Search Tool (BLAST) (Altschul, S.F. et al. 1990. J. Mol. Biol. 215:403; Altschul, S.F. et al. 1997. Nucleic Acid Res. 25:3389-3402) and ClustalW programs both available on the internet. Other suitable programs include GAP, BESTFIT and FASTA in the Wisconsin Genetics Software Package (Genetics Computer Group (GCG), Madison, WI, USA).

Included in the definition of functional homologues are the proteins listed in Fig. 12.

The term "functional fragment" refers to a shortened version of the protein which is a functional variant or functional derivative. A "functional variant" or a "functional derivative" of a protein is a protein the amino acid sequence of which can be derived from the amino acid sequence of the original protein by the substitution, deletion and/or addition of one or more amino acid residues in a way that, in spite of the change in the amino acid sequence, the functional variant retains at least a part of at least one of the biological activities of the original protein that is detectable for a person skilled in the art. A functional variant is generally at least 60% homologous (preferably the amino acid sequence is at least 60% identical), advantageously at least 70% homologous and even more advantageously at least 90% homologous to the protein from which it can be derived. A functional variant may also be any functional part of a protein; the function in the present case being particularly but not exclusively its antigenic property, i.e. that it is capable of eliciting antibodies which block or inhibit the biological function of the full length active protein. Preferably the amino acid sequence differs from the sequence of Fig. 10 (the protein sequence) mainly or only by conservative substitutions. More

preferably the protein comprises an amino acid sequence having 90% or more, still more preferably 95%, sequence identity with the sequence of Fig. 10 and optimally 100% identity with those sequences.

The expression "conservative substitutions" as used with respect to amino acids relates to the substitution of a given amino acid by an amino acid having physicochemical characteristics in the same class. Thus where an amino acid in the sequence as provided in Fig. 10 has a hydrophobic characterising group, a conservative substitution replaces it by another amino acid also having a hydrophobic characterising group; other such classes are those where the characterising group is hydrophilic, cationic, anionic or contains a thiol or thioether. Such substitutions are well known to those of ordinary skill in the art, i.e. see US 5,380,712. Conservative amino acid substitutions may be made, for example within the group of aliphatic non-polar amino acids (Gly, Ala, Pro, Ile, Leu, Val), the group of polar uncharged amino acids (Cys, Ser, Thr, Met, Asn, Gln), the group of polar charged amino acids (Asp, Glu, Lys, Arg) or the group of aromatic amino acids (His, Phe, Tyr, Trp).

The term " immunogenic part" includes reference to any part of the MgtA protein, or a functional homologue or (functional) fragment thereof, which is capable of eliciting an immune response in a mammal. Said immunogenic part preferably corresponds to an antigenic determinant of said pathogen.

As used herein, the term "antigen" refers to a molecule capable of being bound by an antibody or a T cell receptor (TCR) if presented by moleculas of the major histocompatibility complex (MHC). The term "antigen", as used herein, also encompasses T-cell epitopes. A T-cell epitope is recognized by a T-cell receptor in the context of a MHC class I, present on all cells of the body except erythrocytes, or class II, present on immune cells and in particular antigen presenting cells. This recognition event leads to activation of T-cells and subsequent effector mechanisms such as proliferation of the T-cells,

cytokine secretion, perforin secretion etc. An antigen is additionally capable of being recognized by the immune system and/or being capable of inducing a humoral immune response and/or cellular immune response leading to the activation of B- and/or T-lymphocytes. This may, however, require that, at least in certain cases, the antigen contains or is linked to a T-Helper cell epitope and is given in adjuvant. An antigen can have one or more epitopes (B- and T-epitopes). The specific reaction referred to above is meant to indicate that the antigen will preferably react, typically in a highly selective manner, with its corresponding antibody or TCR and not with the multitude of other antibodies or TCRs which may be evoked by other antigens. Antigens as used herein may also be mixtures of several individual antigens.

As used herein, the term "antigenic determinant" is meant to refer to that portion of an antigen that is specifically recognized by either B- or T-lymphocytes. B-lymphocytes respond to foreign antigenic determinants via antibody production, whereas T-lymphocytes are the mediator of cellular immunity. Thus, antigenic determinants or epitopes are those parts of an antigen that are recognized by antibodies, or in the context of an MHC, by T-cell receptors. An antigenic determinant may contain one or more epitopes. Epitopes may be present on the intracellular (inside), transmembrane spanning (transmembrane), as well as extracellular (outside) regions of a protein molecule. It is expected that antigenic determinants are associated with in particular those regions of the transmembrane protein MgtA (the antigen) which are on the outside of the cytoplasmic membrane of the bacteria. These regions can be predicted from the sequence as provided in Fig. 10, by using for instance the software program TMHMM, e.g. version 2.0c, which provides a method for prediction transmembrane helices based on a hidden Markov model. Using this program, it was determined that the 914 amino acids of MgtA were predicted to form a protein having 8 transmembrane helices (residues 81-98, 102-121, 269-288, 298-320, 766-788, 808-830, 851-873, and 883-902. The four 'outside' regions, preferred as antigenic determinants or

immunogenic parts in the present invention, include the residues 99-101, 289-297, 789-807, and 874-882. Also included are the corresponding outside regions from the proteins listed in Fig. 12 and other homologues.

The term "prophylactic or therapeutic treatment of an infection by a meningeal pathogen" refers to both prophylactic or therapeutic treatments wherein the transcytosis of the pathogen is blocked or diminished, but also to treatments wherein antibodies against MgtA recognize the bacteria and will protect the host against infection, either directly through immune clearance, or indirectly by blocking the activity of the protein, thereby inhibiting the in vivo growth of the bacteria. Also, the term refers to blocking the function of the MgtA protein *in vivo* thereby reducing the adhesion abilities of the pathogen with a concomitant reduction in colonisation and invasion capabilities. The term thus includes inducing immune responses in subjects using vaccine formulations of the invention, as well as inhibiting growth of the pathogen *in vivo* by using antibodies of the present invention as an active compound in a pharmaceutical composition administered to the subject.

The term "antibody" refers to molecules which are capable of binding an epitope or antigenic determinant and includes reference to antigen binding forms of antibodies (e. g., Fab, F(ab)2). The term "antibody" frequently refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). However, while various antibody fragments can be defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments such as single chain Fv, chimeric antibodies (i. e., comprising constant and variable regions from different species), humanized antibodies (i. e., comprising a complementarity determining region (CDR) from a non-human source) and heteroconjugate antibodies (e. g., bispecific antibodies). The antibody can be monoclonal or

polyclonal and can be prepared by techniques that are well known in the art such as immunization of a host and collection of sera (polyclonal) (see, e.g., Parker, Radioimmunoassay of Biologically Active Compounds, Prentice-Hall (Englewood Cliffs, N.J., U.S., 1976), Butler, *J. Immunol. Meth.* **7**, 1-24 (1975); Broughton and Strong, *Clin. Chem.* **22,** 726-732 (1976); and Playfair, *et al., Br. Med. Bull.* **30**, 24-31 (1974)) or by preparing continuous hybrid cell lines and collecting the secreted protein (monoclonal) (see, *e.g.*, Kohler *et al* in *Nature* **256**, 495-497 (1975) and *Eur. J. Immunol.* **6**, 511-519 (1976); by Milstein *et al. Nature* **266**, 550-552 (1977); and by Walsh *Nature* **266**, 495 (1977)) or by cloning and expressing nucleotide sequences or mutagenized versions thereof coding at least for the amino acid sequences required for specific binding of natural antibodies. Antibodies may include a complete immunoglobulin or fragment thereof, which immunoglobulins include the various classes and isotypes, such as IgA, IgD, IgE, lgG1, IgG2a, lgG2b and lgG3, IgM, etc. Fragments thereof may include Fab, Fv and F(ab')2, Fab', and the like. In addition, aggregates, polymers, and conjugates of immunoglobulins or their fragments can be used where appropriate so long as binding affinity for a particular molecule is maintained.

As used herein, the term "monoclonal antibody" refers to an antibody composition having a homogeneous antibody population. The term is not limited regarding the species or source of the antibody, nor is it intended to be limited by the manner in which it is made. The term encompasses whole immunoglobulins as well as fragments such as F.sub.ab, F.sub.(ab')2, F.sub.v, and others, such as CDR fragments, which retain the antigen binding function of the antibody. Monoclonal antibodies of any mammalian species can be used in this invention. In practice, however, the antibodies will typically be of rat or murine origin because of the availability of rat or murine cell lines for use in making the required hybrid cell lines or hybridomas to produce monoclonal antibodies.

As used herein, the term "humanized monoclonal antibodies" means that at least a portion of the exposed amino acids in the framework regions of the antibody (or fragment), which do not match with the corresponding amino acids in the most homologous human counterparts, are changed, such as by site directed mutagenesis of the DNA encoding the antibody. Because these exposed amino acids are on the surface of the molecule, this technique is called "resurfacing." Moreover, because the amino acids on the surface of the molecule are the ones most likely to give rise to an immune response, this resurfacing decreases the immunogenicity of the monoclonal antibody when administered to a species whose cell line was not used to generate the antibody, such as a human. The term "humanized monoclonal antibody" also includes chimeric antibody wherein the light and heavy variable regions of a monoclonal antibody generated by a hybridoma from a non-human cell line are each attached, via recombinant technology, to one human light chain constant region and at least one heavy chain constant region, respectively. The preparation of such chimeric (i. e., humanized) antibodies are well known in the art.

The term "specifically recognizing", includes reference to a binding reaction between an antibody and a protein having an epitope recognized by the antigen binding site of the antibody. This binding reaction is determinative of the presence of a protein having the recognized epitope amongst the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to an analyte having the recognized epitope to a substantially greater degree (e. g., at least 2-fold over background) than to substantially all analytes lacking the epitope which are present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies raised to the polypeptides of the present invention can be selected to obtain antibodies specifically recognizing polypeptides of the present invention. The proteins used as immunogens can

be in native conformation or denatured so as to provide a linear epitope. A variety of immunoassay formats may be used to select antibodies specifically recognizing a particular protein (or other analyte). For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York (1988), for a description of immunoassay formats and conditions that can be used to determine selective reactivity.

A "subject" as referred to herein includes reference to is meant to include mammals and other animals, wherein mammals include for example, humans, apes, monkeys, horses, cattle, pigs, goats, dogs, cats, rats, mice, and sheep. The term "non-human animal" is meant to disclaim humans.

The MgtA protein has already been disclosed in WO 02/083855, WO 02/077021 and US 2005/136404. In these publications the protein, amongst many others, has been proposed as an immunogen, which would be useful in vaccines against *Streptococcus pneumoniae*. However, this use of the protein has not been enabled, because nowhere it has been shown that the protein is antigenic and thus would elicit antibody production after injection. Nor has any effect of these elicited antibodies been disclosed. Thus, at best, the cited documents would serve as an invitation to do further research, while the chances for success would be unpredictable.

Methods for purification of MgtA proteins and amino acid sequencing.

The MgtA protein may be used to produce vaccines or antibodies of the invention. A suitable source of MgtA protein is for instance *S. pneumoniae*. The protein may be used with or without the presence of detergents and or lipid analogs non-purified (associated with in intact cells), partially purified (associated with membrane fragments or other cellular constituents), or purified,(i.e. isolated and essentially free of other cellular constituents).

Having prepared purified or partially purified MgtA protein it is possible to prepare a substantially pure preparation of MgtA protein. Although numerous methods and strategies for protein purification are known in the art it will be most convenient to purify MgtA protein by either electrophoresis using for instance a sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE) or by affinity chromatography. Each of these methods will be described below.

MgtA protein may be separated from other proteins by electrophoresis using for instance Tricine-SDS-PAGE (Schagger and Von Jagow (1987) *Analytical Biochemistry* 166, 368-379) or Glycine-SDS-PAGE (Laemmli (1970) *Nature* 227, 680-685). Other electrophoresis systems that are capable of resolving the various proteins comprised in a bacterial lysate, or transcribed from its genome and expressed in a suitable expression system, may of course also be employed, such as non-denaturing gel electrophoresis. The area of the PAGE gel including the target protein may be excised and the target polypeptides may be eluted therefrom. The protein of interest may be identified by its mobility relative to reference polypeptides in a gel. To increase purity the eluted protein may be run on a second SDS-PAGE gel and eluted a second time. The protein or peptide contained in the excised gel fragment may then be eluted again and is suitable for use in immunization or in protein sequencing.

MgtA protein may also be purified by affinity chromatography using an antibody (such as a monoclonal antibody) that specifically binds to a MgtA protein. The antibody may be covalently coupled to solid supports such as celluloses, polystyrene, polyacrylamide, cross-linked dextran, beaded agarose or controlled pore glass using bifunctional coupling agents that react with functional groups on the support and functional groups (*i.e.*, reactive amino acid side chains) on the antibody molecule. Such methods are readily available to the skilled person. The resulting antibody-bearing solid phase is contacted with purified or partially purified MgtA protein under reducing conditions using pH, ionic strength, temperature and residence times that permit the

MgtA protein to bind to the immobilized antibody. The protein is eluted from the column by passing an eluent that dissociates hydrogen bonds through the bed. Buffers at specific pH or NaCl solutions above about 2 M are commonly used eluents.

Methods for carrying out affinity chromatography using antibodies as well as other methods for immunoaffinity purification of proteins are well known in the art (see *e.g.*, Harlow and Lane, (1988) Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

With the teachings provided herein, the skilled person is capable of isolating a MgtA protein, determining the amino acid sequence of for instance the N-terminal part of said protein, designing a set of degenerate probes (for the degeneracy of the genetic code) to hybridise with the DNA coding for a region of said protein, using these probes on an array of genes in a genomic library produced from the meningeal pathogen, obtaining positive hybridisations and locating the corresponding genes. The skilled person is then capable of identifying the structural region of the gene and optionally upstream and downstream sequences thereof. Thereafter the skilled person is capable of establishing the correct sequence of the amino acid residues that form the protein in any meningeal pathogen, and establish the presence of functional homologues of MgtA.

Antibody production

Antibodies, either monoclonal or polyclonal, can be generated to a purified or partially purified protein or peptide fragment of a meningeal pathogen in a variety of ways known to those skilled in the art including injection of the protein as an antigen in animals, by hybridoma fusion, and by recombinant methods involving bacteria or phage systems (see Harlow and Lane (1988) *supra.*; Marks *et al.*, (1992) *Journal of Biological Chemistry*, 267, 16007-16010; Marks *et al.*, (1992) *Biotechnology* 10: 779:783; Lowman *et al.*, (1991) *Biochem.* 30(45): 10832-8; Lerner *et al.*, (1992) *Science* 258:1313-1314,

each of which reference discloses suitable methods).

Antibodies against MgtA or functional homologues thereof, may be produced by immunizing an appropriate vertebrate, preferably mammalian host, *e.g.*, rabbits, goats, rats and mice or chicken with the protein alone or in conjunction with an adjuvant. Usually two or more immunizations will be involved, and the blood or spleen will be harvested a few days after the last injection. For polyclonal antisera, the immunoglobulins may be precipitated, isolated and (affinity) purified. For monoclonal antibodies, the splenocytes will normally be fused with an immortalized lymphocyte, *e.g.*, a myeloid line, under selective conditions for hybridomas. The hybridomas may then be cloned under limiting dilution conditions and their supernatants screened for antibodies having the desired specificity. Techniques for producing (monoclonal) antibodies and methods for their preparation and use in various procedures are well known in the literature (see *e.g.* U.S. Pat. Nos. 4,381,292, 4,451,570, and 4,618,577; Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.; Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D.,Seidman, J.G., Smith, J.A., Struhl, K. eds. (1998) *Current protocols in molecular biology.* V.B. Chanda, series ed. New York: John Wiley & Sons; Rose, N., DeMacrio, E., Fahey, J., Friedman, H., Penn, G. (1997) *Manual of Clinical Laboratory Immunology.* American Soc. Microbiology Press, Washington, D.C Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M. Strober, W. (Eds.) (1997) *Current Protocols in Immunology.* John Wiley & Sons Inc. Baltimore). Typically, an antibody directed against a protein will have a binding affinity of at least $1x10^5$-$1x10^7$ M^{-1}.

A recombinant MgtA protein or functional homologues thereof, such as may be obtained by expressing a MgtA-encoding genomic sequence in a suitable expression system, is preferred as the antigen in methods for producing an antibody. However, purified proteins may also be used, as well as protein fragments. Antigens suitable for antibody production include any

fragment of MgtA protein that elicits an immune response in a mammal exposed to said protein. Preferred antigens of the invention include those fragments that comprise the antigenic determinenants, although any region of the MgtA protein may in principle be used.

Methods for cloning genomic sequences, for manipulating the genomic sequences to and from expression vectors, and for expressing the protein encoded by the genomic sequence in a heterologous host are well-known, and these techniques can be used to provide the expression vectors, host cells, and the cloned genomic sequences encoding MgtA protein, functional homologues or fragments thereof, which sequences are to be expressed in a host to produce antibodies for use in methods of the present invention (see for instance Sambrook, J., Russell D.W., Sambrook, J. (2001) *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Plainview, N.Y., and Ausubel, *et al.*, *supra*).

A variety of expression systems may be used to produce antigens for use in methods of the present invention. For instance, a variety of expression vectors suitable to produce proteins in *E.coli*, *B.subtilis*, yeast, insect cells, plant cells and mammalian cells have been described, any of which might be used to produce an antigen suitable to produce an anti-MgtA antibody or fragment thereof. Of course *Streptococcus pneumoniae* itself may also be used as an expression vector for this purpose.

One use of antibodies of the invention is to provide active ingredients for a pharmaceutical composition capable of inhibiting growth of a meningeal pathogen. Another use of antibodies of the invention is to screen cDNA expression libraries for identifying clones containing cDNA inserts that encode proteins of interest or structurally-related, immuno-cross-reactive proteins. Such screening of cDNA expression libraries is well known in the art (see *e.g.* Young R.A., Davis, R.W. (1983) *Proc. Natl. Acad. Sci. U.S.A.* 80:1194-1198), to which reference is made in this context, as well as other published sources. Another use of these antibodies is for use in affinity chromatography for

purification of MgtA protein or functional homologues thereof. These antibodies are also useful for assaying for infection with meningeal pathogens.

MgtA Antigen Epitopes

The MgtA antigen epitopes of this invention, which alone or together form an MgtA-based antigenic determinant of the meningeal pathogen, are molecules that are immunoreactive with anti-MgtA monoclonal antibodies and whose binding to a MgtA antigen located on the surface of a meningeal bacterial pathogen cell prevents the growth and/or transcytosis of said cell. Systematic techniques for identifying these epitopes are known in the art, as described in US 4,708,871, which is incorporated herein by reference. Typically, these epitopes are short amino acid sequences. These sequences may be embedded in the sequence of longer peptides or proteins, as long as they are accessible.

The epitopes of the invention may be prepared by standard peptide synthesis techniques, such as solid-phase synthesis. Alternatively, the sequences of the invention may be incorporated into larger peptides or proteins by recombinant methods. This is most easily accomplished by preparing a DNA cassette which encodes the sequence of interest, and ligating the cassette into DNA encoding the protein to be modified at the appropriate site. The sequence DNA may be synthesized by standard synthetic techniques, or may be excised from the phage pIII gene using the appropriate restriction enzymes. Epitopes identified herein may be prepared by simple solid-phase techniques. The minimum binding sequence may be determined systematically for each epitope by standard methods, for example, employing the method described in US 4,708,871. Briefly, one may synthesize a set of overlapping oligopeptides derived from the MgtA antigen bound to a solid phase array of pins, with a unique oligopeptide on each pin. The pins are arranged to match the format of a 96-well microtiter plate, permitting one to assay all pins simultaneously, e.g., for binding to an anti-MgtA monoclonal antibody. Using this method, one

may readily determine the binding affinity for every possible subset of consecutive amino acids.

Antibody Formulations and Methods of Administration

The antibodies of this invention are administered at a concentration that is therapeutically effective to prevent or treat infections by meningeal pathogens. To accomplish this goal, the antibodies may be formulated using a variety of acceptable excipients known in the art. Typically, the antibodies are administered by injection, either intravenously or intraperitoneally. Methods to accomplish this administration are known to those of ordinary skill in the art. It may also be possible to obtain compositions which may be topically or orally administered, or which may be capable of transmission across mucous membranes.

Before administration to patients, formulants (components other than the active ingredient in a product have many functions, such as carrier and excipients) may be added to the antibodies. A liquid formulation is preferred. For example, these formulants may include oils, polymers, vitamins, carbohydrates, amino acids, salts, buffers, albumin, surfactants, or bulking agents.

Additionally, antibodies can be chemically modified by covalent conjugation to a polymer to increase their circulating half-life, for example. Preferred polymers are polyethylene glycol (PEG) and polyoxyethylated polyols, such as polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG). The PEG has a preferred average molecular weight between 1,000 and 40,000, more preferably between 2,000 and 20,000, most preferably between 3,000 and 12,000.

Another drug delivery system for increasing circulatory half-life is the liposome. Methods of preparing liposome delivery systems are discussed in Gabizon et al., Cancer Research (1982) 42:4734; Cafiso, Biochem. Biophys. Acta (1981) 649:129; and Szoka, Ann. Rev. Biophys. Eng. (1980) 9:467. Other

drug delivery systems are known in the art and are described in e.g., Poznansky et al., *Drug Delivery Systems* (R. L. Juliano, Ed., Oxford, N.Y. 1980), pp. 253-315; M. L. Poznansky, Pharm. Revs. (1984) 36:277.

After a liquid pharmaceutical composition is prepared, it is preferably lyophilized to prevent degradation and to preserve sterility. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent (Ringer's solution, distilled water, or sterile saline, for example) which may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

As stated above, the antibodies and compositions of this invention are used to treat mammalian, and preferably human subjects to prevent or treat meningeal pathogenic infections. The preferred route of administration is parenterally. In parenteral administration, the compositions of this invention will be formulated in a unit dosage injectable form such as a solution, suspension or emulsion, in association with a pharmaceutically acceptable parenteral vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are saline, Ringer's solution, dextrose solution, and Hanks' solution. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. A preferred vehicle is 5% dextrose in saline. The vehicle may contain minor amounts of additives such as substances that enhance isotonicity and chemical stability, including buffers and preservatives.

The dosage and mode of administration will depend on the individual. Generally, the compositions are administered so that antibodies are given at a dose between 1 μg/kg and 20 mg/kg, more preferably between 20 μg/kg and 10 mg/kg, most preferably between 1 and 7 mg/kg. Preferably, it is given as a bolus dose, to increase circulating levels by 10-20 fold and for 4-6 hours after the bolus dose. Continuous infusion may also be used after the bolus dose. If

so, the antibodies may be infused at a dose between 5 and 20 μg/kg/minute, more preferably between 7 and 15 μg/kg/minute.

The antibody of the present invention may be used prior to infection as a precaution, or after infection has occurred as a therapeutic treatment. Preferably, the therapeutic use of the antibodies as described herein or fragments thereof include administration prior or during the acute invasive phase of the disease.

Vaccine Formulations and Methods of Administration

The vaccine antigens of this invention are administered at a concentration that is therapeutically effective to prevent or treat infections by meningeal pathogens. To accomplish this goal, the vaccines may be formulated using a variety of acceptable excipients known in the art. Typically, the vaccines are administered by injection, either intravenously or intraperitoneally. Methods to accomplish this administration are known to those of ordinary skill in the art.

Preferably the vaccine contains at least 50 μg of antigenic mass per dose, and most preferably 80 μg per dose. The antigenic mass being the mass of the antigen protein. Vaccines according to the present invention with an antigenic mass up to 275 μg per dose could even be prepared, and such vaccines may still not elicit local reactions at the injection site. Of course even more micrograms of antigen can be put in a vaccine dose of a vaccine according to the invention, but if the protection obtained with the vaccine is not improved with a higher dose the increase in antigenic load only results in the vaccine being more expensive than necessary. In addition an increasing dose of antigen may eventually lead to unacceptable local reactions at the injection site, which should be avoided.

A vaccine according to the invention may contain a (partially) purified or recombinant MgtA protein or an antigenic part thereof, wherein said recombinant protein is preferably produced by way of expression from an

expression vector in suitable host cells, such as bacterial, fungal, insect or mammalian cells, said expression vector containing the *mgtA* gene sequence or an immunogenic part thereof under control of a suitable promoter. Suitable protein expression systems are known in the art and may be used as applied nromally by a person skilled in the art to prepare a vaccine component according to the invention.

A vaccine according to the invention may further comprise a suitable adjuvant. Many adjuvant systems are known in the art, for example commonly used oil in water adjuvant systems. Any suitable oil may be used, for example a mineral oil known in the art for use in adjuvantia. The oil phase may also contain a suitable mixture of different oils, either mineral or non-mineral. Suitable adjuvantia may also comprise vitamin E, optionally mixed with one or more oils. The water phase of an oil in water adjuvanted vaccine will contain the antigenic material. Suitable formulations will usually comprise from about 25-60% oil phase (40-75% water phase). Examples of suitable formulations may comprise 30% water phase and 70% oil phase or 50% of each.

The vaccine formulations of the present invention may be used in prophylactic methods of the invention by immunizating an animal by introducing said formulations into an animal subcutaneously, intramuscularly, intranasally, intradermally, intravenously, transdermally, transmucosally, orally, or directly into a lymph node. In another embodiment, the composition may be applied locally, near a local pathogen reservoir against which one would like to vaccinate.

The present invention further provides a method for the manufacture of a vaccine intended for the protection of a subject against infection by a meningeal pathogen, wherein said vaccine is combined with a pharmaceutically acceptable diluent, carrier, excipient or adjuvant therefore such that a formulation is provided which can provide a dose of at least 20 µg/of MgtA in a single administration event.

A vaccine (prepared by a method) according to the invention can be used in a method to protect a subject against infection by a meningeal pathogen.

To provide adequate protection the vaccine is preferably administered in a 2 shot vaccination regimen, whereby the first shot (priming vaccination) and second shot (boosting vaccination) are given to the subject with a interval of about 3 weeks. In this way the subject will have obtained full protection against infection by a meningeal pathogen. The vaccination is very favourable for young children.

The vaccine of the present invention can also advantageously comprise other antigens or antibodies which would be useful to combat infection for the same pathogen or multiple pathogens . Such antigens or antibodies can be any antigens or antibodies which are commonly used to prevent or treat the infection. Further, the vaccine of the invention can be advantageously co-administered with any antibiotic treatment. The vaccine of the invention will inhibit the growth of the bacteria, in particular *S. pneumoniae* and prevent transcytosis, which means that the bacteria will be present in the body in places (such as the circulation), where they are prone to antibiotic treatment.

The vaccine formulations and pharmaceutical compositions of the present invention can thus be used for preventing transcytosis, and thus are ideal for preventing or treating meningitis.

Further, the vaccine formulations and pharmaceutical compositions of the present invention will inhibit the growth of meningeal pathogens, and will therefore be suited for prevention or treatment of pneumonia, sepsis, pericarditis and any other infection caused by the meningeal pathogens, whereby the vaccine formulations and pharmaceutical compositions of the invention can be co-administered with other immunogenic determinants or with antibiotics.

EXAMPLES

Materials & Methods

Bacterial strains and growth conditions

Strains used in this study are listed in Table 1 and were stored in 10 % glycerol at –80 °C. *S. pneumoniae* was grown as standing cultures in M17 broth supplemented with 0.5 % glucose (GM17) at 37 °C. For growth on plates, 3 % defibrinated sheep blood (Johnny Rottier, Kloosterzande, The Netherlands) was added to GM17 agar. When needed the antibiotics erythromycin, kanamycin, chloramphenicol and tripmethoprim were added at concentrations of, respectively, 0.25 ug/ml, 500 ug/ml, 2 µg/ml and 15 ug/ml for *S. pneumoniae.*

Table 1: Used strains and plasmids in this study

Strains/Plasmid	**Description**	**Reference or source**
S. pneumoniae		
D39	Serotype 2 strain	Avery *et al.*, *Mol. Med.* 1:344 (1995)
R6	Unencapsulated serotype 2; labstrain	Hoskins et al., *J. Bacteriol.* 183:5709 (2001)
D39*Δcps*	D39 lacking capsule locus, Kan^R	Example 1
D39*ΔcpsΔmgtA*	D39*Δcps* lacking *mgtA*, Kan^R, Ery^R	Example 1
D39*ΔcpsnisRK*	D39 *ΔcpsΔbgaA::nisRK*, Kan^R, $Trmp^R$	Example 1
D39*ΔcpsΔmgtAnisRK*	D39 *ΔcpsΔmgtAΔbgaA::nisRK*, Kan^R, Ery^R, $Trmp^R$	Example 1
L. lactis		
NZ9000	MG1363 *ΔpepN::nisRK*	Kuipers *et al.*, *Journal of Biotechnology* 64:15 (1998)
rPlasmids		
pNZ8048	Cm^R; nisin-inducible P*nisA*	De Ruyter *et al.*, *Appl. Environ. Microbiol.* 62:3662 (1996)
pNZ8048::*mgtA*	Cm^R; *mgtA* under nisin-inducible P*nisA*	Example 1
pTK3	Ery^R $Trmp^R$; containing 3'and 5' fragments of D39 *bgaA*, harbouring *nisRK* genes in between	Kloosterman *et al.* *Microbiology* 152:351 (2006)

Kan^R: Kanamycin resistance, Ery^R: Erythromycin resistance, $Trmp^R$: Trimethoprim resistance, Cm^R: Chloramphenicol resistance.

Strain and plasmid construction

Production of Δ*cps* mutant in D39

S. pneumoniae D39Δ*cps* was produced by replacement of the capsule locus by a kanamycin resistance gene, as described by Pearce et al. (29). The resulting Δ*cps* mutation (see Figure 2) was confirmed by PCR, using primers annealing upstream of *dexB* and at the 3' site of *kanR* (d*cps*.control.fw/Kana2) and using primers annealing at the 5' site of *kanR* and downstream of *aliA* (Kana 1/d*cps*.control.rev) (Table 2), resulting in fragments of 2033 and 2408 bp, respectively.

Generation of Δ*mgtA* (sp1551) mutant in D39Δ*cps*

For the production of a knockout strain of *mgtA*, the 3' and 5' flancking regions of *mgtA* (SPD_1383,sp1551) in *S. pneumoniae* D39Δ*cps* were amplified using primers SP1550for/SP1550rev and SP1552for/SP1552rev (Table 2),, respectively. The erythromycin resistance gene was amplified from plasmid pORI13 by making use of the primers Eryfor and Eryrev. The 3 different PCR fragments were fused by making use of overlapping sequences for the erythromycin resistance gene in the primers SP1550rev and SP1552for, using the outward primers SP1550for and SP1552rev for amplification (Table 2),. This resulting PCR product of 2052 bp was transferred to D39Δ*cps* and the bacteria were plated on GM17 plates containing 3% sheep blood, erythromycin (0.25 ug/ml) and 10 mM $MgCl_2$. The deletion of *mgtA* from the genome (see Figure 3) was confirmed by PCR using SP1550fw and SP1552rev (Table 2), and Southern blotting.

Generation of *nisRK* integration in D39Δ*cps* and D39Δ*cps*Δ*mgtA*

For the production of D39Δ*cpsnisRK* and D39Δ*cps*Δ*mgtAnisRK*, the nisRK genes were amplified by making use of the primers *bgaA*-1 and *bgaA*-4 (Table 2), used with pTK3 as a template . The resulting PCR product was

integrated via double cross-over in the *bgaA* locus, and the correct replacement was confirmed by Southern blotting.

Generation of pNZ8048:*mgtA*

To be able to complement the mgtA knockout mutant, a plasmid was constructed containing the *mgtA* gene under control of a nisin inducible promoter (P*nisA*) (see Figure 4). The *mgtA* gene was amplified from the chromosome of R6 by making use of the primers mgtacomplsta and mgtastop (Table 2),. The resulting 2749 bp fragment was digested with NcoI and XbaI and ligated into the complementary sites of pNZ8048. This ligation mix was transferred to *L. lactis* NZ9000. When restriction analysis and sequencing confirmed that the right construct was produced, the plasmid pNZ8048::*mgtA* was transferred to D39Δ*cps*Δ*mgtAnisRK* for complementation studies of the *mgtA* knockout mutant.

Table 2: Primers used in this study

Primer	Nucleotide sequence (5' to 3')	Restriction site
Dcap.control.fw	GGCAGACAGAGTTAGGAGTTGAG	
Dcap.control.rev	CCCAGTCTTCGCCAGCAGC	
Kana.1	GTGAATTGGAGTTCGTCTTG	
Kana.2	TATGGACAGTTGCGGATGTA	
bgaA-1	GCTCTAGACAAATCGTTGAACGAGGTGG	XbaI
bgaA-4	GAAGATCTGTAATTTGATAGTCTTGACG	BglII
Sp1550 for	GCACCTGCTTGCCAGAATAG	
Sp1550rev	AGTTATCGGCATAATCGTTATCGGATGTCAGC ATATCAAT	
Sp1552for	GAGATCTAATCGATGCATGCCGGAATGACGAC ACTATCAC	
Sp1552rev	GCACCAAGAGGATCAATGAC	
Eryfor	TAACGATTATGCCGATAACT	
Eryrev	GCATGCATCGATTAGATCTC	
mgtacomplsta	CATGCCATGGCAGAAAGAGAGTCTGTTTTGC	NcoI
*mgtA*stop	TCTAGACCTTAAATCGCATTCTTG	XbaI

Growth experiments with D39Δ*cps* and D39Δ*cps*Δ*mgtA*

Overnight cultures of *S. pneumoniae* used for growth experiments were diluted 50 times in GM-17 and grown until OD_{600} 0.2. Until use, the samples were supplemented with 10% glycerol and stored at -80°C. Growth experiments were performed in 94 wells microtiter plates containing 5 µl bacterial culture and 195 µl GM17 containing various concentrations of different metal ions as described in the results. Growth of the strains was followed by OD_{595} measurements (GENios, Tecan) for 15 hours using an interval time of 30 minutes 37°C ±0.5°C . Prior to every measurement the plate was shaked for 3 seconds with a normal intensity and orbital mode.

Endothelial cell line

In this study, immortalized Human Brain Microvascular Endothelial Cells (HBMEC) were used up to passage 36. The cells were obtained from Kwang S. Kim (Johns Hopkins Univ School of Medicine, Baltimore, USA) Cells were cultivated in RPMI1640 (Biochrom), supplemented with 10% FCS (Biochrom), 10% Nu-serum (BD Biosciences), 2 mM L-glutamin (Gibco), 1 mM Na-pyruvate (Gibco), 1% MEM-vitamines (Gibco) and 1% non-essential amino acids (Gibco) at 37°C, 5% CO2. The cells were subcultured every third or fourth day, by first washing the cells with PBS followed by incubation with 1.5 ml prewarmed Trypsin/EDTA (Sigma). Following the removal of trypsin after 0.5 minutes, the cells were incubated at 37C until they lifted off from the culture flask (usually 3 to 4 minutes). They were then resuspended in fresh medium and seeded in the right dilution (usually 1:4 or 1:6) for further growth.

Endothelial cell adherence and invasion

For adherence and invasion studies of HBMEC were seeded in a concentration of $1*10^4$ cells per well and incubated for 2 days. When the monolayer was 100% confluent the cell layer was washed twice with 500 ul

PBS/well and incubated for 1 hour with culture medium containing 10 ng/ml TNFα to activate the expression of the PAF receptor. $1*10^7$ CFU/well *S. pneumoniae* from an aliquot of OD600O0.2 was washed with 1 ml DPBS with Mg^{2+} and Ca^{2+} (Cambrex) and added to each well in 500 ul cell culture medium, also containing 10 ng/ml TNFα. After 2 hours of incubation, HBMEC cells were washed 2 times with DPBS with Mg and Ca (Cambrex) to remove unadhered bacteria and lysed by 5 minutes incubation in 100 ul DPBS/0.1% Triton X-100. Serial dilutions of harvested bacteria were plated on Columbia agar plates containing 3% sheep blood and 10 mM $MgCl_2$.

To kill extracellular *S. pneumoniae*, the monolayers in the remaining wells were washed twice with DPBS containing Mg^{2+} and Ca^{2+}, and the medium was supplemented with 200 ug/ml gentamycin. After 1 hour of incubation the medium was plated on Columbia agar plates containing 3% sheep blood and 10 mM $MgCl_2$ to ensure that the antibiotic concentration was sufficient to kill the extracellular bacteria. The monolayer was washed twice with DPBS containing Mg^{2+} and Ca^{2+} and lysed by 3 minutes incubation in 100 ul DPBS/0.1%Triton X-100. Serial dilutions of harvested bacteria were plated on Columbia agar plates containing 3% sheep blood and 10 mM $MgCl_2$. To quantify the amount and viability of the detached HBMEC cells when 1E8 *S. pneumoniae* D39F*cps* or D39F*cps*F*mgtA* was added, trypan blue (0.4%) was used in a 1:1 dilution. The culture medium was analysed after 2 or 3 hours of infection, for the presence of detached HBMEC by microscopy.

Transcytosis assay

To study the transcytosis of *S. pneumoniae* through HBMEC, a double chamber culture system was set up using Transwell polycarbonate filters (3um pore size, Cat. no. 3402, Corning Costar Corp.) placed into a 12 wells plate (Ring A et al. J. Clin. Invest 102, 347 (1998)). The HBMEC cells were seeded at a concentration of approximately $1*10^5$ cells/well and these wells were cultivated for 3 days in a petridish to prevent growth of the HBMEC cells on

the wrong side of the filter. Routinely 0.5 ml of fluid was kept in the upper chamber and 1.5 ml in the lower chamber throughout the experiment.

Monolayers were incubated for 30 minutes in culture medium containing 10 ng/ml TNFα to activate the expression of the PAF receptor (Ring et al J. Clin. Invest. 1998). The monolayers were infected for 2 hours with $1*10^7$ CFU/well of unencapsulated *S. pneumoniae* strains from an aliquot of $OD_{600}\approx0.2$ as described before. After two hours the monolayer was washed twice with DPBS with Mg^{2+} and Ca^{2+} and antibiotic-containing medium (10 ug/ml penicillin G and 200 ug/ml gentamycin) was added to both chambers for 1 hour to kill extracellular bacteria. The monolayers were washed again twice with DPBS with Mg^{2+} and Ca^{2+} and culture medium containing 0.04 ug/ml penicillin G (Sigma) was added to the upper chamber of the transwell® system. Culture medium without antibiotics was added to the lower chamber of the system to let the bacteria transcytose when possible. During this assay, at various stages monolayers were sacrificed to analyse the number of adhered or invaded bacteria, as described in the previous section. Serial dilutions of these samples were plated on Columbia agar plates containing 3% sheep blood and 10 mM $MgCl_2$.

Permeability measurements

To determine the stability of the monolayer of HBMEC during the transcytosis assay, the diffusion of Lucifer Yellow through the monolayer at different stages of the experiment was monitored. Therefore 0.5 ml of 400 ug/ml Lucifer Yellow dipotassium salt (Sigma) was added to the upper chamber and 1.5 ml of DPBS with Mg^{2+} and Ca^{2+} was added to the lower chamber. After 5 minutes of incubation at room temperature, the fluorescence of the solution in the lower chamber was measured at 535 nm emmision and 485 nm excitation wavelength, in a 96 wells plate.

Results

MgtA is the main Mg^{2+} transporter in _S. pneumoniae_ under _in vitro_ growth conditions.

ORF SP1551 (SPD_1383, spr1401) putatively encodes a cation-transporter domain, a P-type ATP-ase domain and haloacid dehalogenase-like hydrolase domain and has been designated as *mgtA*. Comparison of the Salmonella MgtA amino acid sequence, which has been shown to be a Mg^{2+} transporter, with the *S. pneumoniae* genome, showed that SPD_1383 is the ORF with the highest similarity with the salmonella *mgtA* gene of all transporters in the genome. To determine whether SP1551(spr1401, SPD_1383) is indeed an *mgtA* homologue the gene was deleted from the genome of R6 using PCR mutagenesis. Interestingly, colonies obtained after transformation grew slow and remained small and this effect was reversed after addition of extra $MgCl_2$ (1 mM) to the blood plates. The deletion of ORF SP1551 was confirmed by Southern blot and subsequently growth of the parent strains and the mutants was compared in GM17 broth. As on plates growth of the R6 mutants was poor, but could be partially restored by the addition of 5 mM $MgCl_2$ to the growth medium, addition of ten times more $MgCl_2$ (50 mM) did not further improve growth (Fig. 5). This indicates that *mgtA* is the main Mg^{2+} importer of *S. pneumoniae* under in vitro growth conditions, but that there are also other low affinity transporters present that can partially compensate for the deletion of *mgtA* when an excess of $MgCl_2$ is present.

Addition of either $CaCl_2$, $MnCl_2$, $CuCl_2$ and $ZnCl_2$ did not restore the growth of the *mgtA* mutant in GM17, whereas the parent strain grew normal under these conditions (data not shown) (Fig. 6). The initial increase in OD observed in the medium where $MnCl_2$ was added, was due to complex formation of the Mn^{2+} and a component in the GM17, as the same was observed in medium without bacteria (data not shown). Lower concentrations

of MnCl2 did not show a restoration of growth of the mutant.These data indicate that mgtA specifically transports magnesium.

To study the effect of the deletion of *mgtA* on the interaction of *S. pneumoniae* and HBMEC, we first transferred the deletion into strain D39*Δcps* as deletion of the capsule greatly enhances the adherence of *S. pneumoniae* to eukaryotic cells. Again colonies were small on plate and grew slower than the parent strain. As in strain R6, growth of the mutant in broth was poor, and this growth defect could be reversed by the addition of $MgCl_2$ to the medium (Fig 7).

Interestingly, the D39*ΔcpsΔmgtA* mutant does not seem as impaired as the R6 mutant, which indicates that in D39 there are either other transporters, they have other affinities or they are differentially regulated. To determine whether this phenotype was solely due to the deletion of *mgtA* and not secondary effects, the *mgtA* gene was cloned into the pNZ8048 vector (see above) and introduced in strain D39*Δcps*Δbga*AnisRKΔmgtA*. As a control the plasmid pNZ8048 without an insert was also introduced into this strain.

After transformation, colonies that had received the plasmid with the *mgtA* gene grew better than those that had received plasmid pNZ8048, which indicated that the heterologous expression of the *mgtA* gene in the *ΔmgtA* mutant restored growth to wild type levels. This was confirmed when the growth of D39*ΔcpsΔbgaAnisRKΔmgtA* containing pNZ8048 and D39*ΔcpsΔbgaAnisRKΔmgtA* containing p*mgtA* was compared to that of D39*ΔcpsΔbgaAnisRK* in GM17 broth (Fig. 7). As observed before, growth of the mutant containing pNZ8048 was impaired in GM17. In contrast, growth of the mutant was completely restored to wild type levels when the plasmid contained the *mgtA* gene (Fig. 7). This demonstrates that the phenotype of the *ΔmgtA* mutant is solely due to the disruption of the *mgtA* gene.

A *mgtA* mutant is unable to translocate monolayers of Human Brain Microvascular Endothelial cells

Human Brain Microvascular Endothelial Cells (HBMEC) were grown as described previously (Stins et al. 2001. Microb. Pathog. 30:19) into a tight monolayer in a transwell® system that consists of a membrane with a pore size that allows for the passage of bacteria but not eukaryotic cells. The bacteria are co-incubated with the cells in the upper compartment of this system, which enables them to adhere to and subsequently invade the endothelial cells. The ability to translocate is assessed by following the appearance of bacteria in the lower compartment over time (Fig 1B). To increase the efficiency of adherence and invasion, unencapsulated strains were used. Co-incubation of D39*Δcps* with the HBMEC cells in a transwell® system resulted in the appearance of the bacteria in the lower compartment over time, while the stability of the monolayer was unaffected, demonstrating that the bacteria went through the endothelial cells before ending up in the lower compartment (data not shown).

Subsequently, equal amounts of strains D39*Δcps* and D39*ΔcpsΔmgtA* were added to the HBMEC and adhesion to, invasion, survival in and translocation of the endothelial cells of both strains was compared.

Adhesion of the *ΔmgtA* mutant was about one log lower than that of the parent strain and, subsequently invasion was slightly lower than that of the parent strain (Fig 8A). However, the percentage of adherent bacteria that invaded and survived were similar in both strains, indicating that the mutant is not impaired in invasion of and survival in HBMEC. This is in contrast to what was found when the transcytosis of both strains was compared. Over time, as observed before, the parent strain appeared in the lower compartment. However, the mutant failed completely to reach the lower compartment (Fig. 8B), indicating that it is unable to traverse the cell layer. This was not due to a trivial reason such as the mutant sticking to the membrane as it was able to traverse the transwell when no endothelial cells were present (data not shown).

To determine that this effect was also solely due to the mutation of *mgtA*, the same strains as for the complementation assay in GM17 were used in the transcytosis assay. To ensure optimal *mgtA* expression, aliquots of all three strains were grown with nisin present in the growth medium. In these experiments, the *mgtA* mutant containing the empty vector was unable to cross the eukaryotic cell layer. Strain D39 *Δcps bgaA*::NisRK and strain D39*bgaA*:;NisRK *ΔcpsΔmgtA* containing the plasmid with the *mgtA* gene did cross the eukaryotic cell layer as they appeared in the lower compartment over time (Figure 8C), demonstrating that the observed defect in translocation is indeed due to the deletion of *mgtA*.

To further demonstrate that it is the uptake of magnesium which is important for the translocation of *S. pneumoniae* over the cell layer and not some other putative function of *mgtA*, we determined what happened to the mutant when extra magnesium was added to the culture medium while the bacteria were co- incubated with the HBMEC. Addition of Mg^{2+}, will ensure that the bacteria are constantly in a condition of high Mg concentration as the medium will also be present in the vesicles when the bacteria are taken up. As is clear in figure 8D, addition of 10 mM $MgCl_2$ to the tissue culture medium in the upper compartment restored translocation of the mutant. This indicates that the Mg^{2+} uptake by *MgtA* is important for transcytosis.

Coinfection of HBMEC with the *mgtA* mutant and parent strain does not restore translocation of the mutant.

To determine whether the simultaneous presence of the wildtype and *ΔmgtA* mutant could restore translocation of the mutant, the HBMEC were infected with mixtures of the parent strain and the mutant in various ratios. To determine the ratio between the mutant and the parent strain, bacteria were plated in replicate, once on normal Columbia agar plates and once on Columbia agar plates with erythrymocin, to specifically detect the mutant. To ensure that all *mgtA* mutants were detected, 10 mM $MgCl_2$ was added to the

all plates as well. As can be observed in Figure 9 even a 100 fold surplus of the wildtype did not result in the appearance of the mutant in the lower compartment, indicating that the role of *mgtA* in the translocation process cannot be complemented by the presence of bacteria that posses a functioning protein.

Furthermore, it seems that a surplus of the mutant inhibits the translocation of the parent strain.

Example 2 Antibodies against MgtA

Primers were designed to clone the *mgtA* gene of D39 into pNZ8048 with a C-terminal 6xHis-tag. Primer sequences:

mgtA-hisreverse

CTAGTCTAGACCTTAATGGTGATGGTGATGGTGAATCGCATTCTTG

MgtAcomplforward:

CATGCCATGGCAGAAAGAGAGTCTGTTTTGC

The protein was overproduced in *L. lactis* (de Ruyter *et al.*, 1996, *Appl Environ Microbiol* 62: 3662-3667) (Figure 13a) using the same sample preparation for protein gels and western blotting procedures as described before (Kloosterman *et al.*, 2006, *Microbiology* 152: 351-359). Subsequently, the protein was purified using FPLC and a His-trap column (HIS-Trap column 5 ml: GE Healthcare 17-5248-02) as follows:

- Induction of NZ9000 pNZ8048::mgtA::his6 at OD600 = 0.5 with 3 ng/ml nisin (2 hours)
- Spin down, lyse cells with French press after lysozyme treatment
- Spin down cell debriss, use supernatant for membrane isolation by ultracentrifugation

- Take up membrane pellet in solubilization bfr (300 mM NaCl, 20 mM NaPi pH 8.0, 0.2% Tween-20, 10% glycerol, + 6M ureum) for extraction of membrane proteins at 4°C
- Spin down membranes, use supernatant for purification with FPLC
- Load sample on Ni-NTA column, wash column with solubilization bfr
- Elution of MgtA::his by using elution bfr (= solubilization bfr + 300 mM imidazol + 6M ureum)
- Dialysis against elution buffer without ureum
- Concentration and desalting of the protein using 70 % ethanol.

The purified protein was put on gel and shown to contain MgtA-his (Figure 13B). One mg was sent to Eurogentec (Eurogentec Nederland b.v., Avenue Ceramique 223, 6221KX, Maastricht, THE NETHERLANDS. website: eurogentec.com) to generate antibodies in two Rabbits using their superspeedy program, according to their specifications.

Pre-immune, intermediate and final bleed serum of two rabbits was tested against the purified protein, wild type *S. pneumoniae* and the ΔmgtA mutant on Western blot.

An antibody response against the protein was observed in both rabbits in the intermediate and in the final bleeds and not in the pre-immune bleed (Figure 14). Signal in the final bleed was strongest. There was also a cross reactive band against another protein (not MgtA as it was not present in the *mgtA* mutant), this could be removed by absorbing the serum with an acetone powder generated from either *L. lactis* or D39Δ*mgtA* (Figure 15). This shows that the MgtA protein is immunogenic.

The serum was subsequently tested against several different strains of different serotypes and reacted in all cases with a protein of the correct height, indicating that the MgtA protein is present in all these strains and well conserved (Figure 16).

The serum was subsequently also tested against several other bacterial strains, *L. lactis* (1 strain), *S.aureus* (2 strains), *S.epidermidis* (2 strains)(Figure 17) and *S. pyogenes* (2 strains) (Figure 18). The serum recognized a protein of the correct height in both *S. aureus* strains and one *S. pyogenes* strain, but not in *L. lactis* and *S. epidermidis*. Thus, antibodies raised against the *S. pneumoniae* MgtA protein are also specifically recognizing homologous proteins in other pathogens.

Claims

1. A vaccine formulation providing protection against infection of a subject by a meningeal pathogen, said formulation comprising an effective amount of the protein MgtA or a functional homologue or an immunogenic part thereof together with at least one of a pharmaceutically acceptable diluent, carrier, excipient or adjuvant therefore.

2. A formulation according to claim 1, wherein said immunogenic part is an antigenic determinant of said pathogen.

3. A formulation according to claim 1 or 2, wherein said formulation comprises an essentially cell-free preparation of said pathogen comprising said protein or said part thereof.

4. A formulation according to claim 2 or 3, wherein said immunogenic part of said protein comprises a part that is exposed on the outer membrane of said pathogen.

5. A formulation according to any one of claims 1-4, wherein said formulation provides protections against meningitis, pneumonia, and/or sepsis caused by a meningeal pathogen.

6. A formulation according to any one of the preceding claims, wherein the formulation provides protections against meningeal streptococci, preferably selected from the group consisting of β-haemolytic *Streptococcus* spp. belonging to Lancefield group C, *S. acidominimus, S agalactiae, S. bovis, S. constellatus, S. equinus, S. equisimilis, S. mitis, S. oralis, S. pneumoniae, S. pyogenes, S. salivarius, S. sanguis, S. suis* and *S. uberis*, more preferably selected form the group consisting of *S agalactiae, S. pneumoniae, S. pyogenes, S. suis* and *S. uberis*.

7. The protein MgtA or an immunogenic part thereof, for use as a vaccine.

8. An anti-MgtA antibody or fragment thereof, preferably a humanized anti-MgtA antibody or fragment thereof.

9. An anti-MgtA antibody or fragment thereof, preferably a humanized anti-MgtA antibody or fragment thereof, for use as a medicament for the prophylactic or therapeutic treatment of an infection by a meningeal pathogen in a mammalian subject, preferably a human subject.

10. Use of anti-MgtA antibody or fragment thereof, preferably a humanized anti-MgtA antibody or fragment thereof, for the manufacture of a medicament for the prophylactic or therapeutic treatment of an infection by a meningeal pathogen in a mammalian subject, preferably a human subject.

11. A pharmaceutical composition comprising an anti-MgtA antibody or fragment thereof, preferably a humanized anti-MgtA antibody or fragment thereof, and a pharmaceutically acceptable carrier.

12. A method for prophylactic or therapeutic treatment of an infection by a meningeal pathogen in a mammalian subject, preferably a human subject comprising administering to said subject in need of such treatment an effective amount of a vaccine formulation as defined in any one of claims 1-6 and/or an effective amount of a pharmaceutical composition as defined in claim 11.

13. A method for preparing an MgtA-based vaccine formulation, the said method comprising bringing into association, an effective amount of the protein MgtA or an immunogenic part thereof and at least one of a pharmaceutically acceptable diluent, carrier, excipient or adjuvant therefore.

14. A method for preparing a pharmaceutical composition as defined in claim 11, the said method comprising bringing into association, an effective amount of an anti-MgtA antibody, preferably a humanized anti-MgtA antibody, or fragment thereof, and a pharmaceutically acceptable carrier.

Figure 1

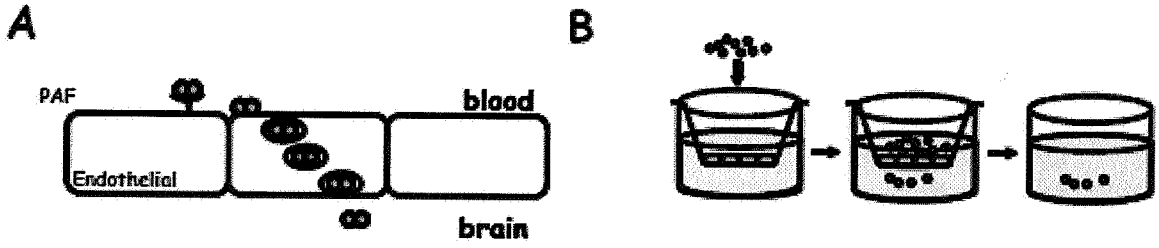


Figure 2

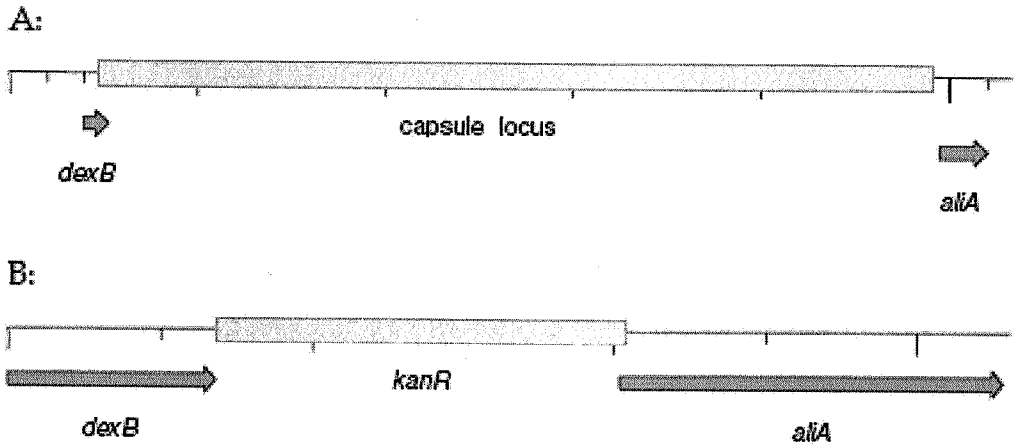


Figure 3

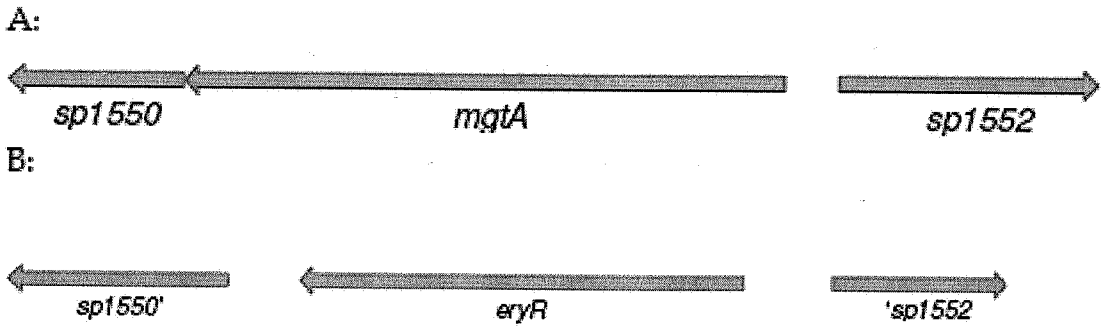


Figure 4

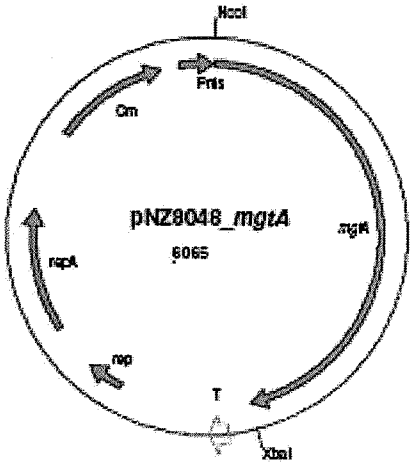


Figure 5

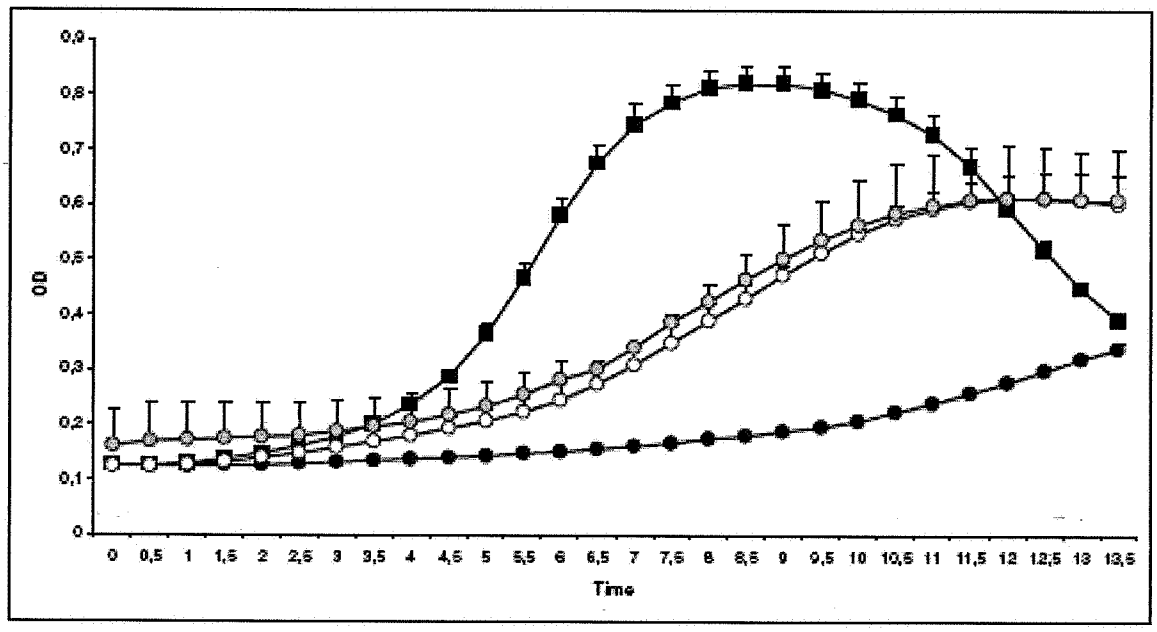


Figure 6

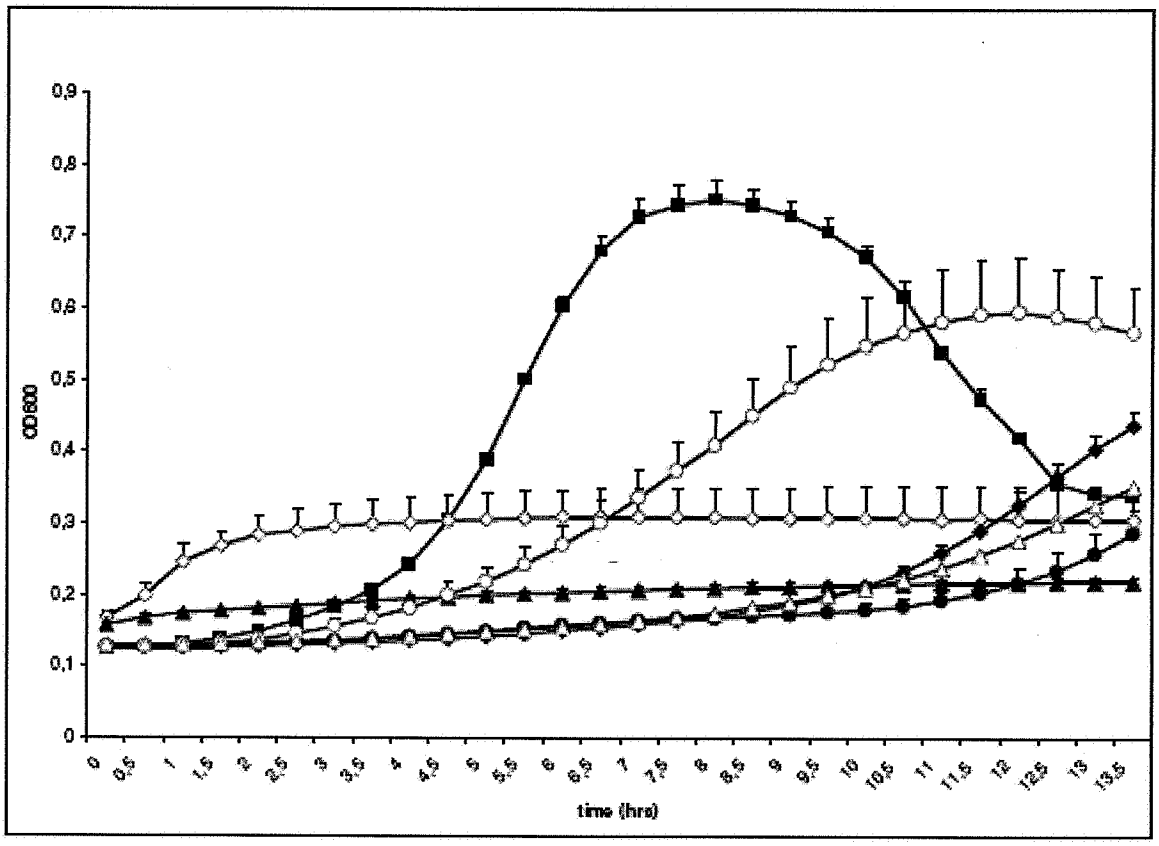

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0,3
0,2
0,1
0
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time (hrs)

Figure 7

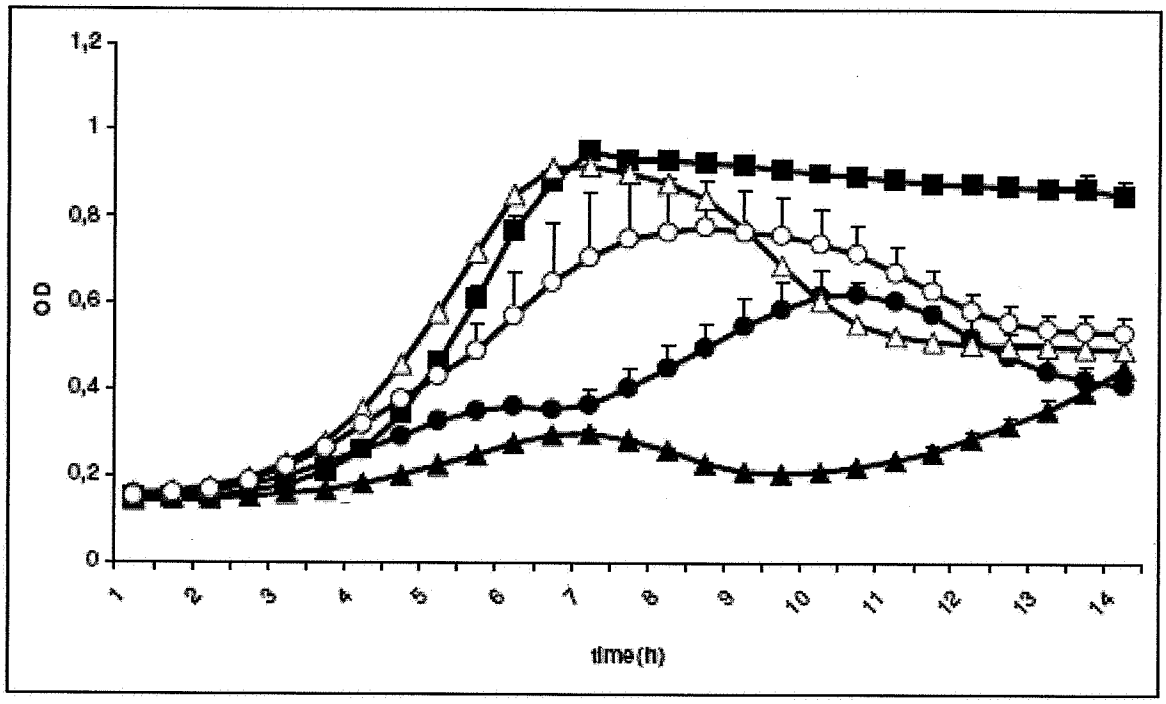


Figure 8

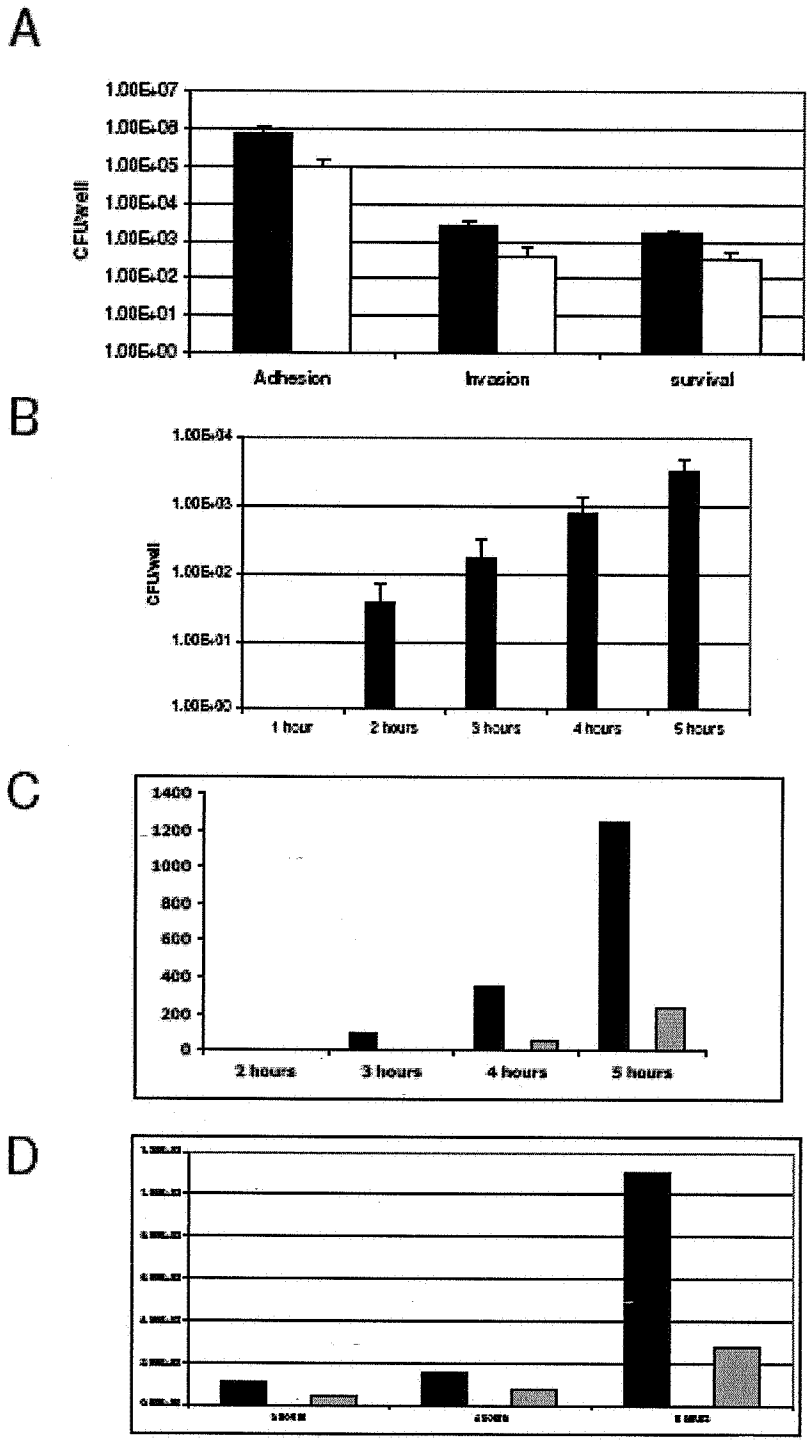

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Adhesion
Invasion
survival
B
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1.00E+02
1.00E+01
1.00E+00
CFU/well
1 hour
2 hours
3 hours
4 hours
5 hours
C
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800
600
400
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0
2 hours
3 hours
4 hours
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D

Figure 9

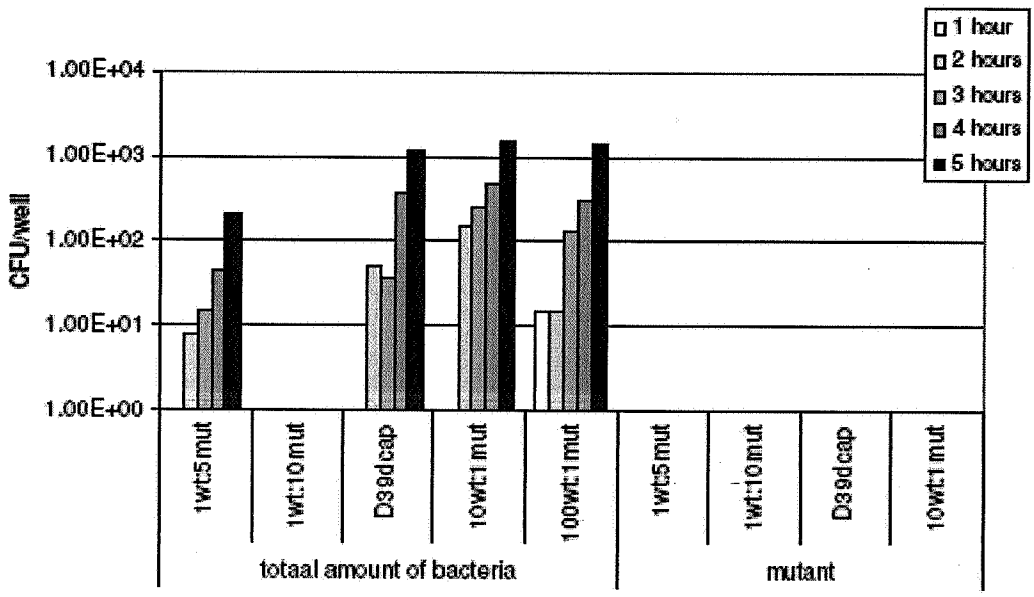

1 hour
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3 hours
4 hours
5 hours
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1.00E+01
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CFU/well
1wt:5 mut
1wt:10 mut
D39dcap
10wt:1 mut
100wt:1 mut
1wt:5 mut
1wt:10 mut
D39dcap
10wt:1 mut
totaal amount of bacteria
mutant

Figure 10

>SP1551 Protein Sequence >SP1551 15901394 cation-transporting ATPase, E1-E2 family [Streptococcus pneumoniae TIGR4]

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KNAI

SPD_1383 protein sequence cation-transporting ATPase, E1-E2 family protein [Streptococcus pneumoniae D39.
LOCUS ABJ54610

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addnfatiiv aveegrkvfs niqktiqyll santaevlti flstlfgwdv lqpvhllwin
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aypvhvgdnh aihadaltma fatlgliqlf haynvksvyq siltvgpfks ktfnwsilvs
fillmativv eplegifhvt kldlsqwgiv magsfsmiii veivkfiqrk lgfdknai

Figure 11

>SP1551 DNA Sequence >SP1551 15901394 cation-transporting ATPase, E1-E2 family [Streptococcus pneumoniae TIGR4]

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TGGTGTTGAACCTGCGGAACCTGGTGTCATGAATCATAAACCACGTGGACGCAAGGCAAGCTTCTTCTCA
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SPD_1383 DNA sequence cation-transporting ATPase, E1-E2 family protein [Streptococcus pneumoniae D39.
LOCUS

ATGACAGAAAGAGAGTCTGTTTTGCACACAATGTCAAGGAGGAGACACATGTCAAAAGAA
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GCCCGCGTTCTTCGTGATGGACACATGGCAGAGATTGACTCTAAAGAATTGGTACCAGGC
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GATGTGGAAGGTAACTTGATTGGTGACCCAACCGAAACAGCCTTTATCCAATATGCCTTG
GACAAGGGCTATGATGTCAAAGGTTTCTTAGAGAAATATCCTCGTGTAGCTGAATTGCCA
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Figure 12 CLUSTAL W (1.83) multiple sequence alignment

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spyM18_0689             ----------------MSKEQRHEAFYTQSEETVLAQLETSREGLTSAQAKERLAEYGRN 44
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SpyM3_0440              ----------------MSKEQRHEAFYTQSEETVLAQLETSREGLTSAQAKERLAEYGRN 44
SAJ_0627                ----------------MLKEQKKSLFYTQGQEEVLTSLESSREGLSTTEAKNRLEMYGRN 44
SAK_0664                ----------------MLKEQKKSLFYTQGQEEVLTSLESSREGLSTTEAKNRLEMYGRN 44
SAM_0540                ----------------MLKEQKKSLFYTQGQEEVLTSLESSREGLSTTEAKNRLEMYGRN 44
SAN_0617                ----------------MLKEQKKSLFYTQGQEEVLTSLESSREGLSTTEAKNRLEMYGRN 44
SAG_0514                ----------------MLKEQKKSLFYTQGQEEVLTSLESSREGLSTTEAKNRLEMYGRN 44
SP_1551                 MTERESVLHTMSRRRHMSKEQKRQAFYTQSPEEVLQAVDATEQGLSSSEAEKRLAEFGHN 60
SPN05340                MTERESVLHTMSRRRHMSKEQKRQAFYTQSPEEVLQAVDATEQGLSSSEAEKRLAEFGHN 60
spr1410                 ----------------MSKEQKRQAFYTQSPEEVLQAVDATEQGLSSSEAEKRLAEFGHN 44
SMT0794                 ----------------MSKEQKRQAFYTQSPEEVLKAVDATEQGLSSSEAEKRLAEFGHN 44
                                        * ***::. ****. * **  :::::.:**::::*::**  :*:*

M5005_Spy0516           ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
SPy0623                 ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
spyM18_0689             ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
MGAS2096_Spy0528        ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
MGAS9429_Spy0507        ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
M28_Spy0495             ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
MGAS10750_Spy0535       ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
MGAS10270_Spy0510       ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
M6_Spy0537              ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
SpyM3_0440              ELDEGEKRSLFMKFLDQFKDLMIIILIVAALLSVLTEGMEGLTDAIIILAVVILNAAFGV 104
SAJ_0627                ELEEGKKRSLIAKFFDQFKDLMIIILLVAAALSVITEGMHGLTDALIILAVVILNAAFGV 104
SAK_0664                ELEEGKKRSLIAKFFDQFKDLMIIILLVAAALSVITEGMHGLTDALIILAVVILNAAFGV 104
SAM_0540                ELEEGKKRSLIAKFFDQFKDLMIIILLVAAALSVITEGMHGLTDALIILAVVILNAAFGV 104
SAN_0617                ELEEGKKRSLIAKFFDQFKDLMIIILLVAAALSVITEGMHGLTDALIILAVVILNAAFGV 104
SAG_0514                ELEEGKKRSLIAKFFDQFKDLMIIILLVAAALSVITEGMHGLTDALIILAVVILNAAFGV 104
SP_1551                 ELEEGEKRSILVKFIEQFKDLMIIILVAAAILSVVTSGGEDIADAIIILAVVIINAAFGV 120
SPN05340                ELEEGEKRSILVKFIEQFKDLMIIILVAAAILSVVTSGGEDIADAIIILAVVIINAAFGV 120
spr1410                 ELEEGEKRSILVKFIEQFKDLMIIILVAAAILSVVTSGGEDIADAIIILAVVIINAAFGV 104
SMT0794                 ELEEGEKRSILVKFIEQFKDLMIIILVAAAILSVVTSGGEDIADAIIILAVVIINAAFGV 104
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SPy0623                 YQEGQAEAAIEALKSMSSPLARIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
spyM18_0689             YQEGQAEAAIEALKSMSSPLARIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
MGAS2096_Spy0528        YQEGQAEAAIEALKSMSSPLARIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
MGAS9429_Spy0507        YQEGQAEAAIEALKSMSSPLACIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
M28_Spy0495             YQEGQAEAAIEALKSMSSPLARIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
MGAS10750_Spy0535       YQEGQAEAAIEALKSMSSPLARIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
MGAS10270_Spy0510       YQEGQAEAAIEALKSMSSPLARIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
M6_Spy0537              YQEGQAEAAIEALKSMSSPLARIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
SpyM3_0440              YQEGQAEAAIEALKSMSSPLARIRRDGHVTEIDSKELVPGDIVLLEAGDVVPADLRLLEA 164
SAJ_0627                YQEGQAEAAIEALKDMSSPIARVRRDGHTIEVDSKELVPGDLVMLEAGDVVPADLRLLEA 164
SAK_0664                YQEGQAEAAIEALKDMSSPIARVRRDGHTIEVDSKELVPGDLVMLEAGDVVPADLRLLEA 164
SAM_0540                YQEGQAEAAIEALKDMSSPIARVRRDGHTIEVDSKELVPGDLVMLEAGDVVPADLRLLEA 164
SAN_0617                YQEGQAEAAIEALKDMSSPIARVRRDGHTIEVDSKELVPGDLVMLEAGDVVPADLRLLEA 164
SAG_0514                YQEGQAEAAIEALKDMSSPIARVRRDGHTIEVDSKELVPGDLVMLEAGDVVPADLRLLEA 164
SP_1551                 YQEGKAEEAIEALKSMSSPVARVLRDGHMAEIDSKELVPGDIVALEAGDVVPADLRLIEA 180
SPN05340                YQEGKAEEAIEALKSMSSPVARVLRDGHMAEIDSKELVPGDIVALEAGDVVPADLRLIEA 180
spr1410                 YQEGKAEEAIEALKSMSSPVARVLRDGHMAEIDSKELVPGDIVALEAGDVVPADLRLIEA 164
SMT0794                 YQEGKAEEAIEALKSMSSPAARVLRDGHMAEIDSKELVPGDIVSLEAGDVVPADLRLLEA 164
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spyM18_0689             NSLKIEEAALTGESVPVEKDLSTAVSEDAGIGDRVNMGYQNSNVTYGRGIGVITNTGMYT 224
MGAS2096_Spy0528        NSLKIEEAALTGESVPVEKDLSTAVSEDAGIGDRVNMGYQNSNVTYGRGIGVITNTGMYT 224
MGAS9429_Spy0507        NSLKIEEAALTGESVPVEKDLSTAVSEDAGIGDRVNMGYQNSNVTYGRGIGVITNTGMYT 224
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MGAS10270_Spy0510    NSLKIEEAALTGESVPVEKDLSTAVSEDAGIGDRVNMGYQNSNVTYGRGIGVITNTGMYT 224
M6_Spy0537           NSLKIEEAALTGESVPVEKDLSTAVSEDASIGDRVNMGYQNSNVTYGRGIGVITNTGMYT 224
SpyM3_0440           NSLKIEEAALTGESVPVEKDLSTAVSKDAGIGDRVNMGYQNSNVTYGRGIGVITNTGMYT 224
SAJ_0627             ASLKIEEAALTGESVPVEKDISQVVAEDAGIGDRVNMAYQNSNVTYGRGYGVVTNTGMYT 224
SAK_0664             ASLKIEEAALTGESVPVEKDISQVVAEDAGIGDRVNMAYQNSNVTYGRGYGVVTNTGMYT 224
SAM_0540             ASLKIEEAALTGESVPVEKDISQVVAEDAGIGDRVNMAYQNSNVTYGRGYGVVTNTGMYT 224
SAN_0617             ASLKIEEAALTGESVPVEKDISQVVAEDAGIGDRVNMAYQNSNVTYGRGYGVVTNTGMYT 224
SAG_0514             ASLKIEEAALTGESVPVEKDISQVVAEDAGIGDRVNMAYQNSNVTYGRGYGVVTNTGMYT 224
SP_1551              NSLKIEEAALTGESVPVEKDLSVELATDAGIGDRVNMAFQNSNVTYGRGMGVVVNTGMYT 240
SPN05340             NSLKIEEAALTGESVPVEKDLSVELATDAGIGDRVNMAFQNSNVTYGRGMGVVVNTGMYT 240
spr1410              NSLKIEEAALTGESVPVEKDLSVDLATDAGIGDRVNMAFQNSNVTYGRGMGVVVNTGMYT 224
SMT0794              NSLKIEEAALTGESVPVEKDLTVELAADAGIGDRVNMAFQNSNVTYGRGMGVVVNTGMYT 224
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M5005_Spy0516        EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
SPy0623              EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
spyM18_0689          EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
MGAS2096_Spy0528     EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
MGAS9429_Spy0507     EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
M28_Spy0495          EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
MGAS10750_Spy0535    EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
MGAS10270_Spy0510    EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
M6_Spy0537           EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
SpyM3_0440           EVGHIAGMLANADETDTPLKQNLDNLSKILTYAILVIAAVTFAVGVFLRGQHPLEGLMTS 284
SAJ_0627             EVGKIADMLANADESETPLKQSLVQLSKLLTYLIVIIAVITFLVGIFVRKEGWIEGLMTS 284
SAK_0664             EVGKIADMLANADESETPLKQSLVQLSKLLTYLIVIIAVITFLVGIFVRKEGWIEGLMTS 284
SAM_0540             EVGKIADMLANADESETPLKQSLVQLSKLLTYLIVIIAVITFLVGIFVRKEGWIEGLMTS 284
SAN_0617             EVGKIADMLANADESETPLKQSLVQLSKLLTYLIVIIAVITFLVGIFVRKEGWIEGLMTS 284
SAG_0514             EVGKIADMLANADESETPLKQSLVQLSKLLTYLIVIIAVITFLVGIFVRKEGWIEGLMTS 284
SP_1551              EVGHIAGMLQDADETDTPLKQNLNNLSKVLTYAILVIALVTFVVGVFIQGKNPLGELLTS 300
SPN05340             EVGHIAGMLQDADETDTPLKQNLNNLSKVLTYAILVIALVTFVVGVFIQGKNPLGELLTS 300
spr1410              EVGHIAGMLQDADETDTPLKQNLNNLSKVLTYAILVIALVTFVVGVFIQGKNPLGELLTS 284
SMT0794              EVGHIAGMLQDADETDTPLKQNLNNLSKVLTYAILVIALVTFVVGVFIQGKNPLGELMTS 284
                     ***:**.** :***::*****.* :***:*** *::** :** **:*:: :  :  *:**

M5005_Spy0516        VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
SPy0623              VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
spyM18_0689          VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
MGAS2096_Spy0528     VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
MGAS9429_Spy0507     VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
M28_Spy0495          VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
MGAS10750_Spy0535    VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
MGAS10270_Spy0510    VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
M6_Spy0537           VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
SpyM3_0440           VALAVAAIPEGLPAIVTVVLSLGTQVLAKRNAIIRKLPAVETLGSTEIIASDKTGTLTMN 344
SAJ_0627             VALAVAAIPEGLPAIVTIVLSMGTKTLAKRNSIVRKLPAVETLGSTEIIASDKTGTLTMN 344
SAK_0664             VALAVAAIPEGLPAIVTIVLSMGTKTLAKRNSIVRKLPAVETLGSTEIIASDKTGTLTMN 344
SAM_0540             VALAVAAIPEGLPAIVTIVLSMGTKTLAKRNSIVRKLPAVETLGSTEIIASDKTGTLTMN 344
SAN_0617             VALAVAAIPEGLPAIVTIVLSMGTKTLAKRNSIVRKLPAVETLGSTEIIASDKTGTLTMN 344
SAG_0514             VALAVAAIPEGLPAIVTIVLSMGTKTLAKRNSIVRKLPAVETLGSTEIIASDKTGTLTMN 344
SP_1551              VALAVAAIPEGLPAIVTIVLSLGTQVLAKRHSIVRKLPAVETLGSTEIIASDKTGTLTMN 360
SPN05340             VALAVAAIPEGLPAIVTIVLSLGTQVLAKRHSIVRKLPAVETLGSTEIIASDKTGTLTMN 360
spr1410              VALAVAAIPEGLPAIVTIVLSLGTQVLAKRHSIVRKLPAVETLGSTEIIASDKTGTLTMN 344
SMT0794              VALAVAAIPEGLPAIVTIVLALGTQVLAKRNSIVRKLPAVETLGSTEIIASDKTGTLTMN 344
                     ******************:**::**:.****::*:**************************

M5005_Spy0516        QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVEFGL 402
SPy0623              QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVEFGL 402
spyM18_0689          QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKSIGDPTETALVQFGL 402
MGAS2096_Spy0528     QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVQFGL 402
MGAS9429_Spy0507     QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVQFGL 402
M28_Spy0495          QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVQFGL 402
MGAS10750_Spy0535    QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVQFGL 402
MGAS10270_Spy0510    QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVQFGL 402
M6_Spy0537           QMTVEKVYTNGTLQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVQFGL 402
SpyM3_0440           QMTVEKVYTNGALQSSSADIAFDNTT--LRVMNFANDTKVDPSGKLIGDPTETALVQFGL 402
SAJ_0627             QMTVEKVYTNGVLQSSSEEISVDNNT--LRIMNFSNDTKIDPSGKLIGDPTETALVQFGL 402
SAK_0664             QMTVEKVYTNGVLQSSSEEISVDNNT--LRIMNFSNDTKIDPSGKLIGDPTETALVQFGL 402
SAM_0540             QMTVEKVYTNGVLQSSSEEISVDNNT--LRIMNFSNDTKIDPSGKLIGDPTETALVQFGL 402
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SAN_0617                QMTVEKVYTNGVLQSSSEEISVDNNT--LRIMNFSNDTKIDPSGKLIGDPTETALVQFGL 402
SAG_0514                QMTVEKVYTNGVLQSSSEEISVDNNT--LRIMNFSNDTKIDPSGKLIGDPTETALVQFGL 402
SP_1551                 KMTVEKVFYDAVLHDSADDIELGLEMPLLRSVVLANDTKIDVEGNLIGDPTETAFIQYAL 420
SPN05340                KMTVEKVFYDAVLHDSADDIELGLEMPLLRSVVLANDTKIDVEGNLIGDPTETAFIQYAL 420
spr1410                 KMTVEKVFYDAVLHDSADDIELGLEMPLLRSVVLANDTKIDVEGNLIGDPTETAFIQYAL 404
SMT0794                 KMTVEKVFYDAILHDSADDIELGLEMPLLRSVVLANDTKIDVEGNLIGDPTETAFIQYAL 404
                        :******: :. *:.*: :* ..     ** : ::****:* .*: ********:::::.*

M5005_Spy0516           DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
SPy0623                 DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
spyM18_0689             DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
MGAS2096_Spy0528        DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
MGAS9429_Spy0507        DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
M28_Spy0495             DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
MGAS10750_Spy0535       DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
MGAS10270_Spy0510       DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
M6_Spy0537              DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
SpyM3_0440              DHNFDVREAMVAEPRVAELPFDSDRKLMSTIHKQADGKYFIAVKGAPDQLLKRVTQIEEN 462
SAJ_0627                DKNFDVREVLKNEPRVAELPFDSDRKLMSTIHKESDGRYFIAVKGAPDQLLKRVTKIEDN 462
SAK_0664                DKNFDVREVLKNEPRVAELPFDSDRKLMSTIHKESDGRYFIAVKGAPDQLLKRVTKIEDN 462
SAM_0540                DKNFDVREVLKNEPRVAELPFDSDRKLMSTIHKESDGRYFIAVKGAPDQLLKRVTKIEDN 462
SAN_0617                DKNFDVREVLKNEPRVAELPFDSDRKLMSTIHKESDGRYFIAVKGAPDQLLKRVTKIEDN 462
SAG_0514                DKNFDVREVLKNEPRVAELPFDSDRKLMSTIHKESDGRYFIAVKGAPDQLLKRVTKIEDN 462
SP_1551                 DKGYDVKGFLEKYPRVAELPFDSDRKLMSTVHPLPDGRFLVAVKGAPDQLLKRCLLRDKA 480
SPN05340                DKGYDVKGFLEKYPRVAELPFDSDRKLMSTVHPLPDGRFLVAVKGAPDQLLKRCLLRDKA 480
spr1410                 DKGYDVKGFLEKYPRVAELPFDSDRKLMSTVHPLPDSRFLVAVKGAPDQLLKRCLLRDKA 464
SMT0794                 DKGYDVKGFLDKYPRVAELPFDSDRKLMSTVHPLPDGKFLVAVKGAPDQLLKRCVLRDKA 464
                        *:.:**:  :   *****************:*  .*.::::************    :.

M5005_Spy0516           GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
SPy0623                 GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
spyM18_0689             GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
MGAS2096_Spy0528        GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
MGAS9429_Spy0507        GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
M28_Spy0495             GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
MGAS10750_Spy0535       GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
MGAS10270_Spy0510       GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
M6_Spy0537              GQIRSITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
SpyM3_0440              GQIRPITDADKKTILDTNKSLAKQALRVLMMAYKYSDALPT-LETEIVEANLVFSGLVGM 521
SAJ_0627                GLVRDITAEDKEAILNTNKELAKQALRVLMMAYKYETQIPT-LETDIVESDLVFSGLVGM 521
SAK_0664                GLVRDITAEDKEAILNTNKELAKQALRVLMMAYKYETQIPT-LETDIVESDLVFSGLVGM 521
SAM_0540                GLVRDITAEDKEAILNTNKELAKQALRVLMMAYKYETQIPT-LETDIVESDLVFSGLVGM 521
SAN_0617                GLVRDITAEDKEAILNTNKELAKQALRVLMMAYKYETQIPT-LETDIVESDLVFSGLVGM 521
SAG_0514                GLVRDITAEDKEAILNTNKELAKQALRVLMMAYKYETQIPS-LETDIVESDLVFSGLVGM 521
SP_1551                 GDIAPIDEKVTNLIRTNNSEMAHQALRVLAGAYKIIDSIPENLTSEELENDLIFTGLIGM 540
SPN05340                GDIAPIDEKVTNLIHTNNSEMAHQALRVLAGAYKIIDSIPENLTSEELENDLIFTGLIGM 540
spr1410                 GDIAPIDEKVTNLIHTNNSEMAHQALRVLAGAYKIIDSIPENLTSEELENDLIFTGLIGM 524
SMT0794                 GDIAPIDEKVTNLIHTNNSEMAHQALRVLAGAYKIIDSIPENLTSEELENDLIFTGLIGM 524
                        * :  *    .: *  .*..:*:******  ***    :*  * :: :* :*:*:**:**

M5005_Spy0516           IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
SPy0623                 IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
spyM18_0689             IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
MGAS2096_Spy0528        IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
MGAS9429_Spy0507        IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
M28_Spy0495             IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
MGAS10750_Spy0535       IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
MGAS10270_Spy0510       IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
M6_Spy0537              IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
SpyM3_0440              IDPERPEAAQAVKVAKEAGIRPIMITGDHQDTAKAIAKRLGIIEE-DGVDHVFTGAELNE 580
SAJ_0627                IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEDHVFTGAELNE 581
SAK_0664                IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEDHVFTGAELNE 581
SAM_0540                IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEDHVFTGAELNE 581
SAN_0617                IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEDHVFTGAELNE 581
SAG_0514                IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEDHVFTGAELNE 581
SP_1551                 IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEGHVLTGAELNE 600
SPN05340                IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEGHVLTGAELNE 600
spr1410                 IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEGHVLTGAELNE 584
SMT0794                 IDPERPEAAEAVRVAKEAGIRPIMITGDHQDTAEAIAKRLGIIDANDTEGHVLTGAELNE 584
                        *********:**:********************:*********:  *  .**:*******
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M5005_Spy0516          LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVAMTGDGVNDAPSLKTADIGIGM 640
SPy0623                LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVAMTGDGVNDAPSLKTADIGIGM 640
spyM18_0689            LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVAMTGDGVNDAPSLKTADIGIGM 640
MGAS2096_Spy0528       LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVGMTGDGVNDAPSLKTADIGIGM 640
MGAS9429_Spy0507       LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVGMTGDGVNDAPSLKTADIGIGM 640
M28_Spy0495            LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVAMTGDGVNDAPSLKTADIGIGM 640
MGAS10750_Spy0535      LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVAMTGDGVNDAPSLKTADIGIGM 640
MGAS10270_Spy0510      LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVAMTGDGVNDAPSLKTADIGIGM 640
M6_Spy0537             LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVAMTGDGVNDAPSLKTADIGIGM 640
SpyM3_0440             LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNEGKVVAMTGDGVNDAPSLKTADIGIGM 640
SAJ_0627               LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNDGKVVAMTGDGVNDAPSLKTADIGIGM 641
SAK_0664               LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNDGKVVAMTGDGVNDAPSLKTADIGIGM 641
SAM_0540               LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNDGKVVAMTGDGVNDAPSLKTADIGIGM 641
SAN_0617               LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNDGKVVAMTGDGVNDAPSLKTADIGIGM 641
SAG_0514               LSDEEFQKVFKQYSVYARVSPEHKVRIVKAWQNDGKVVAMTGDGVNDAPSLKTADIGIGM 641
SP_1551                LSDEEFEKVVGQYSVYARVSPEHKVRIVKAWQKQGKVVAMTGDGVNDAPALKTADIGIGM 660
SPN05340               LSDEEFEKVVGQYSVYARVSPEHKVRIVKAWQKQGKVVAMTGDGVNDAPALKTADIGIGM 660
spr1410                LSDEEFEKVVGQYSVYARVSPEHKVRIVKAWQKQGKVVAMTGDGVNDAPALKTADIGIGM 644
SMT0794                LSDEDFEKVVGQYSVYARVSPEHKVRIVKAWQKQGKVVAMTGDGVNDAPALKTADIGIGM 644
                       ****:*:**. ********************::****.**********:********** 

M5005_Spy0516          GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
SPy0623                GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
spyM18_0689            GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
MGAS2096_Spy0528       GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
MGAS9429_Spy0507       GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
M28_Spy0495            GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
MGAS10750_Spy0535      GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
MGAS10270_Spy0510      GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
M6_Spy0537             GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
SpyM3_0440             GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANMAEVFTIFLAT 700
SAJ_0627               GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKSIQYLLSANMAEVFTIFFAT 701
SAK_0664               GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKSIQYLLSANMAEVFTIFFAT 701
SAM_0540               GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKSIQYLLSANMAEVFTIFFAT 701
SAN_0617               GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKSIQYLLSANMAEVFTIFFAT 701
SAG_0514               GITGTEVSKGASDMVLADDNFATIIVAVEEGRKVFSNIQKSIQYLLSANMAEVFTIFFAT 701
SP_1551                GITGTEVSKGASDMILADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANTAEVLTIFLST 720
SPN05340               GITGTEVSKGASDMILADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANTAEVLTIFLST 720
spr1410                GITGTEVSKGASDMILADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANTAEVLTIFLST 704
SMT0794                GITGTEVSKGASDMILADDNFATIIVAVEEGRKVFSNIQKTIQYLLSANTAEVLTIFLST 704
                       **************:************************:******** ***:***::* 

M5005_Spy0516          LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
SPy0623                LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
spyM18_0689            LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
MGAS2096_Spy0528       LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
MGAS9429_Spy0507       LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
M28_Spy0495            LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
MGAS10750_Spy0535      LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
MGAS10270_Spy0510      LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
M6_Spy0537             LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
SpyM3_0440             LFGWDVLQPVHLLWINLVTDTLPAIALGVEPAEPGVMKHKPRGRKSSFFDGGVKEAILYQ 760
SAJ_0627               LLGWDVLAPVHLLWINLVTDTLPAIALGVEPAEPGVMTHKPRGRQSNFFDGGVMGAIIYQ 761
SAK_0664               LLGWDVLAPVHLLWINLVTDTLPAIALGVEPAEPGVMTHKPRGRQSNFFDGGVMGAIIYQ 761
SAM_0540               LLGWDVLAPVHLLWINLVTDTLPAIALGVEPAEPGVMTHKPRGRQSNFFDGGVMGAIIYQ 761
SAN_0617               LLGWDVLAPVHLLWINLVTDTLPAIALGVEPAEPGVMTHKPRGRQSNFFDGGVMGAIIYQ 761
SAG_0514               LLGWDVLAPVHLLWINLVTDTLPAIALGVEPAEPGVMTHKPRGRQSNFFDGGVMGAIIYQ 761
SP_1551                LFGWDVLQPVHLLWINLVTDTFPAIALGVEPAEPGVMNHKPRGRKASFFSGGVLSSIIYQ 780
SPN05340               LFGWDVLQPVHLLWINLVTDTFPAIALGVEPAEPGVMNHKPRGRKASFFSGGVLSSIIYQ 780
spr1410                LFGWDVLQPVHLLWINLVTDTFPAIALGVEPAEPGVMNHKPRGRKASFFSGGVLSSIIYQ 764
SMT0794                LFGWDVLQPVHLLWINLVTDTFPAIALGVEPAEPGVMNHKPRGRKASFFSGGVVSSIIYQ 764
                       *:***** *************:***************.******::.**.***  :*:** 

M5005_Spy0516          GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
SPy0623                GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
spyM18_0689            GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
MGAS2096_Spy0528       GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
MGAS9429_Spy0507       GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
M28_Spy0495            GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
MGAS10750_Spy0535      GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
MGAS10270_Spy0510      GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
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M6_Spy0537              GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
SpyM3_0440              GAFQTILVLGVYGFALMFPEHTS-YHDVHADALTMAYVTLGLIQLVHAYNVKSVYQSIFT 819
SAJ_0627                GILQTILVLGVYGWALMYPEHAG-YRMIHADALTMAFATLGLIQLVHAFNVKSVYQSIFT 820
SAK_0664                GILQTILVLGVYGWALMYPEHAG-YRMIHADALTMAFATLGLIQLVHAFNVKSVYQSIFT 820
SAM_0540                GILQTILVLGVYGWALMYPEHAG-YRMIHADALTMAFATLGLIQLVHAFNVKSVYQSIFT 820
SAN_0617                GILQTILVLGVYGWALMYPEHAG-YRMIHADALTMAFATLGLIQLVHAFNVKSVYQSIFT 820
SAG_0514                GILQTILVLGVYGWALMYPEHAG-YRMIHADALTMAFATLGLIQLVHAFNVKSVYQSIFT 820
SP_1551                 GVLQAALVMSVYGLAIAYPVHVGDNHAIHADALTMAFATLGLIQLFHAYNVKSVYQSILT 840
SPN05340                GVLQAALVMSVYGLAIAYPVHVGDNHAIHADALTMAFATLGLIQLFHAYNVKSVYQSILT 840
spr1410                 GVLQAALVMSVYGLAIAYPVHVGDNHAIHADALTMAFATLGLIQLFHAYNVKSVYQSILT 824
SMT0794                 GVLQAAIVMSVYGLAIAYPVHVGDNHAIHADALTMAFATLGLIQLFHAYNVKSVYQSILT 824
                        * :*: :*:.*** *: :* *..  : :********:.*******.**:*********:*

M5005_Spy0516           VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
SPy0623                 VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
spyM18_0689             VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
MGAS2096_Spy0528        VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
MGAS9429_Spy0507        VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
M28_Spy0495             VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
MGAS10750_Spy0535       VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
MGAS10270_Spy0510       VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
M6_Spy0537              VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
SpyM3_0440              VGLFKNKLFNYSIPVAFVALMATVVVPGFNQFFHVTHLTITQWLVVIIGSLLMVVLVELV 879
SAJ_0627                VGAFKNRTFNWSIPVAFILLMVTIVVPGFNKLFHVTHLSSTQWLTVVIGSLLMVVLTEIV 880
SAK_0664                VGAFKNRTFNWSIPVAFILLMVTIVVPGFNKLFHVTHLSSTQWLTVVIGSLLMVVLTEIV 880
SAM_0540                VGAFKNRTFNWSIPVAFILLMVTIVVPGFNKLFHVTHLSSTQWLTVVIGSLLMVVLTEIV 880
SAN_0617                VGAFKNRTFNWSIPVAFILLMVTIVVPGFNKLFHVTHLSSTQWLTVVIGSLLMVVLTEIV 880
SAG_0514                VGAFKNRTFNWSIPVAFILLMVTIVVPGFNKLFHVTHLSSTQWLTVVIGSLLMVVLTEIV 880
SP_1551                 VGPFKSKTFNWSILVSFILLMATIVVEPLEGIFHVTKLDLSQWGIVMAGSFSMIIIVEIV 900
SPN05340                VGPFKSKTFNWSILVSFILLMATIVVEPLEGIFHVTKLDLSQWGIVMAGSFSMIIIVEIV 900
spr1410                 VGPFKSKTFNWSILVSFILLMATIVVEPLEGIFHVTKLDLSQWGIVMAGSFSMIIIVEIV 884
SMT0794                 VGPFKSKTFNWSILVSFILLMATIVVEPLEGIFHVTKLDLSQWGIVMGGSFSMIIIVEIV 884
                        ** **.: **:** *:*: **.*:**  :: :****:*  :**  *: **: *:::.*:*

M5005_Spy0516           KAVQRSLGQDEKAI 893
SPy0623                 KAVQRSLGQDEKAI 893
spyM18_0689             KAVQRSLGQDEKAI 893
MGAS2096_Spy0528        KAVQRSLGQDEKAI 893
MGAS9429_Spy0507        KAVQRSLGQDEKAI 893
M28_Spy0495             KAVQRSLGQDEKAI 893
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SP_1551                 KFIQRKLGFDKNAI 914
SPN05340                KFIQRKLGFDKNAI 914
spr1410                 KFIQRKLGFDKNAI 898
SMT0794                 KFIQRKLGFDKNAI 898
                        * :**.** *::**
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Figure 13A

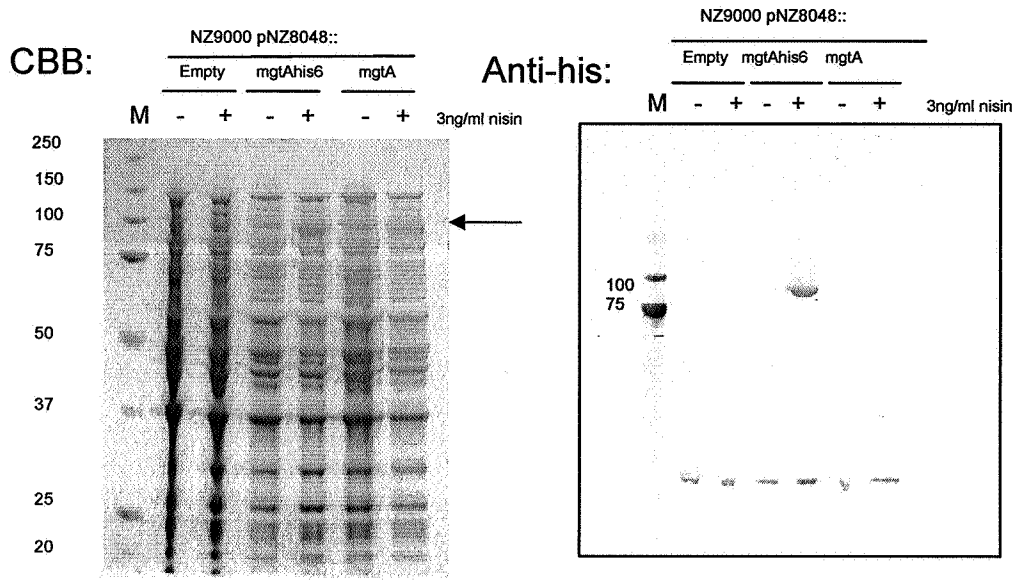
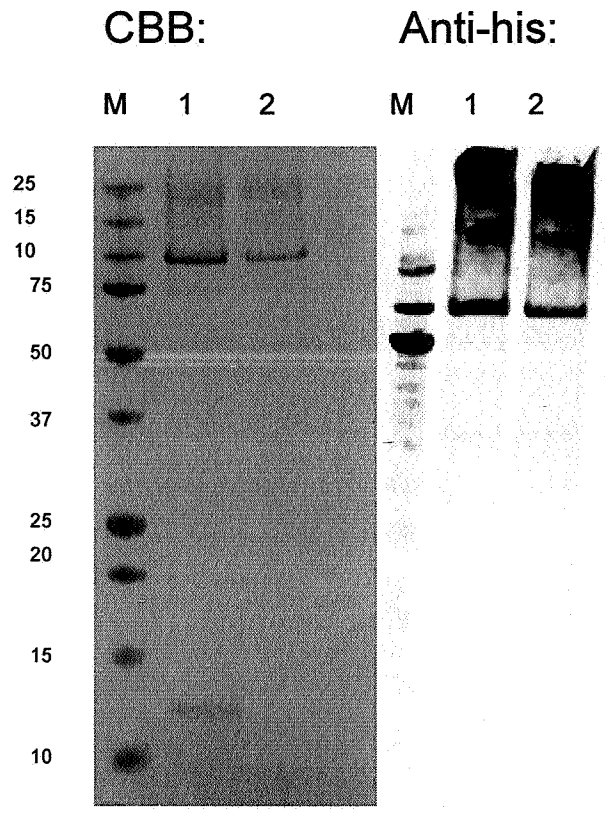

CBB:
NZ9000 pNZ8048::
Empty
mgtAhis6
mgtA
M - + - + - +
3ng/ml nisin
250
150
100
75
50
37
25
20
Anti-his:
NZ9000 pNZ8048::
Empty
mgtAhis6
mgtA
M - + - + - +
3ng/ml nisin
100
75

Figure 13B



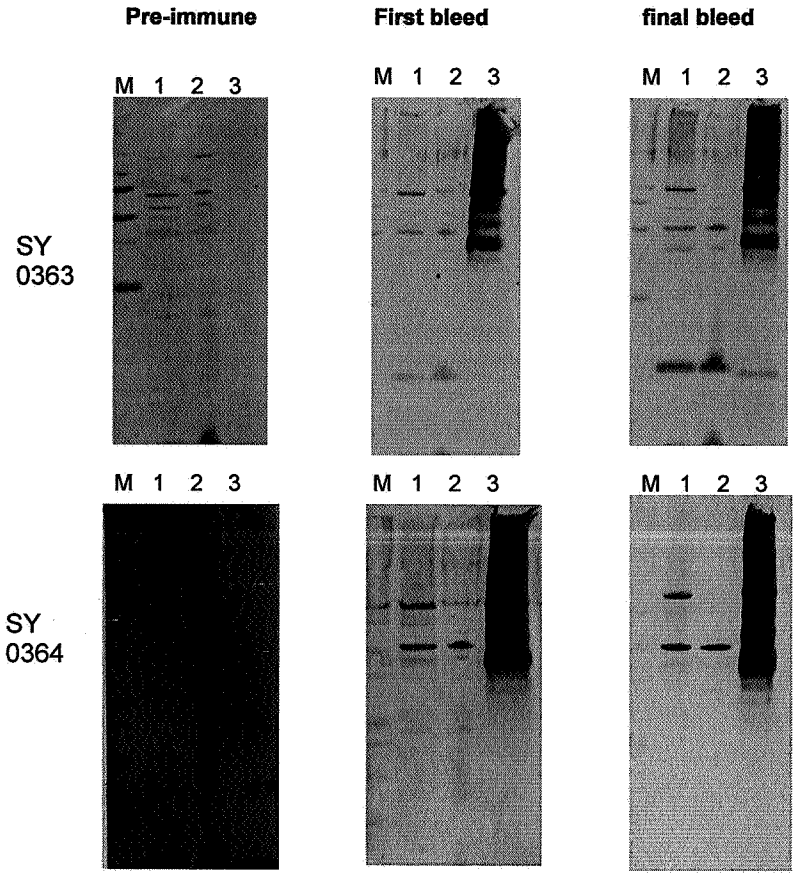


Figure 14

1 = D39Δ*cps nisRK*
2 = D39Δ*cps*Δ*mgtA nisRK*
3 = purified protein MgtA::his

Anti-MgtA 1:5000, preabsorbed against:
D39Δ*mgtA* NZ9000

Figure 15

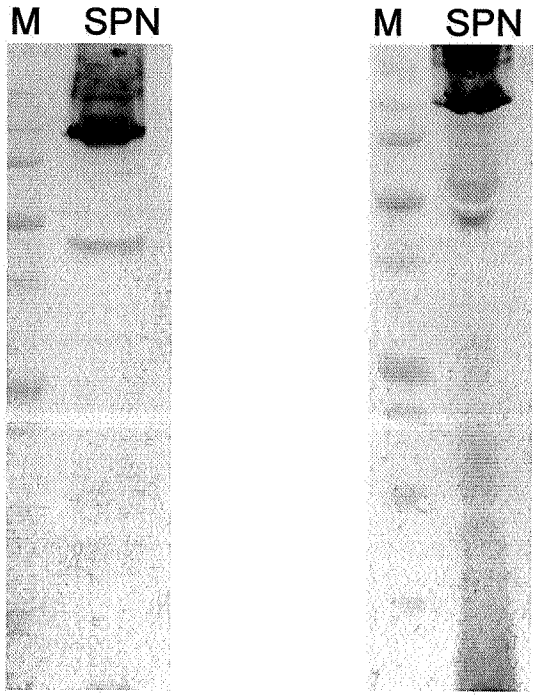
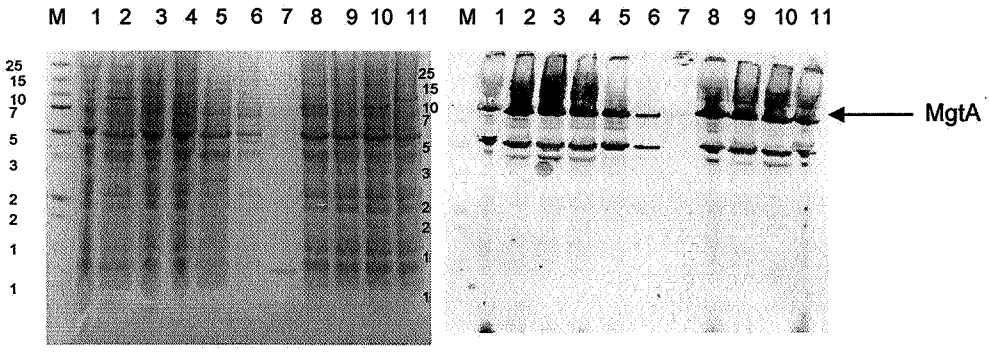
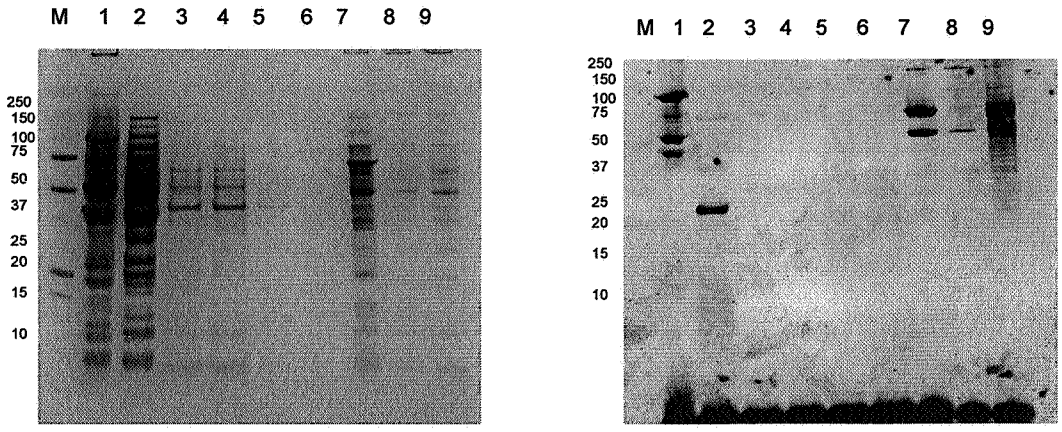


Figure 16



1= D39
2= Tigr4
3 = G54
4 = 670-7B
5 = 23F
6 = 6A14
7 = 6A17
8 = 6A23
9 = 6B20
10 = 6B23
11 = 6B29

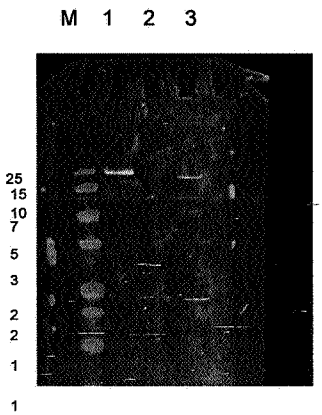
Figure 17



1= S.pneumoniae
2 = L. lactis
3= S.epidermidis
4 = S.epidermidis
5 = S.epidermidis
6 = S.epidermidis
7 = S. aureus
8 = S. aureus
9 = S.aureus

The serum was tested against L. lactis, S. epidermidis and S.aureus lab strains.
It reacts with a protein at the correct height in S.aureus and not with L. lactis and S.epidermidis.
The serum is not pre-absorbed which explains the lower molecular band

Figure 18



1= D39
2= S. pyogenes
3 = S. pyogenes

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2008/050210

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/09 C07K16/12 C07K14/315

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 C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/083855 A (AMERICAN CYANAMID CO [US]; ZAGURSKY ROBERT JOHN [US]; MASI AMY WADHAMS) 24 October 2002 (2002-10-24) cited in the application abstract page 11, lines 16-21 page 19, line 16 - page 20, line 9 page 20, line 6 - page 21, line 9 page 20, line 21 - page 21, line 17 page 64, line 12 - page 65, line 20 page 85, line 15 - page 90, line 24 page 100, lines 5-8 page 100, lines 17-22 claims 42,45,68,71-75,86,87,92 ----- -/--	1-14

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 September 2008	15/09/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Noë, Veerle

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2008/050210

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 application No
PCT/NL2008/050210

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