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Patent Application Publication

Arico et al.

MENINGOCOCCUS ADHESINS NADA, APP AND ORF 40

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ABSTRACT
NadA, App and ORF40 function as adhesins in N. meningitidis. Adhesion can be modulated by targeting these three proteins. NadA allelic variants are disclosed. Autoproteolytic cleavage of App is disclosed, as is removal of the activity by mutagenesis. App is processed and secreted into culture medium when expressed in E. coli. Mature App proteins are disclosed. Knockout mutants are disclosed. Vesicles from non-Neisserial hosts with heterologous adhesin expression are disclosed.
FIGURE 1

E. coli- ORF40
Purified protein

Whole cells
Western blot

Whole-cell immunoblot

E. coli- pET

E. coli- ORF40

FACS
**FIGURE 2**

Western blot

- **E. coli-pET**
- **E. coli-App**
- **E. coli-AppNL**
- Purified protein

Whole-cell immunoblot

FACS
FIGURE 3

Whole cells
OMV preparation

SDS-PAGE

E.coli- pET
E.coli- NadA
E.coli- nadANL

Purified protein

Whole-cell immunoblot
FIGURE 4

ORF40

APP

NADH

HSF POSITIVE CONTROL
**FIGURE 6A**

MFI

0 100 200 300

**FIGURE 6B**

NadA, GNA2132

0 100 200 300 400

**FIGURE 7**

- Hsf
- Hia
- ORF40

- Leader peptide
- Membrane domain
- Repeat regions
- Internal repeat region

**FIGURE 8**

- Hap
- App

- Active site

- Pro-rich domain

- Leader peptide
- IgA-protease-like domain
- Membrane domain
FIGURE 9A

ALLELE1 1: SMKHFSKVLTTAILATFCSGALAATSDDVKAATVATTAAYNNGQE1: 50
ALLELE2 1: SMKHFSKVLTTAILATFCSGALAATNDDDVKAATVADAAANYNGQE1: 50
ALLELE3 1: SMKHFSKVLTTAILATFCSGALAATNDDDVKAATVIAAAANYNGQE1: 50

ALLELE1 51: NGFKAGETIYDIGECDTTIKKDATAADVEADDFKGLGLKKVKVTNLTKTVN: 100
ALLELE2 51: NGFKAGETIYDIGECDTTIKKDATAADVEADDFKGLGLKKVKVTNLTKTVN: 100
ALLELE3 51: NGFKAGETIYDIGECDTTIKKDATAADVEADDFKGLGLKKVKVTNLTKTVN: 100

ALLELE1 101: ENKQNVDAKVKAASEIELKLTTHLADTDAALADTDAALADTDAALADTDAALDNALNKLGE: 150
ALLELE2 101: ENKQNVDAKVKAASEIELKLTTHLADTDAALADTDAALADTDAALADTDAALDNALNKLGE: 143
ALLELE3 101: ENKQNVDAKVKAASEIELKLTTHLADTDAALADTDAALADTDAALADTDAALDNALNKLGE: 150

ALLELE1 151: NITTPEETKTNIVKIDKLEAVADTVKHAEAFNDIADSLDETNKADV: 200
ALLELE2 144: NITTPEETKTNIVKIDKLEAVADTVKHAEAFNDIADSLDETNKADV: 193
ALLELE3 151: NITTPEETKTNIVKIDKLEAVADTVKHAEAFNDIADSLDETNKADV: 200

ALLELE1 201: AVKTANAEKQTAETKQNVDVKAAEATAAGKEAAAGTANNTAADKAEEV: 250
ALLELE2 194: AVKTANAEKQTAETKQNVDVKAAEATAAGKEAAAGTANNTAADKAEEV: 243
ALLELE3 201: AVKTANAEKQTAETKQNVDVKAAEATAAGKEAAAGTANNTAADKAEEV: 250

ALLELE1 251: AAKVTDIKADIATNKIAAKNNAAAAGTANNTAADKAEEV: 273
ALLELE2 244: AAKVTDIKADIATNKIAAKNNAAAAGTANNTAADKAEEV: 293
ALLELE3 251: AAKVTDIKADIATNKIAAKNNAAAAGTANNTAADKAEEV: 300

ALLELE1 273: .................VKSLSLEWTLLRTKERTQGGLAEQALSGILFPQY: 307
ALLELE2 294: DTRLASAEKSLHETRLNGDLTVSDLRTKERTQGGLAEQALSGILFPQY: 343
ALLELE3 301: DTRLASAEKSLHETRLNGDLTVSDLRTKERTQGGLAEQALSGILFPQY: 350

ALLELE1 308: VGRFNTAAVGYKSESAVAIGTFRTENPAAKAGVAVGTSRSSAYH: 357
ALLELE2 344: VGRFNTAAVGYKSESAVAIGTFRTENPAAKAGVAVGTSRSSAYH: 393
ALLELE3 351: VGRFNTAAVGYKSESAVAIGTFRTENPAAKAGVAVGTSRSSAYH: 400

ALLELE1 358: VGVNYEY: 364
ALLELE2 394: VGVNYEY: 400
ALLELE3 401: VGVNYEY: 407
**FIGURE 9B**

--- Leader ---

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<tr>
<td>ALLELE_3</td>
<td>MKHPPSKULTTAILATFCGALATNDDDVKKAAATVAAAYNNQEQING</td>
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<td>\ldots.S\ldots.V.</td>
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<td>\ldots.G\ldots.Q.</td>
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<td>KQNVDAVKAAESEIREKLTKLADTDAALDADTAALDAALTNAINKLGENI</td>
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<td>\ldots.K.</td>
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<td>ALLELE_2</td>
<td>\ldots.</td>
</tr>
<tr>
<td>ALLELE_1</td>
<td>\ldots.</td>
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</tbody>
</table>

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**FIGURE 23**

![Graph showing IL-1α levels in pg/ml for different conditions: control, NadA, NadA boiled, NadA + anti-CD14, and NadA boiled + anti-CD14.](graph)

**FIGURE 24**

![Images showing different samples or conditions labeled as FIGURE 24A and FIGURE 24B.](images)
**FIGURE 25**

**FIGURE 25A**

**FIGURE 25B**

**FIGURE 25C**

**FIGURE 26**

![Diagram of catalytic triad and associated amino acid sequences]

- **Catalytic triad**
  - H
  - D
  - S

- **ATP/GTP binding site**
  - 285

- **Passenger domain**
  - 140

**BOX 1**

- **BOX 2**
  - B. bronchiseptica
  - S. marcescens
  - H. influenzae

**Consensus**

- $X_{45}(A,S)$ hyd-
**FIGURE 27**

- **pET-App**  
  aa: 1-1454
- **pET-App S267A**  
  aa: 1-1454
- **pET-Appβ**  
  aa: 1-23 (Leader Iga protease)  
  + 1077-1454
- **pET-App-His**  
  aa: 43-1454
- **pET-Appα-His**  
  aa: 43-1084

**FIGURE 28**

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</table>

**FIGURE 29**

- pET-AppS267A
- pET-App
- pET-App-His
- pET
**FIGURE 30**

pET-App

pET-App-His

pET

**FIGURE 31**

<table>
<thead>
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</tr>
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<tbody>
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</table>
MENINGOCOCCUS ADHESINS NADA, APP AND ORF 40

[0001] All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

[0002] This invention is in the field of biochemistry and, in particular, the biochemistry of the pathogenic bacteria in the genus Neisseria (e.g. N. meningitidis and N. gonorrhoeae).

BACKGROUND ART


[0004] Sequence data alone, however, does not reveal everything about this pathogen. Objects of the present invention include: (a) to provide ways of intervening in Neisseria biochemistry; (b) to provide new uses for known Neisseria proteins; (c) to provide alternative and improved forms of known Neisseria proteins, such as enzymatically inactive forms of known proteins or proteolytic products of known proteins; and (d) to provide materials useful for studying and modulating Neisseria adhesion.

DISCLOSURE OF THE INVENTION

Nomenclature Used Herein

[0005] ‘ORF40’ is disclosed in example 1 of WO99/36544. Sequences from serogroups A and B of N. meningitidis are disclosed (SEQ IDs 1 to 6 therein). Other forms of the protein are disclosed in WO99/31132 and WO99/58683, and can also be found in GenBank (see gi accession numbers: 11352902, 7228562, 14578015, 12958107, 7228586, 7228572, 7228594, 7228588, 14578013, 7228568, 7228546, 7228548, 7228592, 14578009, 7228558, 7228600, 7228596, 7228542, 7228574, 7228552, 7228554, 14578023, 14578021, 11354080, 7228584 & 7228590).

[0006] ‘App’ (adhesion and penetration protein) is disclosed as ‘ORF1’ in example 77 of WO99/24578. Sequences from serogroups A and B of N. meningitidis and from N. gonorrhoeae are disclosed (SEQ IDs 647 to 654 therein). Other forms of the protein are disclosed in WO99/55873, and can also be found in GenBank (see gi accession numbers: 11280386, 7227246, 11071865, 6977941, 11071863, 11280378, 7379205).

[0007] ‘NAD’ (Neisserial adhesin A) from serogroup B of N. meningitidis is disclosed as protein ‘961’ in WO99/57280 (SEQ IDs 2943 & 2944) and as ‘NM21994’ by Tettelin et al. (see also GenBank accession numbers: 11352904 & 7227256) and in FIG. 9 herein.

[0008] These proteins are preferably expressed other than as a fusion protein (e.g. without GST, MBP, his-tag or similar).

[0009] Preferred proteins for use according to the invention are those of serogroup B N. meningitidis strain MC58, strain 2996 or strain 394/98 (a New Zealand strain). It will be appreciated, however, that the invention is not in general limited by strain—references to a particular protein (e.g. ‘ORF40’, ‘App’ etc.) may be taken to include that protein from any strain. In general, therefore, reference to any particular protein includes proteins which share sequence identity with one of the sequences disclosed above. The degree of ‘sequence identity’ is preferably greater than 50% (e.g. 60%, 70%, 80%, 90%, 95%, 99% or more). This includes mutants and allelic variants. In the context of the present invention, sequence identity is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPMSRCH program (Oxford Molecular), using an affine gap search with parameters gap open penalty=12 and gap extension penalty=1. Typically, 50% identity or more between two proteins is considered to be an indication of functional equivalence.

[0010] The naming conventions used in WO99/24578, WO99/36544 and WO99/57280 are also used herein (e.g. ‘ORF4’, ‘ORF40’, ‘ORF4-1’ etc. as used in WO99/24578 and WO99/36544; ‘m919’, ‘g919’ and ‘a919’ etc. as used in WO99/57280).

Secreted App

[0011] It has been found that, when expressed in E. coli without a GST or his-tag fusion partner, App is exported to the outer membrane as a precursor of about 160 kDa, where it is processed and secreted into the culture.

[0012] The invention therefore provides a method for purifying processed App protein, comprising the steps of: expressing a gene encoding App protein in a non-Neisserial host cell; and purifying processed App protein from the culture medium.

[0013] The invention also provides purified protein obtainable by this process.

[0014] The App protein preferably includes its wild-type 42 residue signal peptide at the N-terminus i.e. no N-terminus fusion partner is used. It is also preferred not to include a C-terminus fusion partner.

[0015] To purify the protein from the culture medium, the culture can be centrifuged and the protein can be recovered from the supernatant.

[0016] The non-Neisserial host cell is preferably a bacterium and is most preferably E. coli.

[0017] Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of a coding sequence (e.g. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the
RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the lac operon in *Escherichia coli* (*E. coli*) [Raibaud et al. (1984) *Annu. Rev. Genet.* 18:173]. Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.


**[0019]** In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or bacteriophage promoter may be joined with the operator sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter. [U.S. Pat. No. 4,551,433]. For example, the tac promoter is a hybrid trp-lac promoter comprised of both trp promoter and lac operon sequences that is regulated by the lac repressor [Amann et al. (1983) *Gene* 25:167; de Boer et al. (1983) *Proc. Natl. Acad. Sci.* 80:21]. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase promoter system is an example of a coupled promoter system [Studier et al. (1986) *J. Mol. Biol.* 189:113; Tabor et al. (1985) *Proc. Natl. Acad. Sci.* 82:1074]. In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an *E. coli* operator region (EP-O-4 267 851).

**[0020]** In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In *E. coli*, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and includes an initiation codon (ATG) and a sequence-3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon [Shine et al. (1975) *Nature* 254:34]. The SD sequence is thought to promote binding of mRNA to the ribosome by the pairing of bases between the SD sequence and the 3' and of *E. coli* 16S RNA [Stea et al. (1979) “Genetic signals and nucleotide sequences in messenger RNA.” *In Biological Regulation and Development: Gene Expression* (ed. R. F. Goldberger)]. To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site [Sambrook et al. (1989) “Expression of cloned genes in *Escherichia coli*.” *In Molecular Cloning: A Laboratory Manual*].

**[0021]** A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by in vitro incubation with cyanogen bromide or by either in vivo or in vitro incubation with a bacterial methionine N-terminal peptidase (EP-A-0219237).

**[0022]** Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Transcription termination sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with strong promoters, such as the trp gene in *E. coli* as well as other biosynthetic genes.

**[0023]** Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (eg. plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will have a replication system, thus allowing it to be maintained in a prokaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host.

**[0024]** Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from reorganizations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various *Bacillus* strains integrate into the *Bacillus* chromosome (EP-A-0127328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

**[0025]** Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline [Davies et al. (1978) *Annu. Rev. Microbiol.* 32:469]. Selectable markers may also include biosynthetic genes, such as those in the histidine, tryptophan, and leucine biosynthetic pathways.

**[0026]** Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an integrating vector, as described above.

**[0027]** Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many bacteria. For example, expression vectors have been developed for, inter alia, the following bacteria: *Bacillus subtilis* [Palva et al.

Adherence Proteins

Example 22 of international patent application WO01/64922 discloses that E. coli which expresses protein NadA can adhere to human epithelial cells. This adherence activity has been further studied and it has also been found for App and ORF40.

The invention provides methods for preventing the attachment of Neisserial cells to epithelial cells.

References to a “Neisserial cell” in this section include any species of the bacterial genus Neisseria, including N. gonorrhoeae and N. lactamica. Preferably, however, the species is N. meningitidis. The N. meningitidis may be from any serogroup, including serogroups A, C, W135 and Y. Most preferably, however, it is N. meningitidis serogroup B.

References to an “epithelial cell” in this section include any cell found in or derived from the epithelium of a mammal. The cell may be in vitro (e.g. in cell culture) or in vivo. Preferred epithelial cells are from the nasopharynx. The cells are most preferably human cells.

Blocking the Neisseria-Epithelium Interaction

The invention provides a method for preventing the attachment of a Neisserial cell to an epithelial cell, wherein the ability of one or more App, ORF40 and/or NadA to bind to the epithelial cell is blocked.

The ability to bind may be blocked in various ways but, most conveniently, an antibody specific for App, ORF40 and/or NadA is used. The invention also provides antibody which is specific for App, ORF40 or NadA. This antibody preferably has an affinity for App, ORF40 and/or NadA of at least 10⁻⁶ M or 10⁻⁷ M, 10⁻⁸ M, 10⁻⁹ M or tighter.

Antibodies for use in accordance with the invention may be polyclonal, but are preferably monoclonal. It will be appreciated that the term “antibody” includes whole antibodies (e.g. IgG, IgA etc.), derivatives of whole antibodies which retain the antigen-binding sites (e.g. F_\text{ab}, F_\text{ab}′, F_\text{c}, etc.), single chain antibodies (e.g. scFv), chimeric antibodies, CDRI-grafted antibodies, humanised antibodies, univalent antibodies, human monoclonal antibodies [e.g. Green (1999) J. Immunol. Methods 231:11-23; Kipriyanov & Little (1999) Mol. Biotechnol. 12:173-201 etc.] and the like. Humanised antibodies may be preferable to those which are fully human [e.g. Fletcher (2001) Nature Biotechnology 19:395-96].

As an alternative to using antibodies, antagonists of the interaction between App, ORF40 or NadA and its receptor on the epithelial cell may be used. As a further alternative, a soluble form of the epithelial cell receptor may be used as a decoy. These can be produced by removing the receptor’s transmembrane and, optionally, cytoplasmic regions [e.g. EP-B2-0139417, EP-A-0609580 etc.].

The antibodies, antagonists and soluble receptors of the invention may be used as medicaments to prevent the attachment of a Neisserial cell to an epithelial cell.

Inhibiting Expression of the Neisseria Gene

The invention provides a method for preventing the attachment of a Neisserial cell to an epithelial cell, wherein protein expression from one or more of App, ORF40 and/or NadA is inhibited. The inhibition may be at the level of transcription and/or translation.


The invention also provides nucleic acid comprising a fragment of x or more nucleotides from nucleic acid which encodes App, ORF40 or NadA, wherein x is at least 8 (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30 or more). The nucleic acid will typically be single-stranded.

The nucleic acid is preferably the formula S^a(N)−^{ba}(N)x(N)−^{ba}(N)_x, wherein 0≤a≤15, 0≤b≤15, N is any nucleotide, and X is a fragment of a nucleic acid which encodes App, ORF40 or NadA. X preferably comprises at least 8 nucleotides (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30 or more). The values of α and β may independently be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15. Each individual nucleotide N in the (N)x(N)−^{ba}(N)x(N)−^{ba}(N)_x-portions of the nucleic acid may be the same or different. The length of the nucleic acid (i.e. a+b+length of X) is preferably less than 100 (e.g. less than 90, 80, 70, 60, 50, 40, 30 etc.).
It will be appreciated that the term “nucleic acid” includes DNA, RNA, DNA/RNA hybrids, DNA and RNA analogues such as those containing modified backbones (with modifications in the sugar and/or phosphates e.g. phosphorothioates, phosphoramidites etc.), and also peptide nucleic acids (PNA) and any other polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases etc. Nucleic acid according to the invention can be prepared in many ways (e.g. by chemical synthesis, from genomic or cDNA libraries, from the organism itself etc.) and can take various forms (e.g. single stranded, double stranded, vectors, probes etc.).

The antisense nucleic acids of the invention may be used as medicaments to prevent the attachment of a Neisseria cell to an epithelial cell.

Knockout of the Neisserial Gene

The invention provides a method for preventing the attachment of a Neisseria cell to an epithelial cell, wherein one or more of App, ORF40 and/or NadA is knocked out.

The invention also provides a Neisseria bacterium in which one or more of App, ORF40 and/or NadA has been knocked out.

Techniques for producing knockout bacteria are well known, and knockout Neisseria have been reported [e.g. Moe et al. (2001) Infect. Immun. 69:3762-3771; Seifert (1997) Gene 188:215-220; Zhu et al. (2000) J. Bacteriol. 182:439-447 etc.].

The knockout mutation may be situated in the coding region of the gene or may lie within its transcriptional control regions (e.g. within its promoter).

The knockout mutation will reduce the level of mRNA encoding App, ORF40 and/or NadA to <1% of that produced by the wild-type bacterium, preferably <0.5%, more preferably <0.1%, and most preferably to 0%.

The knockout mutants of the invention may be used as immunogenic compositions (e.g. as vaccines) to prevent Neisserial infection. Such a vaccine may include the mutant as a live attenuated bacterium.

Mutagenesis of the Neisserial Gene

The invention provides a method for preventing the attachment of a Neisserial cell to an epithelial cell, wherein one or more of App, ORF40 and/or NadA has a mutation which inhibits its activity.

The invention also provides a mutant protein, wherein the mutant protein comprises the amino acid sequence of App, ORF40 and/or NadA, or a fragment thereof, but wherein one or more amino acids of said amino acid sequence is/are mutated (e.g. see below for App).

The amino acids which is/are mutated preferably result in the reduction or removal of an activity of App, ORF40 and/or NadA which is responsible directly or indirectly for adhesion to epithelial cells. For example, the mutation may inhibit an enzymatic activity or may remove a binding site in the protein.

The invention also provides nucleic acid encoding this mutant protein.

The invention also provides a method for producing this nucleic acid, comprising the steps of: (a) providing source nucleic acid encoding App, ORF40 or NadA, and (b) performing mutagenesis (e.g. site-directed mutagenesis) on said source nucleic acid to provide nucleic acid encoding a mutant protein.

Mutation may involve deletion, substitution, and/or insertion, any of which may be involve one or more amino acids. As an alternative, the mutation may involve truncation.


Mutagenesis may be specifically targeted to nucleic acid encoding App, ORF40 and/or NadA. Alternatively, mutagenesis may be global or random (e.g. by irradiation, chemical mutagenesis etc.), which will typically be followed by screening bacteria for those in which a mutation has been introduced into App, ORF40 and/or NadA. Such screening may be by hybridisation assays (e.g. Southern or Northern blots etc.), primer-based amplification (e.g. PCR), sequencing, proteomics, aberrant SDS-PAGE gel migration etc.

The mutant proteins and nucleic acids of the invention may be used as immunogenic compositions (e.g. as vaccines) to prevent Neisserial infection.

Screening Methods

The invention also provides methods for screening compounds to identify those (antagonists) which inhibit the binding of a Neisserial cell to an epithelial cell.

Potential antagonists for screening include small organic molecules, peptides, polypeptides, lipids, metals, nucleotides, nucleosides, polyamines, antibodies, and derivatives thereof. Small organic molecules have a molecular weight between 50 and about 2,500 daltons, and most preferably in the range 200-800 daltons. Complex mixtures of substances, such as extracts containing natural products, compound libraries or the products of mixed combinatorial syntheses also contain potential antagonists.

Typically, App, ORF40 and/or NadA protein is incubated with an epithelial cell and a test compound, and the mixture is then tested to see if the interaction between the protein and the epithelial cell has been inhibited.

Inhibition will, of course, be determined relative to a standard (e.g. the native protein/cell interaction). Preferably, the standard is a control value measured in the absence of the test compound. It will be appreciated that the standard may have been determined before performing the method, or may be determined during or after the method has been performed. It may also be an absolute standard.

The protein, cell and compound may be mixed in any order.

For preferred high-throughput screening methods, all the biochemical steps for this assay are performed in a single solution in, for instance, a test tube or microtitre plate, and the test compounds are analysed initially at a single compound concentration. For the purposes of high-throughput screening, the experimental conditions are adjusted to
achieve a proportion of test compounds identified as "positive" compounds from amongst the total compounds screened.

[0065] Other methods which may be used include, for example, reverse two hybrid screening [e.g. Vidal & Eudo (1999) TIBTECH 17:374-381] in which the inhibition of the Neisseria:receptor interaction is reported as a failure to activate transcription.

[0066] The method may also simply involve incubating one or more test compound(s) with App, ORF40 and/or NadA and determining if they interact. Compounds that interact with the protein can then be tested for their ability to block an interaction between the protein and an epithelial cell.

[0067] The invention also provides a compound identified using these methods. These can be used to treat or prevent Neisserial infection. The compound preferably has an affinity for App, ORF40 and/or NadA of at least 10^{-7} M e.g. 10^{-8} M, 10^{-9} M, 10^{-10} M or tighter.

[0068] The invention also provides a composition comprising (a) an E. coli bacterium which expresses App and/or ORF40 (and, optionally, NadA) and (b) an epithelial cell (e.g. a human epithelial cell).

Expression in Outer Membrane Vesicles (OMVs)

[0069] International patent application WO01/52885 discloses that the addition of further defined components to OMV vaccines significantly broadens their efficacy.


[0071] It has now been found that OMVs prepared from E. coli which express a heterologous Neisseria gene can give better results in standard immunogenicity tests than the antigens in purified form.

[0072] The invention therefore provides a method for preparing an OMV from a non-Neisserial host cell, characterised in that said cell expresses a gene encoding App, ORF40 or NadA protein.

[0073] The invention also provides (a) OMVs obtainable by this process, and (b) an outer membrane vesicle from a non-Neisserial host cell, characterised in that said cell expresses a gene encoding App, ORF40 or NadA protein.

[0074] The non-Neisserial host cell is preferably a bacterial and is most preferably E. coli.

[0075] More generally, the invention provides a method for preparing an OMV from a non-Neisserial host cell, characterised in that said cell expresses a gene encoding one or more of the following proteins:

[0076] (A) Even SEQ IDs 2-892 from WO99/24578;

[0077] (B) Even SEQ IDs 2-90 from WO99/36544;

[0078] (C) Even SEQ IDs 2-3020 from WO99/57280;

[0079] (D) Even SEQ IDs 3040-3114 from WO99/57280;

[0080] (E) SEQ IDs 3115-3241 from WO99/57280;

[0081] (F) The 2160 proteins NMB0001 to NMB2160 from Tettelin et al. [supra];

[0082] (G) A protein comprising the amino acid sequence of one or more of (A) to (F);

[0083] (H) A protein sharing sequence identity with the amino acid sequence of one or more of (A) to (F); and

[0084] (I) A protein comprising a fragment of one or more of (A) to (F).

[0085] Similarly, the invention also provides (a) OMVs obtainable by this process, and (b) an outer membrane vesicle from a non-Neisserial host cell, characterised in that said cell expresses a gene encoding one or more of proteins (A) to (I) described above.

[0086] The degree of “sequence identity” referred to in (H) is preferably greater than 50% (e.g. 60%, 70%, 80%, 90%, 95%, 99% or more) and this includes mutants and allelic variants.

[0087] The ‘fragment’ referred to in (I) should comprise at least n consecutive amino acids from one or more of (A) to (F) and, depending on the particular sequence, is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100 or more). Preferably the fragment comprises an epitope from one or more of (A) to (F). Preferred fragments are those disclosed in WO00/71574 and WO01/04316.

[0088] Preferred proteins for (A) to (F) are found in N. meningitidis serogroup B.

Mutants of App

[0089] Amino acid 267 of SEQ ID 650 of WO99/24578 (SEQ ID 32 herein) is a serine. App is believed to be a serine protease and this serine is believed to be a catalytic residue at its active site. It will be appreciated that standard sequence alignment techniques will reveal the amino acid corresponding to this Ser-267 for any other App sequence (e.g. Ser-260 in SEQ ID 652 of WO99/24578, Ser-267 in SEQ ID 654 etc.).

[0090] The invention provides a protein comprising the amino acid sequence of App, except that one or more of amino acids Ser-267, Asp-158 and His-115 (numbered according to SEQ ID 32) is/are mutated. The mutation may be a deletion, an insertion or, preferably, a substitution. The substitution is preferably with one of the 19 other naturally-occurring amino acids and is more preferably with glycine, alanine, tyrosine or lysine.

[0091] App is believed to cleaved at a site between amino acids 1063 and 1171 (numbered according to SEQ ID 32). It will be appreciated that standard sequence alignment techniques will reveal the amino acids corresponding to these two residues for any other App sequence.

[0092] The invention provides a protein comprising the amino acid sequence of App, except that one or more amino acid(s) between Ser-1064 and Arg-1171 (numbered according to SEQ ID 32) is mutated. The mutation may be a deletion, an insertion, truncation or, preferably, a substitution. The substitution is preferably with one of the 19 other naturally-occurring amino acids. The residue which is mutated is preferably S-1064, D-1065, K-1066, L-1067, G-1068, K-1069, A-1070, E-1071, A-1072, K-1073, K-1074, Q-1075, A-1076, E-1077, K-1078, D-1079, N-1080, A-1081, Q-1082, S-1083, L-1084, D-1085, A-1086, L-1087, F-1088, A-1089, A-1090, G-1091, R-1092, D-1093, A-1094, V-1095, E-1096, K-1097, T-1098, E-1099, S-1100, V-1101, A-1102, E-1103, P-1104,

[0093] App is alternatively believed to cleaved at amino acid 956 and/or amino acid 1178 (numbered according to SEQ ID 32). It will be appreciated that standard sequence alignment techniques will reveal the amino acids corresponding to these residues for any other App sequence.

[0094] The invention provides a protein comprising the amino acid sequence of App, except that one or more of amino acids Phe-956, Asn-957, Ala-1178 & Asn-1179 (numbered according to SEQ ID 32) is mutated. The mutation may be a deletion, an insertion, truncation or, preferably, a substitution. The substitution is preferably with one of the 19 other naturally-occurring amino acids.

[0095] The invention also provides nucleic acid encoding these mutant proteins.

[0096] The invention also provides a method for producing this nucleic acid, comprising the steps of: (a) providing source nucleic acid encoding App, ORF40 or NadA, and (b) performing mutagenesis (e.g. site-directed mutagenesis) on said source nucleic acid to provide nucleic acid encoding a mutant protein.

[0097] The invention provides mature App.

[0098] The invention also provides a protein comprising the amino acid sequence of a processed App, wherein said processed App does not comprise the C-terminus domain which is downstream of an autoproteolytic cleavage site in full-length App. For example, based on SEQ ID 32 as full-length App, the invention provides SEQ IDs 33 to 36. C-terminus domains which may be removed during autoproteolysis are SEQ IDs 38 and 39.

[0099] The invention also provides a protein comprising the amino acid sequence of a processed App, wherein the C-terminus of said processed App is Phe-956 (numbered according to SEQ ID 32). For example, the invention provides SEQ IDs 33 and 35. The amino acid corresponding to Phe-956 in other App sequences can be identified by standard sequence alignment techniques.

[0100] The invention also provides a protein comprising the amino acid sequence of a processed App, wherein the C-terminus of said processed App is Ala-1178 (numbered according to SEQ ID 32). For example, the invention provides SEQ IDs 34 and 36. The amino acid corresponding to Ala-1178 in other App sequences can be identified by standard sequence alignment techniques.

[0101] The invention also provides a protein comprising the amino acid sequence of a processed App, wherein said processed App does not comprise SEQ ID 37, 38 or 39.

[0102] The invention also provides a protein comprising an amino acid sequence selected from the group consisting of SEQ IDs 33, 34, 35, 36, 37, 38 & 39.

[0103] The invention also provides a protein comprising an amino acid sequence with at least p % sequence identity to one or more of SEQ IDs 33, 34, 35, 36, 37, 38 & 39. Depending on the particular sequence, the value of p is preferably 50 or more (e.g. 60, 70, 80, 90, 95, 99 or more). These proteins include homologs, orthologs, allelic variants and functional mutants. Typically, 50% identity or more between two proteins is considered to be an indication of functional equivalence. Identity between proteins is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH1 program (Oxford Molecular), using an affine gap search with parameters gap open penalty=−12 and gap extension penalty=−1.

[0104] The invention further provides proteins comprising a fragment of one or more of SEQ IDs 33, 34, 35, 36, 37, 38 & 39. The fragments should comprise at least q consecutive amino acids from the sequences and, depending on the particular sequence, q is 7 or more (e.g. 8, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more). Preferably the fragments comprise one or more epitopes from the sequence.

[0105] The invention also provides nucleic acid encoding these proteins of the invention.

Alleles of NadA

[0106] The invention provides a protein comprising the amino acid sequence of one or more of SEQ IDs 1 to 14.

[0107] The invention also provides a protein comprising an amino acid sequence having at least x % sequence identity to one or more of SEQ IDs 1 to 14. The value of x is at least 50% (e.g. 60%, 70%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, 99.5% or more). This includes variants e.g. allelic variants, homologs, orthologs, paralogs, mutants, etc.

[0108] A preferred allele of NadA for use with the present invention is SEQ ID 3 (or SEQ ID 6).

[0109] The invention also provides a protein comprising a fragment of one or more of SEQ IDs 1 to 14. These should comprise at least n consecutive nucleotides from one or more of SEQ IDs 1 to 14, wherein n is 6 or more (e.g. 7, 8, 9, 10, 11, 12, 14, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350 or more). The fragment may comprise a sequence which is common to SEQ IDs 1 to 14, or may comprise a sequence which is not common to SEQ IDs 1 to 14.

[0110] Preferred fragments comprise one or more epitopes from SEQ IDs 1 to 14. Other preferred fragments are (a) the N-terminal leader peptides of SEQ IDs 1 to 14, (b) SEQ IDs 1 to 14, but without k N-terminal amino acid residue(s), wherein k is 1 or more (e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 50 etc.), and (c) SEQ MSs 1 to 14, but without 1 C-terminal amino acid residue(s), wherein l is 1 or more (e.g. 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 50 etc.). Preferred fragments fall within both (b) and (c) i.e. truncation at both C- and N-termi.

[0111] Preferred fragments within category (b) lack the N-terminal leader peptide. For SEQ IDs 1, 2, 3, 7, 9, 11 & 13 the value of k is thus 23; for SEQ IDs 4, 5, 6, 8, 10, 12 & 14 the value of k is 25. The leader peptide may be replaced with the leader peptide from another protein, by another protein (i.e. to form a fusion protein) or by an alternative N-terminal sequence to allow efficient expression.

[0112] Preferred fragments within category (c) lack the C-terminal membrane anchor. The value of l is thus 54. Minor variants of this C-terminal deletion may be used (e.g. where l is 45, 46, 47, 48, 49, 50, 51, 52, 53, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66).

[0113] Proteins with the N-terminus sequence MKH or MQH are preferred to those with N-terminus sequence MSM.

[0114] The protein of the invention may include the heptad sequence (AA₃A₁A₂A₃A₄A₅A₆A₇A₈), wherein: A₈ is
Leu, Be, Val or Met; each of AA1, AA2, AA3, AA4, AA5, and AA6 may independently be any amino acid; r is an integer of 1 or more (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 etc.). Where r is 2 or more, the meaning of each AA1, AA2, AA3, AA4, AA5, and AA6 may be the same or different in each of the r heptad repeats. The heptad(s) can form a leucine-zipper domain.

[0115] Proteins of the invention can be prepared in many ways e.g. by chemical synthesis (at least in part), by digesting longer polypeptides using proteases, by translation from RNA, by purification from cell culture (e.g. from recombinant expression), from the organism itself (e.g. isolation from prostate tissue), from a cell line source, etc.

[0116] Proteins of the invention can be prepared in various forms e.g. native, fusions, glycosylated, non-glycosylated, lipidated, non-lipidated etc.

[0117] The protein is preferably in the form of an oligomer.

[0118] Proteins of the invention may be attached or immobilised to a solid support.

[0119] Proteins of the invention may comprise a detectable label e.g. a radioactive label, a fluorescent label, or a biotin label. This is particularly useful in immunoassay techniques.

[0120] Proteins of the invention are preferably in isolated or substantially isolated form.

[0121] In general, the proteins of the invention are provided in a non-naturally occurring environment e.g. they are separated from their naturally-occurring environment. In certain embodiments, the subject protein is present in a composition that is enriched for the protein as compared to a control. As such, purified protein is provided, whereby purified is meant that the protein is present in a composition that is substantially free of other expressed proteins, where by substantially free is meant that less than 90%, usually less than 60% and more usually less than 50% of the composition is made up of other expressed proteins.

[0122] The term “protein” refers to amino acid polymers of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example proteins containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modifications known in the art. Proteins can occur as single chains or associated chains.

[0123] Mutants can include amino acid substitutions, additions or deletions. The amino acid substitutions can be conservative amino acid substitutions or substitutions to eliminate non-essential amino acids, such as to alter a glycosylation site, a phosphorylation site or an acetylation site, or to minimize misfolding by substitution or deletion of one or more cysteine residues that are not necessary for function. Conservative amino acid substitutions are those that preserve the general charge, hydrophobicity/hydrophilicity, and/or steric bulk of the amino acid substituted. Variants can be designed so as to retain or have enhanced biological activity of a particular region of the polypeptide (e.g. a functional domain and/or, where the polypeptide is a member of a polypeptide family, a region associated with a consensus sequence). Selection of amino acid alterations for production of variants can be based upon the accessibility (interior vs. exterior) of the amino acid, the thermostability of the variant polypeptide, desired disulfide bridges, desired metal binding sites etc.

[0124] The invention also provides nucleic acid encoding a protein of the invention as defined above. The invention also provides nucleic acid comprising a fragment of at least a consecutive nucleotides from said nucleic acid, wherein n is 10 or more (e.g. 12, 14, 15, 18, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 150, 200, 500 or more).

[0125] Furthermore, the invention provides nucleic acid which can hybridise to nucleic acid encoding a protein of the invention, preferably under “high stringency” conditions (e.g. 65°C in a 0.1xSSC, 0.5% SDS solution).

[0126] Nucleic acids of the invention can be used in hybridisation reactions (e.g. Northern or Southern blots, or in nucleic acid microarrays or “gene chips”) and amplification reactions (e.g. PCR, SDA, SSSR, ICR, TMA, NASBA, etc.) and other nucleic acid techniques.

[0127] Nucleic acids of the invention can be prepared in many ways e.g. by chemical synthesis in whole or part, by digesting longer polynucleotides using nucleases (e.g. restriction enzymes), from genomic or cDNA libraries, from the bacterium itself, etc.

[0128] Nucleic acids of the invention can take various forms e.g. single-stranded, double-stranded, vectors, primers, probes, labelled, unlabelled, etc.

[0129] Nucleic acids of the invention are preferably in isolated or substantially isolated form.

[0130] The invention includes nucleic acid comprising sequences complementary to those described above e.g. for antisense or probing, or for use as primers.

[0131] The term “nucleic acid” includes DNA and RNA, and also their analogues, such as those containing modified backbones, and also peptide nucleic acids (PNA) etc.

[0132] Nucleic acid according to the invention may be labelled e.g. with a radioactive or fluorescent label. This is particularly useful where the nucleic acid is to be used in nucleic acid detection techniques e.g. where the nucleic acid is a primer or as a probe for use in techniques such as PCR, ICR, TMA, NASBA, etc.

[0133] The invention also provides vectors comprising nucleotide sequences of the invention (e.g. cloning or expression vectors, such as those suitable for nucleic acid immunisation) and host cells transformed with such vectors.

Immunisation

[0134] The invention provides an immunogenic composition comprising (a) a Neisserial NadA protein and/or (b) nucleic acid encoding a NadA protein.

[0135] The invention also provides a method for raising an antibody response in a mammal, comprising administering an immunogenic composition of the invention to the mammal. The antibody response is preferably a protective antibody response. The protective antibody preferably blocks the attachment of NadA and/or App to epithelial cells.

[0136] The invention also provides a method for protecting a mammal against a Neisserial infection, comprising administering to the mammal an immunogenic composition of the invention.

[0137] The invention also provides Neisserial NadA protein for use as a medicament.

[0138] The invention also provides the use of a NadA protein in the manufacture of a medicament for preventing Neisserial infection in a mammal.
The invention also provides the use of nucleic acid encoding a NadA protein in the manufacture of a medicament for preventing Neisseria infection in a mammal.

The mammal preferably is a human. The human may be an adult or, preferably, a child.

The NadA protein is preferably a \textit{N. meningitidis} NadA. It preferably comprises the amino acid sequence of one or more of SEQ ID Nos 1 to 14, or an amino acid sequence having sequence identity thereto or comprising a fragment thereof (see above). The NadA protein is preferably in the form of an oligomer (e.g. a dimer, trimer, tetramer or higher). Within SEQ ID Nos 1 to 14, SEQ ID Nos 1 to 12 are preferred, as antibodies against these NadA proteins are bactericidal across the various hypervirulent alleles. Where an immune response against a non-hypervirulent NadA* strain is desired, however, SEQ ID Nos 13 & 14 are preferred. Of course, NadA mixtures are also possible, particularly mixtures containing more than one NadA allele.

Immunogenic compositions of the invention may be used therapeutically (i.e. to treat an existing infection) or prophylactically (i.e. to prevent future infection).

The uses and methods of the invention are particularly useful for treating/protection against infections of \textit{Neisseria meningitidis}, including serogroups A, B, and C. They are particularly useful against strains of \textit{N. meningitidis} from hypervirulent lineages ET-5, EY-38 and cluster A4.

The uses and methods are particularly useful for preventing/treating diseases including, but not limited to, meningitis (particularly bacterial meningitis) and bacteremia.

Efficacy of therapeutic treatment can be tested by monitoring Neisseria infection after administration of the composition of the invention. Efficacy of prophylactic treatment can be tested by monitoring immune responses against NadA after administration of the composition.

The composition of the invention may additionally comprise an antigen which, when administered to a mammal, elicits an immune response which is protective against a lineage III strain of \textit{N. meningitidis}.

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

The invention may be used to elicit systemic and/or mucosal immunity.

Dosage treatment can be a single dose schedule or a multiple dose schedule.

The immunogenic composition of the invention will generally include a pharmaceutically acceptable carrier, which can be any substance that does not itself induce the production of antibodies harmful to the patient receiving the composition, and which can be administered without undue toxicity. Suitable carriers can be large, slowly-metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable carriers can include liquids such as water, saline, glycerol and ethanol. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, can also be present in such vehicles. Liposomes are suitable carriers. A thorough discussion of pharmaceutical carriers is available in Remington (2000) \textit{Remington: The Science and Practice of Pharmacy}. 20th edition, ISBN: 0683306472.

Neisseria infections affect various areas of the body and so the compositions of the invention may be prepared in various forms. For example, the compositions may be prepared as injectables, either as liquid solutions or suspensions. Solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. The composition may be prepared for topical administration e.g. as an ointment, cream or powder. The composition can be prepared for oral administration e.g. as a tablet or capsule, or as a syrup (optionally flavoured). The composition may be prepared for pulmonary administration e.g. as an inhaler, using a fine powder or a spray. The composition may be prepared as a suppository or pessary. The composition may be prepared for nasal, aural or ocular administration e.g. as drops.

The composition is preferably sterile. It is preferably pyrogen-free. It is preferably buffered e.g. at between pH 6 and pH 8, generally around pH 7.

Immunogenic compositions comprise an immunologically effective amount of immunogen, as well as any other of the specified components, as needed. By ‘immunologically effective amount’, it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, age, the toxicologic group of individual to be treated (e.g. non-human primate, primate, etc.), the capacity of the individual’s immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor’s assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through routine trials. Dosage treatment may be a single dose schedule or a multiple dose schedule (e.g. including booster doses). The composition may be administered in conjunction with other immunoregulatory agents.

The immunogenic composition may include an adjuvant. Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: (A) aluminium compounds (e.g. an aluminium hydroxide such as oxyhydroxide, or an aluminium phosphate such as hydroxyphosphate or orthophosphate, aluminium sulphate etc.), or mixtures of different aluminium compounds, with the compounds taking any suitable form (e.g. gel, crystalline, amorphous etc.), and with adsorption being preferred; (B) MF59 (5% Squalene, 0.5% Tween 80, and 0.5% Span 85, formulated into submicron particles using a microfluidizer); (C) liposomes; (D) ISCOMs, which may be devoid of additional detergent; (E) SAF, containing 10% Squalane, 0.4% Tween 80, 5% pluronic-block polymer L121, and thr-MDP, either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion; (F) RibiTm adjuvant system (RAS), (RibImunochem) containing 2% Squalane, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of monophosphoryl lipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL4CWS (Detox™); (G) saponin adjuvants, such as QuilA or QS21, also known as Stimulon™; (H) chitosan; (I) complete Freund’s adjuvant (CFA) and incomplete Freund’s adjuvant (IFA); (J) cytokines, such as interleukins (e.g. IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12), and lipopolysaccharide.
IL-12, etc.), interferons (e.g. interferon-γ), macrophage colony stimulating factor, tumor necrosis factor, etc.; (K) microparticles (i.e. a particle of ~100 nm to ~150 μm in diameter, more preferably ~200 nm to ~30 μm in diameter, and most preferably ~500 nm to ~10 μm in diameter) formed from materials that are biodegradable and non-toxic (e.g. a poly(ε-hydroxy acid), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone etc.); (L) monophosphoryl lipid A (MPL) or 3-O-deacylated MPL (3dMPL); (M) combinations of 3dMPL with, for example, QS21 and/or oil-in-water emulsions; (N) oligonucleotides comprising CpG motifs i.e. containing at least one CG dinucleotide, with 5-methylcytosine optionally being used in place of cytosine; (O) a polyoxyethylene ether or a polyoxyethylene ester; (P) a polyoxyethylene sorbitan ester surfactant in combination with an octoxynol or a polyoxyethylene alkyl ether or ester surfactant in combination with at least one additional non-ionic surfactant such as an octoxynol; (Q) an immunostimulatory oligonucleotide (e.g. a CpG oligonucleotide) and a saponin; (R) an immunostimulant and a particle of metal salt; (S) a saponin and an oil-in-water emulsion; (T) a saponin (e.g. QS21)+3dMPL+IL-12 (optionally+α sterol); (U) E. coli heat-labile enterotoxin ("LT"), or detoxified mutants thereof, such as the K53 or K72 mutants; (V) cholera toxin ("CT"), or detoxified mutants thereof; (W) microparticles (i.e. a particle of ~100 nm to ~150 μm in diameter, more preferably ~200 nm to ~30 μm in diameter, and most preferably ~500 nm to ~10 μm in diameter) formed from materials that are biodegradable and non-toxic (e.g. a poly(ε-hydroxy acid) such as poly(lactide-co-glycolide), a polyhydroxybutyric acid, a polyorthoester, a polyanhydride, a polycaprolactone etc.); and (X) other substances that act as immunostimulating agents to enhance the effectiveness of the composition. Aluminium salts (aluminium phosphates and particularly hydroxyphosphates, and/or hydroxides and particularly oxy-hydroxides) and MF59 are preferred adjuvants for parenteral immunisation. Toxic mutants are preferred mucosal adjuvants.

[0155] Muramyl peptides include N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-κ-isoglutamine (nor-MDP), N-acetylmutamyl-L-alanyl-D-isoglutaminyl-L-alaninam-2-[(1'2'-dipalmitoyl-snglycerol-3-hydroxyphosphoryloxy)-ethylamine MTP-PE], etc.

[0156] Compositions of the invention may comprise antigens (e.g. protective antigens against N. meningitidis or against other organisms) in addition to NadA e.g. DTP antigens, Hib antigen etc.

[0157] Immunogenic compositions of the invention may be used therapeutically (i.e. to treat an existing infection) or prophylactically (i.e. to prevent future infection). Therapeutic immunisation is particularly useful for treating Candida infection in immunocompromised subjects.

[0158] As an alternative to using proteins antigens in the immunogenic compositions of the invention, nucleic acid (preferably DNA e.g. in the form of a plasmid) encoding the antigen may be used.

Disclaimers

[0159] The invention preferably excludes: (a) amino acid and nucleic acid sequences available in public sequence databases (e.g. GenBank or GenESIQ) prior to 26 Jul. 2002 and, more preferably, prior to 27 Jul. 2001; (b) amino acid and nucleic acid sequences disclosed in patent applications having a filing date or, where applicable, a priority date prior to 26 Jul. 2002 and, more preferably, prior to 27 Jul. 2001. In particular, SEQ ID entries in the following patent applications may be excluded: WO99/24578; WO99/36544; WO99/57280; WO00/22430; WO00/66741; WO00/66791; WO00/71574; WO00/71725; WO01/04316; WO01/31019; WO01/37863; WO01/38350; WO01/52885; WO01/64920; WO01/64922.

DEFINITIONS

[0160] The term “comprising” means “including” as well as “consisting of” e.g. a composition “comprising” X may consist exclusively of X or may include something additional e.g. X+Y.

BRIEF DESCRIPTION OF DRAWINGS

[0161] Figs. 1 to 3 show expression data for (1) ORF40 (2) App (3) NadA.

[0162] Figs. 4 to 6 show FACS analysis of proteins involved in adhesion to human cells. In Figs. 4 and 5 (Fig. 6), the data are for, from left to right, ORF40 (A), App (B), NadA (C) and GNA2132 (D).

[0163] Figs. 7 and 8 show homologies of (7) ORF40 and (8) App.

[0164] Fig. 9 shows an alignment of NadA alleles, and Fig. 10 shows the relationship of alleles 1 to 3.

[0165] Fig. 11 shows predicted secondary structure for NadA.

[0166] Fig. 12 shows analysis of sequences upstream and downstream of NadA.

[0167] Fig. 13 shows PCR analysis of NadA expression in different strains of N. meningitidis.

[0168] Fig. 14 shows immunoblot analysis of NadA expression in different strains of N. meningitidis.

[0169] Fig. 15 shows variation of NadA expression with culture time.

[0170] Fig. 16 shows NadA FACS of isogenic capsulated and non-capsulated N. meningitidis cells.

[0171] Fig. 17 shows immunofluorescence results obtained using anti-NadA against Chang cells (17A to 17C) or Hela cells (17D).

[0172] Fig. 18 shows immunofluorescence results obtained using anti-NadA against Chang cells after incubation at (A) 37°C, or (B) 4°C.

[0173] Fig. 19 shows immunofluorescence results for Chang cells treated with saponin.

[0174] Fig. 20 shows immunofluorescence results obtained using monocyes.

[0175] Fig. 21 shows immunofluorescence results obtained using macrophages.

[0176] Fig. 22 shows IL-α secretion by monocyes in response to NadA treatment.

[0177] Fig. 23 shows the effect of anti-CD14 on IL-α secretion by monocyes.

[0178] Fig. 24 shows immunofluorescence results obtained using anti-NadA against E. coli transformed to express NadA.

[0179] Fig. 25 shows staining of the transformed E. coli using (A) anti-NadA (B) anti-E. coli or (C) both.

[0180] Fig. 26 is a schematic representation of App features. The N-terminal leader peptide, the passenger domain and the C-terminal β-domain are indicated. The positions of the serine protease active site, the ATP/GTP binding site, the
two Arginine-rich sites and the Proline-rich region are shown. In BOX 1, cleavage sites are shown. In BOX 2 a comparison of known proteolytic sites of different autotransporters is shown and a consensus signature is derived. Arrows identify the cleavage sites; X—any amino acid; hyd—hydrophobic residues; (A,S)—Alanine or Serine.

FIG. 27 is a schematic representation of the constructs used for studying App.

FIG. 28 shows a western blot of outer membrane and extracellular proteins in E. coli.

FIG. 29 shows FACS analysis of outer membrane and extracellular proteins in E. coli.

FIG. 30 shows immunofluorescence of outer membrane and extracellular proteins in E. coli.

FIG. 31 shows total E. coli proteins analysed by SDS-PAGE.

FIG. 32 shows an immunoblot of crude precipitated culture supernatants using mouse antiserum against App-his.

FIG. 33 shows FACS adhesion data using rabbit antiserum against E. coli. Percentages of cells positive to adhesion are shown near the fluorescence profiles.

FIG. 34 shows immunofluorescence microscopy data showing bacterial adherence and aggregation.

FIG. 35 shows concentration-dependent binding of App-His( ) App-His( ) and NMB2132( ) expressed as net Mean Fluorescence Intensity (MFI).

FIG. 36 shows the effect on binding of App-His (100 μg/ml) of pre-incubation with promase (left-hand columns) or phospholipase A2 (right-hand columns) with increasing concentration of enzyme. Promase was tested at 0.250, 500, 1000 μg/ml; phospholipase A2 was tested at 0, 50, 200, 800 μg/ml.

FIG. 37 is a comparison of cellular binding specificity of App-His protein at 100, 25 or 6.25 μg/ml against various different cells.

FIG. 38 shows association of wild-type or App-knockout N. meningitidis MC58 bacteria.

FIG. 39 shows a western blot analysis of total lysates from N. meningitidis MC58 harvested at 0.5 or 0.8 OD620, lanes 1 & 3 show wild-type MC58 and lanes 2 & 4 show the App knockout.

FIG. 40 shows a western blot analysis of supernatants in parallel to FIG. 39.

MODES FOR CARRYING OUT THE INVENTION

NadA Homology

NadA shows homology to (a) YadA of enteropathogenic Yersinia, a non-pili associated adhesin implicated in virulence [Cornelis (1998) Microbiol. Mol. Biol. Rev. 62:1315-1352.] and (b) UspA2 of Moraxella catarrhalis, a protein involved in serum resistance and a protective antigen [Chen et al. (1999) Infect. Immun. 67:1310-1316.]. Sequence similarity is mainly clustered in the carboxyl terminal region (56-63% identity in the last 70 amino acids). Outside this region the level of identity drops to 23-25%.

YadA and UspA2 have been identified as adhesins [Hoeizy et al. (2000) EMBO J 19:5989-5999]. Both proteins form very stable and difficult-to-dissociate high molecular weight oligomers (150-200 kDa) anchored to the outer membrane. NadA has also been found to form very stable high molecular weight aggregates on the outer membrane of meningococcus.

The amino acid sequence of NadA was analysed [Nielsen et al. (1997) Protein Engineering 10:1-6; Levin & Garner (1988) Biochim. Biophys. Acta 955:283-295; Berger et al. (1995) PNAS USA 92:8259-8263; Bornberg-Bauer et al. (1998) Nucleic Acids Res. 26:2740-2746]. Secondary structure analysis is shown in FIG. 11. The globular N-terminal and amphipathic C-terminus are indicated, as are the positions of the leader peptide (LP) and a membrane anchor. The carboxyl-terminal region (aa 310-362) has a predicted amphipathic β-structure (β-strands shown in black) and a terminal aromatic amino acid, which are typical features of outer membrane anchoring domains. The amino terminal region (aa 23-90) has no defined secondary structure, but the rest of the protein has mainly α-helix propensity (84.6%). Within this region, residues 90-146 and 183-288 have high probability of forming coiled coils. In addition, residues 122-143 contain four leucine residues in the “a” positions of the heptad repeats (L-(x)-(L)-(x)-(L)-(x)-(L)-L) that may form a leucine zipper domain ( ). It is known that both coiled coils and leucine zipper sequences are involved in dimerization and may mediate oligomerisation of monomers via association of two or more alpha helices.

Even though primary structure similarity between NadA, YadA and UspA2 is clustered at the C-terminus, therefore, the overall similarity between the three proteins is conserved at secondary structure level. Putative leucine zippers are present in both NadA and UspA2. NadA, YadA and UspA2 have a carboxyl terminal membrane anchor made by four amphipathic β-strands and an internal α-helical region with propensity to form coiled-coils. In YadA and UspA2 these α-helices have been shown to form coiled-coils regions, which mediate oligomerisation of monomers [Hoeizy et al. (2000) EMBO J 19:5989-5999; Cope et al. (1999) J. Bacteriol. 181:4026-4034].

The absence of cysteine residues in the mature forms of NadA is another feature shared with its homologues.

The Genomic Environment of NadA

The 1086 bp nadA coding region is flanked at the 3’ end by a terminator sequence while at the 5’ end (FIG. 12A) it shows a putative ribosome-binding site (RBS; 5’-AAGG-3’) and a putative promoter region located 8 and 47 base pairs, respectively, upstream the ATG start codon.

By upstream the coding region are nine repeats of the tetranucleotide TAAA (shaded black in FIG. 12A), preceded by a second putative promoter with -10 and -35 regions. Because of the presence of the TAAA repeats, the gene had been listed as one of those that may undergo phase variation, even though the repeats are not in the coding region [Trettin et al.]. The homologous gene UspA2 has a tetranucleotide repeat (AGAT) located in the same position as in nadA, which varies in different strains [Cope et al. (1999) J. Bacteriol. 181:4026-4034].

The G+C content of the nadA gene and its upstream region is lower than average (45% against an average of the rest of the genome, 51.5%), suggesting acquisition of the gene by horizontal transfer.

The NadA gene and its upstream region are not present in the published sequence of the genome of serogroup A, strain Z2491 [Parkhill et al. (2000) Nature 404:502-506]. In the MenA genome, a short sequence of 16 nucleotides with no homologies in the database, replaces the nadA gene (FIG. 12B), whereas the upstream and downstream genes (amb1993 and amb1995) are well conserved (91% and 97% identity). Analysis of the sequences immediately adjacent to the nadA region and absent in the Z2491 serogroup A strain
shows that the segment is flanked by the TCAGAC direct repeats. This may indicate a mechanism of recombination. In the A strain the stretch of 16 nucleotides has a disrupted pair of TCAGAC repeats flanking it.

Variation in NadA Genotype

[0204] Given the difference in nadA expression between serotypes A and B, 175 different strains of *N. meningitidis* were chosen for analysis—150 isolates representative of the five disease-associated serogroups (A, B, C, Y and W-135) and 25 strains isolated from healthy carriers. The analysis also included one strain each of *N. gonorrhoeae*, *N. cinerea* and *N. lactamica*.

[0205] Bacteria were grown overnight at 37° C, in a humidified atmosphere of 5% CO₂ in air on gonococcal (GC) medium agar (Difco) supplemented with Kellogg’s supplement solution (0.2 M D-glucose, 0.03 M L-glutamine, 0.001 M ferric nitrate, and 0.02 M ascorbic acid) (Sigma-Aldrich Chemical Co., St. Louis, Mo.) as previously described [Knap et al. (1988) *Antimicrob. Agents Chemother.* 32:765-767; Roberts et al. (1977) *J. Bacteriol.* 131:557-563]. One loopful of bacteria was dissolved in 500 µl of PBS and chromosomal DNA was prepared as previously described [Tinsley et al. (1996) *PNAS* USA 93:11109-11114].

[0206] The bacteria were screened by PCR and/or dot blot hybridization.

[0207] PCR amplification of the nadA genes was performed on 10 ng of chromosomal DNA using primers, mapping 350 nt upstream and downstream from the coding region (forward primer: SEQID 16; reverse primer: SEQID 17), and Platinum High Taq Polymerase (GIBCO). PCR conditions were: 30 cycles of denaturation at 95° C for 30 s, annealing at 60° C, for 30 s, and extension at 68° C, for 1 min. PCR products were analysed on 1% agarose gel and the sizes were determined using a molecular weight marker 1 Kb Plus DNA Ladder (GIBCO). The amplified fragments were purified on a Qiaquick column (Qiagen) and then automated cyclo-cosequenced (Applied Biosystems model 377) by primer walking on both strands of the amplified fragment.

[0208] For dot blotting, the probe used was the whole nadA gene, as amplified from 2996 strain and labelled with digoxigenin using the Roche DIG High-Prime DNA Labelling and Detection Kit. 10 µl aliquot of cell suspension of each strain were boiled for 10 min. and spotted on nylon membrane (Boehringer). The membranes underwent cross-linking of DNA by 2° exposure to UV light and other standard procedures for preparation and signal detection as reported by the manufacturer.

[0209] The nadA gene was absent in *N. gonorrhoeae* and in the commensal species *N. lactamica* and *N. cinerea*. In *N. meningitidis*, however, 47% of isolates were positive for its presence.

[0210] PCR generated (FIG. 13) a product of 1800 by in NadA* strains MC58 (lane 1), 90/18311 (lane 2) and 2996 (lane 3). It gave a product of 400 by in NadA* strain Z2291 and NG3/88 (lane 5). Some strains (e.g. 93/4286, C4678, 2022, ISS1113) gave a PCR product of 2500 by (lane 4: L93/4286).

[0211] The presence/absence of NadA in *N. meningitidis* was correlated with strain lineage. Strains isolated from invasive meningococcal disease have been classified by multilocus enzyme electrophoresis (MLEE) into a small number of hypervirulent lineages: Electrophoretic Types ET37, ET5, cluster A4, lineage III, subgroups I, III and IV-1 [Achtman (1995) Global epidemiology of meningococcal disease. In Meningococcal disease (Cartwright, ed). John Wiley and Sons, Chichester, England. 159-175; Crouzet (1998) *APMIS* 106:505-25]. Recently, a sequence-based classification, multilocus sequence typing (MLST), has been introduced, which classifies the above strains into Sequence Types ST11, ST32, ST8, ST41, ST1, ST5, ST4, respectively [Maiden et al. (1998) *PNAS* USA 95:3140-3145]. Strains isolated from healthy carriers fall into many different ET and ST types.

[0212] The nadA gene was present in 51 out of 53 strains (96%) of the hypervirulent lineages ET1-5, ET-37 and cluster A4, whereas it was absent in all the tested lineage III strains. Seven of the 25 carrier strains were positive. Most of the serogroup C strains tested were positive even if not belonging to hyper-virulent lineages. The same was true for the serogroup B strains with serotype 2a and 2b. For serogroup A, one strain belonging to subgroup III was positive whereas the other two strains belonging to subgroup IV were negative.

[0213] Lineage III has only recently been introduced in Europe and USA and the geographic segregation in New Zealand for many years could have impaired its ability to acquire novel genes. For instance, mutations may have occurred in the surrounding chromosome regions preventing Lineage III from further recombination events. Another possible explanation is that ET-5, ET-37 and Cluster A4 strains need nadA to achieve peak fitness whereas Lineage III isolates cannot derive any significant benefit from nadA insertion, thus undergoing a negative selection.

[0214] NadA is thus over-represented in three hypervirulent *N. meningitidis* lineages. It appears to be a foreign gene present in a subset of hypervirulent strains.

[0215] NadA Alleles

[0216] As PCR products were differently sized (FIG. 13) and most of the NadA* strains could be grouped in three different sizes, genes were sequenced for 36 strains representative of each size: 26 positive strains, 4 strains with a long PCR product, and 6 NadA* strains.

[0217] In the negative strains, a 16 bp sequence was found which was identical to the sequence present in the published serogroup A genome sequence.

[0218] Analysis of the sequence of the four long PCR product strains revealed an interruption by a single copy of IS1301, interrupting the protein after 162 amino acids with a stop codon. The insertion site was identical in all four strains, but the orientation of IS1301 differed, indicating independent events. The target consensus for IS1301, 5'-ATTTGA-3' was found within the NadA gene at nucleotide 472, generated by an A->G mutation, and was accompanied by a TA duplication.

[0219] In NadA* strains, gene size ranged from 1086 to 1215 bp, with consequent variation of the amino acid sequences of the encoded proteins from 362 to 405 amino acids. It was possible to cluster 22 of the 26 NadA* genes into three well-defined alleles (FIGS. 9 & 10; Table 1). The sequence of the gene within each allele is identical and overall identity between the alleles ranges from 96% to 99%. This level of conservation is surprising and suggests weak selective pressure and/or very recent acquisition of the NadA gene. The latter possibility is consistent with the low G+C content of the genome in this region (see above).
[0220] The sequences shown in FIG. 9A assume that the N-terminus amino acid is the first Met in the open reading frame (SEQ IDs 4 to 6), but the second Met (residue 3 in SEQ IDs 4 to 6) has a better-positions Shine-Dalgarno motif (FIG. 9B). Sequences starting from the second Met codon are thus preferred (SEQ IDs 1 to 3). Allele 1 codes for a protein of 362 amino acids (SEQ ID 1) and includes strain MC58 and all the ET-5 positive strains sequenced. The other five strains belonging to allele I were very recent isolates and they have not been ET-type yet, although serotype and serosubtype classification (B:15:P1.7 and B:4:P1.15) of these strains suggests affiliation of these strains to the ET-5 complex.

Allele 2 codes for a protein of 398 amino acids (SEQ ID 2) resulting from the addition of 2 aa after residue 268 (numbering according to SEQ ID 1), addition of 41 aa after residue 271, and deletion of 7 aa after residue 122, resulting in the deletion of the first heptad repeat of the leucine zipper domain. Leucine residues at a fixed spacing of seven residues commonly identify leucine zippers. One leucine in the repeats has frequently been replaced mostly by Met, Val or Ile. In this case allele 2 could use the Ile upstream or downstream to form the leucine zipper motif.

Allele 3 codes for a protein of 405 amino acids (SEQ ID 3) and, like allele 2, contains 43 extra amino acids at residues 268 and 271 but differs from allele 2 by not having the 7aa deletion after residue 122. Allele 3 is found in serogroup A, B and C strains.

[0221] The remaining 4/26 positive strains (ISS1024, ISS759, 973-1720, 95350; marked with * in Table 1) contain minor variants of alleles 1 to 3.

[0222] Serogroup C strain ISS1024 has a variant of allele 2 with a single heptad repeat deletion at residues 229-235 (SEQ ID 9/8). This sequence was originally classified as a fourth allele but has been re-classified as a variant of allele 2. Allele 2 is thus found in all ET-37 strains, one strain of cluster A4 and three additional non-ET-type serogroup C strains.

[0223] Serogroup C strains ISS759 and 973-1720 both contain a variant of allele 3 with a single amino acid mutation in the leader peptide (SEQ IDs 9/10) resulting from a single nucleotide mutation. Among all allele 3 strains, only 973-1720 belongs to a hypervirulent strain (cluster A4).

[0224] Serogroup B strain 95350 contains a recombinant (chimera) of alleles 1 and 2 (SEQ IDs 11/12), with nVAD/A being a fusion between the N-terminal portion of allele 2 and the C-terminal segment of allele 1. The putative site of recombination is located approximately between residues 141 and 265 of the protein.

[0225] All insertions and deletions happen in the coiled-coil region and involve 7 or 41 amino acids which, representing 2 or 6 turns of the c-helix, allows for variations in length of the coiled coil region without disturbing the overall structure. Furthermore, the deletion in ISS1024 results in the loss of the first heptad repeat of the leucine zipper domain but does not destroy the domain because leucine residues at a fixed spacing of seven residues can be replaced mostly by Met, Val or Ile. In this case allele 2 could use the Ile upstream or downstream to form the leucine zipper motif (FIG. 11).

[0226] Any of these various NadA sequences and alleles can be used in accordance with the invention.

[0227] When sequence analysis was extended to the putative promoter and terminator regions (50 bp upstream, 350 bp downstream), variations were found only in the in the 5' region. Three Italian strains (ISS1071, ISS832 and ISS1104) differed for a single base mutation while in strain 961-5945 there was a 7 base differences (indicated with * in FIG. 10). Variations were also found in the 5' regions where the TAAA tetranucleotide was repeated from 4 to 12 times in different strains (Table 1). The number of repeats was variable also within each allele (Table 1).

[0228] Further work was performed on carrier strains isolated from healthy individuals by oro-pharyngeal swab. Some strains, even if described as carriers, belong to hypervirulent clusters, and NadA was found in all such carrier strains as described above (i.e. allele 1 in the ET-5 strains and allele 2 in the ET-37 strains).

[0229] NadA was also found in five carrier strains (N1228, N1265, N1266, N1262, N1282) which do not belong to a hypervirulent cluster. These five strains shared a sequence (SEQ IDs 13 & 14) which was not found in strains isolated from patients. This allele is referred to as ‘allele C’ (carrier).

[0230] An alignment of allele C with alleles 1 to 3 is shown in FIG. 9C. Disruption in the coiled-coil segments of the protein is evident.

[0231] Unlike alleles 1 to 3, allele C protein does not readily form a high molecular aggregate when expressed in E. coli. Like alleles 1 to 3, however, allele C is exposed on the surface of N. meningitidis, because it is a target for bactericidal antibody raised against itself. However, these antibodies are not bactericidal against strains carrying alleles 1 to 3; similarly, antibodies raised against alleles 1 to 3 are not bactericidal against allele C strains.

NadA Oligomers on the Cell Surface

[0232] W991/64922 reports that NadA forms oligomeric structures. To study NadA oligomers in more detail, whole cell lysates of N. meningitidis were probed by Western blot.

[0233] Bacterial colonies [strains MC58 (allele 1), 90/18311 (allele 2), 2996 (allele 3), 953/4286 (ISS1301 insertion) and NG3/88 (nadA-)] were grown to stationary phase in GC broth supplemented with 0.3% glucose. Samples were taken at different times, pelleted by centrifugation at 3000g for 10 min, and resuspended in PBS and thawed/frozen up to bacterial lysis. Equal amounts of proteins were subjected to SDS-PAGE on 12.5% polyacrylamide gels and electrophoresis onto nitrocellulose membranes.

[0234] To prepare anti-NadA polyclonal serum, recombinant NadA was expressed and purified. Sequences encoding the three nadA alleles (allele 1: aa 24-362; allele 2: aa 24-343; allele 3: aa 24-350), were amplified by PCR on chromosomal DNA and cloned into pET21b+ vector (Novagen). The plasmids were transformed in E. coli BL21 (DE3) to express the proteins as C-terminal histidine fusions. Protein expression was induced at 30°C by adding 1 mM IPTG at OD750nm 0.5 and growing the bacteria for an additional 3 h; expression was evaluated by SDS-PAGE. Recombinant fusion proteins were
purified by affinity chromatography on Ni²⁺-conjugated chelating fast-flow Sepharose 4B resin. 20 μg of purified protein was used to immunise six-week-old CD1 female mice (4 to 6 per group). Proteins were given intraperitoneally, with complete Freund’s adjuvant (CFA) for the first dose and incomplete Freund’s adjuvant (IFA) for the second (day 21) and third (day 35) booster doses. Bleed out samples were taken on day 49 and used for the serological analysis.

The blots showed a high molecular weight reactive band in strains MC58 (FIG. 14, lane 1), 90/18311 (lane 2) and 2996 (lane 3). The band was absent in strain NG3/88 (lane 5). Bovine of the sample buffer up to 40 minutes did not change the pattern. The different size of the proteins was consistent with the size of the alleles. Given the expected size ranging from 35 to 40 kDa of monomeric proteins, the high MW of the observed band could be explained by the presence of an oligomeric form of NadA. This possibility is supported by the fact that in a strain containing the IS1301 insertion, coding for a shorter protein of 162 amino acids and lacking most of the coiled-coil region, the high MW reactive band was absent and replaced by a band of 14.5 kDa (FIG. 14, lane 4), consistent with the predicted molecular weight of the processed monomeric protein.

Although the oligomeric protein was found in all strains containing a functional gene, expression levels varied from strain to strain (Table I). Moreover, the amount of NadA protein varied within the same strain during growth.

Four different strains (MC58, 2996, C11, F6124), chosen as representative of diverse overall NadA expression level, were followed during growth up to stationary phase. FIG. 15 shows growth of two of the tested strains (15A: MC58, with low NadA expression; 15B: 2996, with high NadA expression), with the curve showing OD500. Western blots of samples taken at each point of the OD500 growth curve showed that the NadA band was barely visible at the beginning of the growth and became more intense during growth up to its maximum, at stationary phase. All strains analysed showed the same growth-phase dependent behaviour.

High MW NadA was also seen in western blots of outer membrane vesicles, consistent with NadA being anchored to the outer membrane.

Similarly, FACS analysis on live bacteria during log-phase growth showed that NadA was available for antibody binding on the surface of the bacteria. FACS intensity in a strain with a polysaccharide capsule (strain NMB) was reduced 1 log in comparison to an isogenic non-encapsulated mutant strain (M7), but the protein was surface-exposed and available for binding in both strains (FIG. 16).

NadA forms surface-exposed oligomers, which are stable to heat, SDS and reduction with β-mercaptoethanol. As the mature form of the lacks cysteine residues, disulphide bond formation cannot be involved in this phenomenon; rather this is consistent with the predicted coiled-coil structure and the presence of leucine zipper motifs that might mediate intermolecular interactions between monomers [Lupas (1996) Trends Biochem. Sci. 21:375-382; O’Shea et al. (1991) Science 254:539-544]. The size of the oligomers is approximately 170 kDa, suggesting a tetrameric structure [W001/64922]. However, a rigid coiled-coil structure is likely to have an anomalous migration is SDS PAGE and therefore the 170 kDa form may be a trimer.

Protective Immunogenicity

Polyclonal anti-NadA serum was tested for bactericidal activity as previously described [Pizza et al. (2000);

Peeters et al. (1999) Vaccine 17:2702-2712], with pooled baby rabbit serum (Cedarlane) used as complement source. Serum bactericidal titer, was defined as the serum dilution resulting in a 50% decrease in colony forming units (CFU) per ml after 60 minutes incubation of bacteria in the reaction mixture, compared to control CFU per ml at time 0. Typically, bacteria incubated with the negative control antibody in the presence of complement showed a 150 to 200% increase in CFU/ml during the 60 min. of incubation.

Results were as follows:

<table>
<thead>
<tr>
<th>Strain</th>
<th>NadA expression</th>
<th>Allele</th>
<th>Bactericidal titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>2996</td>
<td>+++</td>
<td>3</td>
<td>32768</td>
</tr>
<tr>
<td>C11</td>
<td>+++</td>
<td>3</td>
<td>16384</td>
</tr>
<tr>
<td>F6124</td>
<td>+</td>
<td>3</td>
<td>4096</td>
</tr>
<tr>
<td>MC58</td>
<td>+</td>
<td>1</td>
<td>8192</td>
</tr>
<tr>
<td>B2232</td>
<td>-</td>
<td>-</td>
<td>&lt;4</td>
</tr>
<tr>
<td>NG3/88</td>
<td>-</td>
<td>-</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

As shown, the serum induced complement-mediated killing of all strains that have the nadA gene, and was inactive against the strains that do not have the gene. However, bactericidal titres varied between strains. Titres were higher against strains expressing higher amounts of protein. This result was confirmed when titres were determined in the early and late phase of growth (FIG. 15).

To check whether the differences in the bactericidal activity were due to different allele sequences, immune sera, raised against the three NadA types, were produced and used in a cross bactericidal assay. The results obtained with the antisera were similar to those shown above, suggesting that the bactericidal activity is not influenced by the allele diversity but rather to the antigen expression level.

The ability of immune sera to protect animals from bacteremia during infection was also tested, using the infant rat model. The sera were obtained by immunising guinea pigs with 50 μg purified NadA (allele 3). Immunisation of outbred Wistar rats (5 to 7 days old) was performed subcutaneously together CFA for the first dose and IFA for the further three doses (28, 56, 84). Bleed out samples were taken on day 105 and used for the animal protection assay.

Two experiments were performed using two different MenB strains (8047 and 2996). Each strain has been serially passaged three times in infant rats. In experiment 1, groups of four rats were challenged intraperitoneally with 100 μl of a mix of (a) bacteria from strain 8047 (7×10⁸ CFU per rat) and (b) heat inactivated guinea pig antisera or anti-capsule control mAb (SEAM 3 [Van Der Ley et al. (1992) Infect. Immun. 60:3156]). In experiment 2, group of six rats were treated with the control mAb or with different dilutions of guinea pig antisera at time 0. Two hours later, they were challenged with the 2996 bacteria (5.6×10⁹ CFU per rat). In both experiments, blood cultures were obtained 18 h after the challenge by puncturing the heart with a syringe and needle containing approximately 25 U of heparin without preservative. Bacteria numbers in the blood cultures were obtained by plating out 1, 10, and 100 μl of blood onto chocolate agar overnight. For calculation of geometric mean CFU/ml, animals with sterile cultures were assigned a value of 1 CFU/ml.
[0247] Results were as follows:

<table>
<thead>
<tr>
<th>Exp</th>
<th>Treatment</th>
<th>Positive/Total (10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anti-capsular mAb (2 μg/rat)</td>
<td>0/4 0.001</td>
</tr>
<tr>
<td></td>
<td>Anti-NadA antiserum (1:5 dilution)</td>
<td>0/4 0.001</td>
</tr>
<tr>
<td></td>
<td>PBS + 1% BSA</td>
<td>5/5 40.17</td>
</tr>
<tr>
<td>2</td>
<td>Anti-capsular mAb (20 μg/rat)</td>
<td>1/6 0.003</td>
</tr>
<tr>
<td></td>
<td>Anti-NadA antiserum (1:5 dilution)</td>
<td>1/6 0.002</td>
</tr>
<tr>
<td></td>
<td>Anti-NadA antiserum (1:25 dilution)</td>
<td>3/6 0.035</td>
</tr>
<tr>
<td></td>
<td>Pre-immune NadA serum</td>
<td>6/6 1.683</td>
</tr>
</tbody>
</table>

Thus anti-NadA antiserum is highly protective in this assay.

[0249] Overall, therefore, NadA has several attributes of being a good vaccine antigen: (i) it is a surface-exposed molecule, potentially involved in bacterial adhesion; (ii) it is present in at least 50% of the disease-associated strains and in almost 100% of three hypervirulent lineages; (iii) it elicits protective and bactericidal antibodies in laboratory animals; and (iv) each allele induces cross-bactericidal antibodies.

[0250] ORF40 shows homology to Hsf and its allelic variant Hia, both adhesins of Haemophilus influenzae. The different size among Hia, Hsf and ORF40 is in part explained by the presence of three copies of a large repeated domain in Hsf, which is present in single copy in Hia and only partially in ORF40 (Fig. 7). In MenB, ORF40 is found on the outer membrane as a protein of about 200 kDa (cf. predicted MW of 59 kDa for mature protein).

[0251] App shows homology (Fig. 8) to the adhesion and penetration protein Hap of H. influenzae, which is an adhesin with a serine-protease activity that undergoes autoproteolytic cleavage and extracellular release [Hendrixson et al. (1997) Mol Microbiol 26:505-518]. Unrelated surface-associated Hap mediates adherence to epithelial cells and promotes bacterial aggregation and colonisation.

[0252] In N. meningitidis, App is exported to the outer membrane, processed and secreted. Both Hap and App belong to the autotransporter family which comprises proteins from gram-negative bacteria characterized by a distinct mechanism of secretion. This system was first described for IgA1 protease of N. gonorrhoeae, which is considered the prototype of this family. Proteins of the autotransporter family have been implicated in the virulence of many gram-negative pathogens [Henderson & Naturo (2001) Infect Immun 69:1231-1243]. They are synthesized as large precursor proteins comprising at least three functional domains: a typical N-terminal leader sequence, an internal domain (passenger domain) and a C-terminal domain (translocator domain or β-domain). The leader sequence mediates the export (sec-dependent) of the protein to the periplasm. Subsequently the translocator domain inserts into the outer membrane forming a β-barrel pore to allow the export of the passenger domain. Once at the bacterial surface, the passenger domain can be cleaved and released into the environment. Cleavage can occur by an autoproteolytic event directed by protease activity in the passenger domain itself. Passenger domains of autotransporters are widely divergent, reflecting their remarkably disparate roles. On the contrary the β-domains display high degree of conservation consistent with their conserved function.

[0253] App possesses the prevailing domains of the autotransporter proteins as well as the conserved serine protease motif (GDSLGP). It has been shown that this motif is responsible for cleavage of human IgA1 by the Neisseria IgA1 proteases and for autoproteolytic cleavage of Hap protein of H. influenzae. App has been shown to be a conserved antigen among meningococci, to be expressed during infection and carriage, to stimulate B cells and T cells, and to induces a bactericidal antibody response [Hadi et al. (2001) Mol Microbiol 41:611-623; Van Ulsen et al. (2001) FEMS Immunol Med Microbiol 32:53-64].

[0254] In serogroup B strain 2996, App has 1454 amino acids and a predicted MW of 159,965 Da. Fig. 26 shows the protein’s predicted structural features. Three domains can be seen: domain 1 (amino acids 1-42) is the signal peptide; domain 2 is the passenger domain, which is the functionally active protein; domain 3 is the C-terminal translocator domain with β-barrel structure.

[0255] At the N-terminus of the passenger domain, His-115, Asp-158 and Ser-267 correspond to the serine protease catalytic triad His-98, Asp-140 and Ser-243 from Hap [Fink et al. (2001) J Biol Chem 276:39492-39500]. Residues 285-302 are a putative ATP/GTP-binding site (P loop), which suggests a mechanism of energy coupling for outer membrane translocation. Towards the C-terminus of the passenger domain, two Arg-rich regions are present. The first (RRSRR) is residues 934-938 and the second (RRARR) begins at residue 1149. These motifs are reminiscent of known targets for trypsin-like proteolytic cleavage sites such as the one in diphtheria toxin and those upstream of the auto-cleavage sites of H. influenzae Hap, N. gonorrhoeae IgA-protease and B. pertussis PhaB (Fig. 26, box 1). Downstream of the Arg-rich regions are motifs 956-NTL956 and 1177-N956, which are identical or similar to the cleavage sites in autotransporters Ssp (Serratia marcescens), Prn (Bordetella bronchiseptica), Brka (Bordetella pertussis) [Jose et al. (1995) Mol Microbiol 18:378-380] and Hap (H. influenzae) (Fig. 26, box 2). Together, these sequence motifs suggest that the two motifs 956-NTL956 and 1177-N956 and the ARG(R,S,R)RR pattern could act as signals for correct localisation of downstream processing sites.

[0256] Further analysis of the App sequence shows a proline-rich region, where the dipeptide motif PQQ is repeated four times beginning at residue 1156. A search for homology to known protein sequences reveals some similarity to the surface proteins of S. pneumoniae PspA and PspC and to a proline-rich region of the B. pertussis outer membrane protein p69 pertactin, where the (PQQ)₃ motif is located in a loop containing the major immunoprotective epitope.

[0257] Finally, the last three amino acids of App (YRW) are identical to those of Hap where they have been described as crucial for outer membrane localisation and protein stability [Hendrixson et al., 1997].

Expression in E. coli without Fusion Partners

[0258] ORF40, App and NadA full-length genes were cloned in pet21b+ vector and the plasmids were transformed in E. coli BL21(DE3) in order to express the genes under control of T7 promoter. Expression was achieved activating the promoter with IPTG or under non-induced conditions. Localisation and surface-exposure of the proteins were
assayed by cell-fractionation experiments (SDS-PAGE and Western blot), FACS analysis and whole-cell immunoblot. As shown in FIGS. 1 to 3, all the three proteins are translocated to the surface of E. coli:

- [0259] ORF40 is expressed as monomorphic form and possibly forms also multimers (FIG. 1).
- [0260] App is exported to E. coli outer membrane as a precursor of about 160 kDa, that is processed and secreted in the culture supernatant (FIG. 2).
- [0261] NadA is found to be present in the outer membrane fraction as a single high molecular weight band of approximately 180 kDa. This probably corresponds to an oligomeric form of the protein. Such a band is absent in E. coli expressing intracellular NadA (FIG. 3).

- [0262] App expression was studied in more detail.

- [0263] N. meningitidis strain 2996 genomic DNA was prepared as described (Tasley and Nasserif 1996) PNAS USA 93:11109-11114. DNA devoid of the sequence coding for the signal peptide (amino acids 1 to 42) and of the STOP codon was amplified using PCR primers SEQ IDs 18 & 19 followed by digestion with Nhel and Xhol and insertion into the Nhel/Xhol sites of the pET-21b expression vector, to give "pET-App-His" (FIG. 27). This plasmid was introduced into E. coli BL21(DE3) and used for the expression of a C-terminal His-tagged fusion protein which was purified and used to raise antibodies. The full-length app gene was amplified and cloned in a similar way, using PCR primers SEQ IDs 20 & 21, to give plasmid "pET-App".

- [0264] Plasmids were introduced into E. coli BL21(DE3) and expression induced by addition of 1 mM IPTG. The expressed protein was detected by western blotting (FIG. 28, lane 1). To verify that the protein was exported to the E. coli surface, FACS (FIG. 29) and immunofluorescence microscopy (FIG. 30) were used. The FACS analysis showed positive surface expression on the pET-App transformants (full-length gene) but no surface expression with App-His (no signal peptide) or with the empty vector. The immunofluorescence results agreed with FACS. Therefore expression of the full-length app gene resulted in the export of App to the surface of E. coli, but deletion of the first 42 amino acids abolished surface-localisation.

- [0265] Western blot analysis of outer membrane proteins from pET-App transformants revealed a specific reactive band of ~160 kDa (FIG. 28, lane 1), corresponding to the predicted molecular weight of the full-length protein. A corresponding band was missing in the outer membrane fraction from untransformed controls (lane 3). Western blot analysis of culture supernatants revealed a secreted protein of ~100 kDa with pET-App (lane 2) that was absent with the untransformed controls (lane 4). Sometimes a very weak band was also detected at ~140 kDa in pET-App transformants.

- [0266] Therefore the full length app gene when introduced into E. coli induces expression of an App protein which is exported to the outer membrane, cleaved and released into the culture supernatant.

Native Expression Can Influence the Quality of the Immune Response

- [0267] To evaluate the role of protein conformation on induction of an immune response, outer membrane vesicles from E. coli expressing ORF40, App or NadA were isolated and used to immunise mice. Sera were tested for bactericidal activity and results compared with those obtained with the fusion proteins. The bactericidal response (strain 2996) was improved 5-10 fold when the proteins are produced in their "native" form in OMVs:

<table>
<thead>
<tr>
<th>Bactericidal titres*</th>
<th>Antigen</th>
<th>Fusion protein</th>
<th>E. coli OMV</th>
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<tr>
<td>ORF40</td>
<td>256</td>
<td>2048</td>
<td></td>
</tr>
<tr>
<td>App</td>
<td>64</td>
<td>1024</td>
<td></td>
</tr>
<tr>
<td>NadA</td>
<td>32768</td>
<td>&gt;55536</td>
<td></td>
</tr>
</tbody>
</table>

* Titres expressed as the reciprocal of the serum dilution yielding ~50% bacteria killing App Autoproteolytic Cleavage

- [0268] E. coli pET-App transformants secrete a 100 kDa product into culture supernatant and show a 160 kDa surface product. To test whether the secreted App product derives from an autoproteolytic process, one of the putative catalytic residues (Ser-267) was replaced with Ala.

- [0269] The pET-AppS267A mutant was obtained by site-directed mutagenesis using the QuickChange kit (Strategene) and primers SEQ IDs 22 & 23.

- [0270] SDS-PAGE analysis of total proteins from pET-AppS267A transformants (FIG. 31, lane 2) showed a protein similar in size to pET-App transformants (lane 1). The protein was shown to be surface exposed by FACS analysis (FIG. 29). Western blot analysis of culture supernatants showed App in pET-App transformants (FIG. 32, lane 1) but not in pET-AppS267A transformants (lane 2).

- [0271] Mutation of Ser-267 to Ala thus abolishes processing and secretion of the App precursor, which remains cell-associated. These data suggest that App has a serine protease activity that is responsible for autoproteolytic processing and release in the supernatant of the secreted App domain.

- [0272] Cleavage at S55NTL59 would leave a fragment with predicted molecular weight of 104190 Da. Cleavage at S17NSG178 would give a 128798 Da fragment. These two predicted fragments may match the two bands of ~140 and ~100 kDa observed in culture supernatants. Cleavage may occur first to give the ~140 kDa fragment and then second to give the 100 kDa fragment. The β domain of App would thus begin at residue 1177.

NadA, ORF40 and App Function as Adhesins

- [0273] Example 22 of international patent application WO01/64922 discloses that NadA expression in E. coli makes the transformed bacterium adhere to human epithelial cells. The adherent phenotype has been further studied for NadA and also for App and ORF40.

- [0274] E. coli BL21(DE3) bacteria (10⁵ CFU), grown under non-induced or induced conditions, were inoculated onto Chang human epithelial monolayers (10⁵ cells) and incubated at 37°C for 1 or 2 hours. Cells were then incubated with rabbit anti-E. coli and PE-conjugate secondary antibody. Adhesion was detected by FACS as specific fluorescence intensity associated to Chang cells. Positive controls were E. coli DH5α expressing hsf (DH5α/pDH501); negative controls were BL21(DE3)/pET21b and DH5α/p777–. The results in FIG. 4 show that the ability of the recombinant E. coli strains to adhere to cultured epithelial cells is associated with expression of these three proteins.
To confirm that these three proteins are able to promote interaction with host cells, the recombinant proteins themselves were investigated for binding to epithelial cells. 10^5 Chang human epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4, human conjunctiva) were incubated at 4°C for 30 minutes with medium alone or with different concentration of ORF40 (150 μg/ml), App (150 μg/ml) or NadA (300 μg/ml), or with GNA2132 (300 μm/μl) as negative control [see Piazza et al. (2000)] Binding was detected by FACS using polyclonal sera against the single recombinant proteins and a secondary PE-conjugate antibody. The FACS signal shifts (FIG. 5) show that the three proteins are able to bind to human epithelial cells, whereas purified GNA2132 (negative control) does not.

FIG. 6A shows that binding increases in a dose-dependent manner. Binding of NadA reaches a plateau at around 200 μg/ml. GNA2132 fails to bind even at 400 μg/ml (FIG. 6B). Data in FIG. 6 are mean fluorescent intensity (MFI) values plotted against protein concentration (μg/ml).

Using FACS, binding of NadA to cells was also seen with Hep-2 and MOLT-4 cells, but not with HeLa, A549, Hee-1B, Hep-G2, CHO or HUVEC cells. Adhesion to Chang cells could be abolished by treating the cells with pronase, indicating that the human receptor for NadA is a protein.

Adhesion of purified NadA protein to Chang conjunctiva cells was also observed using immunofluorescence microscopy. The protein (lacking its C-terminal anchor domain) was incubated with Chang cells at 37°C, in complete culture medium for 3 hours at various concentrations. Cells were then washed, fixed, and analyzed by laser confocal microscopy after staining with anti-NadA mouse polyclonal antibodies and secondary Texas-red coupled anti-mouse IgG antibodies. No binding was seen at 0 nM (FIG. 17A), but binding was evident at 170 nM (17B) and 280 nM (17C), with clustering evident at higher concentrations. In contrast, no binding of NadA was seen with HeLa cells, even at 280 nM protein (17D).

Binding was much more evident at 37°C. (FIG. 18A) than at 4°C (FIG. 18B). The dot-like structures seen at 4°C, compared to clusters at 37°C, suggest that lateral interactions between NadA monomers are temperature-dependent (stimulated by membrane fluidity).

To distinguish surface and endocytosed protein, saponin detergent was added during the staining procedure. Intracellular clusters having the size of endosomes were more evident (arrow) when saponin was used, but a high proportion of protein remained on the cell surface (FIG. 19).

Immunofluorescence also revealed that NadA binds to monocytes (FIG. 20A). NadA alone (no staining antibody; 20B) and NadA stained with pre-immune serum (20C) were not visible. At high magnification, evidence of uptake into vesicles (either endosomes or phagosomes) was seen.

FIG. 21 shows that murine macrophages (raw 264.7) bind and endocytose NadA (125 nM, 3 hours, 37°C, cells cultured in DMEM).

Heating NadA at 95°C for 15 minutes prior to incubation removed its ability to bind to monocytes, as measured by secretion of IL-α by the cells (FIG. 22). The stimulatory activity of NadA preparations is thus heat-labile. Stimulatory activity was also blocked by the use of anti-CD14 (FIG. 23) or by the removal of NadA from the preparations using bead-immobilised anti-NadA.

Immunofluorescence microscopy was also used to detect binding of E. coli expressing NadA. Transformed E. coli bound strongly (FIG. 24A) whereas untransformed bacteria did not (24B). IL-α release by monocytes was over 1.5x higher using the transformed E. coli than the untransformed bacteria at a bacteria/monocyte ratio of 40:1.

Transformed E. coli were bound to glass cover slips, fixed and double-stained with anti-NadA (FIG. 25A) and anti-E. coli antibodies (25B). When both were used, patches of anti-NadA were visible, suggesting that NadA tends to form aggregates on the bacterial surface, which hamper the interaction of antibodies with other surface antigens.

Looking at App, recombinant E. coli strains were incubated with monolayers of Chang conjunctiva epithelial cells (Wong-Kilbourne derivative, clone 1-5c-4 [human conjunctiva], ATCC CCL 20.2) and adhesion was analysed using FACS. Cells obtained from confluent monolayers were seeded at 10^5 cells per well in 12-well tissue culture plates and incubated for 24 hours. Cultures of bacteria after IPTG induction were washed twice in PBS and resuspended in DMEM+1% FBS to a concentration of 5x10^5 bacteria per ml. Aliquots of 1 ml of each strain were added to monolayer cultures of Chang cells and incubated for 3 hours at 37°C in 5% CO2. Non-adherent bacteria were removed by washing three times with PBS, and 300 μl of cell dissociation solution (Sigma) were added to each microtitre well. Incubation was continued at 37°C for 10 minutes. Cells were harvested and then incubated for 1 hour at 4°C with rabbit polyclonal anti-E. coli antisera (DAKO). Cells were washed twice in PBS+5% FBS and incubated for 30 minutes at 4°C with R-phycocerythrin-conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories). Cells were then washed in PBS+5% FBS and resuspended in 100 μl PBS. Fluorescence was measured with FACSCalibur flow cytometer (Becton Dickinson). For each of fluorescence profile, 10000 cells were analysed.

The results reported in FIG. 33 show pET-App transformants were able to adhere to Chang cells, giving a fluorescence shift of 90.3%. S267A transformants were also able to adhere (91.0%). Untransformed E. coli were unable to adhere to Chang cells (bottom FACS plot).

As for NadA, FACS results were in agreement with immunofluorescence microscopy data. As shown in FIGS. 34A & 34B, pET-App transformants incubated with monolayers demonstrated high levels of adhesion to epithelial cells and visible bacteria-bacteria aggregation. For the S267A mutant, adhesion and bacterial aggregation were increased (34C & 34D). Untransformed controls showed no adhesion (34G). Deletion of the first 42 amino acids also abolished adhesion.

In contrast to Chang epithelial cells, no adhesion was seen when HUVEC endothelial cells were tested with pET-App transformants. To cause sepsis and meningitis, N. meningitidis has to interact with human endothelial cells. App may thus be involved in the first step of colonisation at the level of human respiratory epithelial mucosa, rather than in pathological endothelial colonisation.

Localization and Specificity of App Binding Activity.

To identify the binding region of App, a chimeric protein named Appβ was used. This protein consists of the C-terminal domain of App (amino acids 1077 to 1454) fused to the leader peptide of IgA1 protease of N. gonorrhoeae. The gonococcal leader sequence was chosen because it has been well characterized and is functional in E. coli. Plasmid pET-Appβ contains a 1.1 kbp DNA fragment amplified by PCR using SEQ IDs 26 & 27.
[0291] The pET-Appβ construct was introduced into E. coli BL21(DE3). FACS localisation studies confirmed that Appβ was localized on the E. coli surface. The in vitro adhesion assay using Chang epithelial cells showed adhesion by immunofluorescence (FIGS. 34E & 34F). FACS analysis showed that the pET-App transformants were still able to adhere to epithelial cells but at lower levels (74.2% shift) than pET-App transformants.

[0292] These results indicate that the App binding domain is located in its C-terminal region, in the 100mer fragment between residues 1077 and 1176. Purified recombinant proteins were also studied. App-ct-His consists of the N-terminal portion of App (amino acids 43-1084) fused to a poly-His tag. Plasmid pET-Appα-His contains a Nhel/Xhol 3.1 kb fragment amplified by PCR with SEQ IDs 24 & 25. The binding activity of the purified recombinant App-ct-His was compared to that of App-His by FACS binding assays. Chang cells were incubated with increased concentrations of recombinant App proteins or lipoprotein NMB2132-His (negative control). Binding of App-His (I) increased in a dose-dependent manner and reached a plateau at a concentration of ~50 µg/ml whereas the binding of App-His (II) was very low (FIG. 35). The control NMB2132-His (A) failed to bind Chang cells.

[0294] To explore the biochemical nature of the molecule involved in interaction with App, the Chang cells were treated with pronase or phospholipase A2 before the binding experiments. 105 cells per well were plated in microplates and incubated in FCS-free DMEM at 37°C in 5% CO2 for 30 minutes with (a) pronase at 250, 500, or 1000 µg/ml or (b) phospholipase A2 at 50, 200, or 800 µg/ml. After enzymatic incubation, an equal volume of complete medium was added to each well to stop the reaction. Cells were subsequently mixed with 100 µg/ml App-His or medium alone and incubated for 1 hours at 4°C. As shown in FIG. 36, pronase treatment (left-hand columns) markedly reduced the binding of App-His protein to Chang cells, while treatment with phospholipase A2 (right-hand columns) did not reduce the binding. The receptor for App on Chang cells is thus the proteinaceous.

[0295] Adhesion to different cell lines were also tested (FIG. 37). After incubation of cultured cells with three different concentrations of App-His (100, 25 & 6.25 µg/ml) high level binding to Chang cells and HepC2 cells was seen, a moderate level of binding to A-549 cells, and minimal binding to HeLa cells. No binding was observed to Hec-1-B, Hep-2, 16HBE14o epithelial cell lines or to HUVEC endothelial cells.

App Knockout

[0296] After the work on E. coli suggesting an adhesion role for App, an isogenic mutant strain of N. meningitidis was constructed. The starting strain was MC58. Its app gene was truncated and replaced with an antibiotic cassette by transforming the parent strain with the plasmid pBSUAppERM, which contains a truncated app gene and the ermC gene (erythromycin resistance) for allelic exchange. Briefly, 600 bp of the upstream flanking region including the start codon and 700 bp downstream flanking region including the stop codon were amplified from MC58 using primers SEQ IDs 28 to 31. Fragments were cloned into pBluescript and transformed into E. coli DH5 using standard techniques. Once all subcloning was complete, naturally competent N. meningitidis strain MC58 was transformed by selecting a few colonies grown overnight on GC agar plates and mixing them with 20 µl of 10 mM TrisCl pH8.5 containing 1 µg of plasmid DNA. The mixture was spotted onto a GC agar plate, incubated for 6 hrs at 37°C, 5% CO2 then diluted in PBS and spread on GC agar plates containing 5 µg/ml erythromycin. The deletion app gene in the genome of MC58 was confirmed by PCR. Lack of App expression was confirmed by Western blot analysis.

[0297] Adhesion of wildtype MC58 and the isogenic MC58App mutant strain was evaluated on Chang cells. There was a ~10 fold reduction (ranging from 3- to 27-fold in different experiments) of the association of the knockout mutant compared with the wild type strain (FIG. 38). No difference was observed between the app+ mutant and the parental strain with Hep2 and 16HBE14o cell lines and with HUVEC endothelial cells, confirming that App does not mediate adhesion to these cells.

[0298] No non-pilus adhesins which contribute to adhesion of N. meningitidis in a capsulated background have previously been reported.

[0299] App expression was studied in N. meningitidis MC58. Colonies from plates grown overnight were diluted in GC broth and incubated at 37°C with 5% CO2. Samples were taken when OD620 = 0.5 (mid log phase) and 0.8 (stationary phase) and analysed by western blot. Two bands with apparent molecular weights ~160 and ~140 kDa were detected in whole cells lysates of log phase bacteria (FIG. 39, lane 1), while stationary phase bacteria showed only a faint band at ~140 kDa (lane 3). As expected, no App was observed in the ΔApp mutant (lanes 2 & 4).

[0300] In marked contrast, supernatant samples of wildtype MC58 showed a band at ~140 kDa and its amount was higher in stationary phase than in log phase (FIG. 40, lanes 3 & 1). The stationary phase sample also showed a reactive band at ~100 kDa.

[0301] It will be understood that the invention is described above by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

### TABLE 1

| Strain | Serogroup type: subtype | Clonal group | nadA allele (TAAA) repeats | NadA
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<th>Strain</th>
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<th>(TAA) repeats</th>
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*indicates that the strain carriers a minor variant of the relevant allele
nd = not done

### TABLE II

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65   70   75   80
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100  105  110
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Asn Gly Phe Lys Ala Gly Glu Thr Ile Tyr Asp Ile Asp Glu Asp Gly 50 55 60
Thr Ile Thr Lys Asp Ala Thr Ala Asp Val Glu Ala Asp Asp 65 70 75 80
Phe Lys Gly Leu Gly Leu Lys Val Val Thr Asn Leu Thr Lys Thr 85 90 95
Val Asn Glu Asn Lys Gin Asn Val Asp Ala Lys Val Lys Ala Ala Glu 100 105 110
Ser Glu Ile Glu Lys Leu Thr Thr Leu Leu Ala Asp Thr Asp Ala Ala 115 120 125
Leu Ala Asp Thr Asp Ala Ala Leu Asp Thr Thr Ala Leu Asn 130 135 140
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85 90   95
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Lys | Lys | Ala | Ala | Thr | Val | Ala | Ile | Ala | Ala | Tyr | Asn | Asp | Gly | Gln |
35  |     |     |     | 40  |     |     |     |     |     |     |     |     |     | 45  |
Glu | Ile | Asn | Gly | Phe | Lys | Ala | Gly | Thr | Ile | Tyr | Asp | Ile | Asp | Glu |
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Asp | Gly | Thr | Ile | Thr | Lys | Asp | Ala | Thr | Ala | Ala | Asp | Val | Glu | Ala |
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Ala Ala Leu Ala Asp Thr Asp Ala Leu Asp Ala Thr Thr Asn Ala 130 135 140
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Thr Asn Ile Val Lys Ile Thr Asp Glu Lys Leu Glu Ala Val Ala Asp Thr 165 170 175
Val Asp Lys His Ala Glu Ala Phe Asn Asp Ile Ala Asp Ser Leu Asp 180 190 195 200 205
Glu Thr Asn Thr Lys Ala Asp Glu Ala Val Lys Thr Ala Asn Glu Ala 210 215 220
Lys Gin Thr Ala Glu Glu Thr Lys Gin Asn Val Asp Ala Lys Val Lys 225 230 235 240
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<223> OTHER INFORMATION: variant allele 2 of NadA in strain 1001024
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Phe Cys Ser Gly Ala Leu Ala Ala Thr Asn Asp Asp Val Lys Lys 20 25 30
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<210> SEQ ID NO 8
<211> LENGTH: 393
<212> TYPE: PRT
<213> ORGANISM: Neisseria species
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: variant allele 2 of NadA (first-ATG start) in
-continued

strain 1881024

<400> SEQUENCE: 8

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35
Glu Ile Asn Gly Phe Lys Ala Gly Glu Thr Ile Tyr Asp Ile Asp Glu  55
50
Asp Gly Thr Ile Thr Lys Asp Ala Thr Ala Ala Asp Val Glu Ala  70
65
Asp Asp Phe Lys Gly Leu Gly Leu Lys Val Val Thr Asn Leu Thr  90
Lys Thr Val Asn Glu Asn Lys Asn Val Asp Ala Lys Val Lys Ala  105
100
Ala Glu Ser Glu Ile Glu Lys Leu Thr Thr Lys Leu Ala Asp Thr Asp  120
115
Ala Ala Leu Asp Ala Thr Asn Ala Leu Asn Lys Leu Gly Glu Asn  135
130
Ile Thr Thr Phe Ala Glu Glu Thr Lys Thr Asn Ile Val Lys Ile Asp  150
145
Glu Lys Leu Glu Ala Val Ala Asp Thr Val Asp Lys His Ala Glu Ala  170
165
Phe Asn Asp Ile Ala Asp Ser Leu Asp Glu Thr Asn Thr Lys Ala Asp  185
180
Glu Ala Val Lys Thr Ala Asn Glu Ala Lys Gln Thr Ala Glu Thr  200
205
Lys Gln Asn Val Asp Ala Lys Val Lys Ala Ala Glu Thr Ala Ala Gly  220
215
Thr Ala Asn Thr Ala Ala Asp Lys Ala Glu Ala Val Ala Lys Val  230
225
Thr Asp Ile Lys Ala Asp Ile Ala Thr Asn Lys Asp Asn Ile Ala Lys  250
245
Lys Ala Asn Ser Ala Asp Val Tyr Thr Arg Glu Glu Ser Asp Ser Lys  265
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Phe Val Arg Ile Asp Gly Leu Asn Ala Thr Thr Glu Lys Leu Asp Thr  280
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Arg Leu Ala Ser Ala Glu Lys Ser Ile Thr Glu His Gly Thr Arg Leu  295
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Asn Gly Leu Asp Arg Thr Val Ser Asp Leu Arg Lys Glu Thr Arg Gln  310
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Gly Leu Ala Glu Gln Ala Ala Leu Ser Gly Leu Phe Glu Pro Tyr Asn  335
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Val Gly Arg Phe Asn Val Thr Ala Ala Val Glu Gly Tyr Lys Ser Glu  350
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Ser Ala Val Ala Ile Gly Thr Gly Phe Arg Phe Thr Glu Asn Phe Ala  360
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Ala Lys Ala Gly Val Ala Val Gly Thr Ser Ser Gly Ser Ser Ala Ala  380
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Tyr His Val Gly Val Asn Tyr Glu Trp
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<210> SEQ ID NO: 9
<211> LENGTH: 404
<212> TYPE: PRT
<213> ORGANISM: Neisseria species
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: variant allele 3 of NadA in strains 973-1720 and 198759

<400> SEQUENCE: 9

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Phe Cys Ser Gly Ala Leu Ala Ala Thr Asn Asp Asp Val Lys Lys
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Ala Ala Thr Val Ala Ile Ala Met Tyr Asn Asn Gly Gln Glu Ile Asn
35  40  45

Gly Phe Lys Ala Gly Glu Thr Ile Tyr Asp Ile Asp Glu Asp Gly Thr
50  55  60

Ile Thr Lys Lys Asp Ala Thr Ala Ala Asp Val Glu Ala Asp Asp Phe
65  70  75  80

Lys Gln Leu Gly Leu Lys Lys Val Val Thr Asn Leu Thr Lys Thr Val
85  90  95

Asn Glu Asn Lys Glu Asn Val Asp Ala Lys Val Lys Ala Ala Glu Ser
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Glu Ile Glu Lys Thr Thr Lys Leu Ala Asp Thr Asp Ala Ala Leu
115 120 125

Ala Asp Thr Asp Ala Ala Leu Asp Ala Thr Asn Ala Leu Asn Lys
130 135 140

Leu Gly Glu Asn Ile Thr Thr Phe Ala Glu Glu Thr Thr Asn Ile
145 150 155 160

Val Lys Ile Asp Glu Lys Leu Glu Ala Val Ala Asp Thr Val Asp Lys
165 170 175

His Ala Glu Ala Phe Asn Asp Ile Ala Asp Ser Leu Asp Glu Thr Asn
180 185 190

Thr Lys Ala Asp Glu Ala Val Lys Thr Ala Asn Glu Ala Lys Glu Thr
195 200 205

Ala Glu Glu Thr Lys Glu Asn Val Asp Ala Lys Val Lys Ala Ala Glu
210 215 220

Thr Ala Ala Gly Lys Ala Glu Ala Ala Asp Gly Thr Ala Asn Thr Ala
225 230 235 240

Ala Asp Lys Ala Glu Ala Val Ala Lys Val Thr Asp Ile Lys Ala
245 250 255

Asp Ile Ala Thr Asn Lys Asp Asn Ile Ala Lys Lys Ala Asn Ser Ala
260 265 270

Asp Val Tyr Thr Arg Glu Glu Ser Asp Ser Lys Phe Val Arg Ile Asp
275 280 285

Gly Leu Asn Ala Thr Thr Glu Lys Leu Asp Thr Arg Leu Ala Ser Ala
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Glu Lys Ser Ile Ala Asp His Asp Thr Arg Leu Asn Gly Leu Asp Lys
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**<210> SEQ ID NO 10**

**<211> LENGTH: 407**

**<212> TYPE: PRT**

**<213> ORGANISM: Neisseria species**

**<220> FEATURE:**

**<221> NAME/KEY: MISC_FEATURE**

**<223> OTHER INFORMATION:** variant allele 3 of NodA (first-ATG start) in strains 973–1750 and 109759

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| 290 | Arg Ile Asp Gly Leu Asn Ala Thr Thr Glu Lys Leu Asp Thr Arg Leu |
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| 300 |
| 305 | Ala Ser Ala Glu Lys Ser Ile Ala Asp His Asp Thr Arg Leu Asn Gly |
| 310 |
| 315 |
| 320 | Leu Asp Lys Thr Val Ser Asp Leu Arg Lys Glu Thr Arg Gln Gly Leu |
| 325 |
| 330 |
| 335 | Ala Glu Gln Ala Ala Leu Ser Gly Leu Phe Gln Pro Tyr Asn Val Gly |
| 340 |
| 345 |
| 350 | Asp Ile Ala Asp Ser Ile Ala Asp Ser Lys Ala Asp Glu His Ala Asp |
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| 365 | Val Ala Ile Gly Thr Gly Phe Arg Phe Thr Glu Asn Phe Ala Ala Lys |
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<210> SEQ ID NO 11
<211> LENGTH: 355
<212> TYPE: PRT
<213> ORGANISM: Neisseria species
<220> FEATURE:
<221> NAME/KEY: ""
<222> OTHER INFORMATION: Natale allele 1/2 chimera (strain 95330)
<400> SEQUENCE: 11

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Ala Ala Thr Val Ala Ile Ala Ala Tyr Asn Gly Glu Ile
35   40    45
Asp Gly Phe Lys Ala Gly Glu Thr Ile Tyr Asp Ile Asp Gly Asp Gly
50   55    60
Thr Ile Thr Lys Asp Ala Thr Ala Ala Asp Val Glu Ala Asp Asp
65   70    75    80
Phe Lys Gly Leu Gly Leu Lys Val Val Thr Asn Leu Thr Lys Thr
85   90    95
Val Asn Glu Asn Lys Gln Asn Val Asp Ala Lys Val Lys Ala Ala Glu
100  105   110
Ser Glu Ile Glu Lys Leu Thr Thr Lys Leu Ala Asp Thr Asp Ala Ala
115  120   125
Leu Asp Ala Thr Thr Ala Ala Leu Lys Leu Gly Glu Asn Ile Thr
130  135   140
Thr Phe Ala Glu Glu Thr Lys Thr Asn Ile Val Lys Ile Asp Glu Lys
145  150   155   160
Leu Glu Ala Val Ala Asp Thr Val Asp Lys His Ala Glu Ala Phe Asn
165  170   175
Asp Ile Ala Asp Ser Leu Asp Glu Thr Asn Thr Lys Ala Asp Glu Ala
180  185   190
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**SEQ ID NO 12**
LENGTH: 357
**ORGANISM:** Neisseria species
**FEATURE:**
**NAME/KEY:** Misc feature
**OTHER INFORMATION:** NadA allele 1/2 chimera (strain 95330) (first-ATG start)

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Glu Lys Leu Glu Ala Val Ala Asp Thr Val Asp Lys His Ala Glu Ala
166 170 175

Phe Asp Asp Ile Ala Asp Ser Leu Asp Glu Thr Asn Thr Lys Ala Asp
180 185 190

Glu Ala Val Lys Thr Ala Asn Glu Ala Lys Gln Thr Ala Glu Glu Thr
195 200 205

Lys Gln Asn Val Asp Ala Lys Val Lys Ala Ala Glu Thr Ala Ala Gly
210 215 220

Lys Ala Glu Ala Ala Glu Thr Ala Asn Thr Ala Ala Asp Lys Ala
225 230 235 240

Glu Ala Val Ala Ala Lys Val Thr Asp Ile Lys Ala Asp Ile Ala Thr
245 250 255

Asn Lys Ala Asp Ile Ala Lys Asn Ser Ala Arg Ile Asp Ser Leu Asp
260 265 270

Lys Asn Val Ala Ala Asn Leu Arg Lys Glu Thr Arg Gln Gly Leu Ala Glu
275 280 285

Gln Ala Ala Leu Ser Gly Leu Phe Gln Pro Tyr Asn Val Gly Arg Phe
290 295 300

Asn Val Thr Ala Ala Val Gly Gly Tyr Lys Ser Glu Ser Ala Val Ala
305 310 315 320

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Val Asn Tyr Glu Trp

<210> SEQ ID NO 13
<211> LENGTH: 323
<212> TYPE: PRT
<213> ORGANISM: Neisseria species
<220> FEATURE:  
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: NadA allele C

<400> SEQUENCE: 13

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Ala Lys Ala Ala Leu Val Asn Ser Tyr Asn Thr Gln Asp Ile Asn
35 40 45

Gly Phe Thr Val Gly Asp Thr Ile Tyr Asp Ile Lys Asp Lys Ile
50 55 60

Thr Lys Lys Glu Ala Thr Glu Ala Asp Val Glu Ala Asp Asp Phe Lys
65 70 75 80

Gly Leu Gly Leu Lys Glu Val Val Ala Gin His Asp Glu Ser Leu Ala
85 90 95

Asp Leu Thr Glu Thr Val Asn Glu Asn Ser Glu Ala Leu Val Lys Thr
100 105 110

Ala Ala Val Val Asp Ile Ser Ala Asp Val Lys Ala Asn Thr Ala
115 120 125

Ala Ile Gly Glu Asn Lys Ala Ile Ala Thr Lys Ala Asp Lys Thr
130 135 140
Glu Leu Asp Lys Val Ser Gly Lys Val Thr Glu Asn Glu Thr Ala Ile
145 150 155 160

Gly Lys Lys Ala Asn Ser Ala Asp Val Tyr Thr Lys Ala Glu Val Tyr
165 170 175

Thr Lys Gln Glu Ser Asp Arg Phe Val Lys Ile Ser Asp Gly Ile
180 185 190

Gly Asn Leu Asn Thr Thr Ala Asn Gly Leu Glu Thr Arg Leu Ala Ala
195 200 205

Ala Glu Gln Ser Val Ala Asp His Gly Thr Arg Leu Ala Ser Ala Glu
210 215 220

Lys Ser Ile Thr Glu His Gly Thr Arg Leu Asn Gly Leu Asp Arg Thr
225 230 235 240

Val Ser Asp Leu Arg Lys Glu Thr Arg Gin Gly Leu Ala Glu Gin Ala
245 250 255

Ala Leu Ser Gly Leu Phe Gin Pro Tyr Asn Val Gly Arg Phe Asn Val
260 265 270

Thr Ala Ala Val Gly Tyr Ser Glu Ser Ala Val Ala Ile Gly
275 280 285

Thr Gly Phe Arg Phe Thr Glu Asn Phe Ala Ala Lys Ala Gly Val Ala
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Val Gly Thr Ser Ser Ser Ser Ala Tyr His Val Gly Val Asn
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Tyr Glu Trp

<210> SEQ ID NO 14
<211> LENGTH: 325
<212> TYPE: PRT
<213> ORGANISM: Neisseria species
<220> FEATURER:
<222> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: HaaA allele C (first-ATG start)

<400> SEQUENCE: 14

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1 5 10 15

Ala Ala Leu Ser Gly Ser Ala Met Ala Asp Asn Ala Pro Thr Ala Asp
20 25 30

Glu Ile Ala Lys Ala Leu Val Asn Ser Tyr Asn Asn Thr Gln Asp
35 40 45

Ile Asn Gly Phe Thr Val Gly Asp Thr Ile Tyr Asp Ile Lys Asn Asp
50 55 60

Lys Ile Thr Lys Lys Glu Ala Thr Glu Ala Asp Val Glu Ala Asp Asp
65 70 75 80

Phe Lys Gly Leu Gly Leu Lys Glu Val Val Ala Gin His Asp Gin Ser
85 90 95

Leu Ala Asp Leu Thr Glu Thr Val Asn Glu Asn Ser Glu Ala Leu Val
100 105 110

Lys Thr Ala Ala Val Asn Ser Ile Ser Ala Asp Val Lys Ala Asn
115 120 125

Thr Ala Ala Ile Gly Glu Asn Lys Ala Ala Ile Ala Thr Lys Ala Asp
130 135 140

Lys Thr Glu Leu Asp Lys Val Ser Gly Lys Val Thr Glu Asn Glu Thr
145 150 155 160
 Ala Ile Gly Lys Lys Ala Asn Ser Ala Asp Val Tyr Thr Lys Ala Glu
165  170  175
Val Tyr Thr Lys Glu Ser Asp Arg Arg Phe Val Lys Ile Ser Asp
180  185  190
Gly Ile Gly Asn Leu Asn Thr Ala Asn Gly Leu Glu Thr Arg Leu
195   200  205
Ala Ala Ala Glu Ser Val Ala Asp His Gly Thr Arg Leu Ala Ser
210   215  220
Ala Glu Lys Ser Ile Thr Glu His Gly Thr Arg Leu Asn Gly Leu Asp
225   230  235  240
Arg Thr Val Ser Asp Leu Arg Lys Glu Thr Arg Glu Gly Leu Ala Glu
245   250  255
Gln Ala Ala Leu Ser Gly Leu Leu Pro Tyr Asn Val Gly Arg Phe
260   265  270
Asn Val Thr Ala Ala Val Gly Gly Tyr Lys Ser Glu Ser Ala Val Ala
275   280  285
Ile Gly Thr Gly Phe Arg Phe Thr Glu Val Phe Ala Ala Lys Ala Gly
290   295  300
Val Ala Val Gly Thr Ser Ser Gly Ser Ser Ala Ala Tyr His Val Gly
305   310  315  320
Val Asn Tyr Glu Trp
325

<210> SEQ ID NO 15
<211> LENGTH: 971
<212> TYPE: DNA
<213> ORGANISM: Neisseria species
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: coding sequence for SEQ ID 13
<400> SEQUENCE: 15
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gcactggcag acaagcgcac cacagctgac gaaatgcca aagcgcaag gttaaacgcc 120
tacctaca caccaaccat cacagcaacct acagtcgggag acacctctca gacatttaa 180
aatgacaga ttccaaagaa agaagcctca gaaagccttttgtaaa gaatcctca 240
ggctgtgtc tgaagaaagt cttggctcaaa cagacatcaac cctgtgccg ctagcagm 300
cgcgtcaag aacacagcgaa gcatgtcaa aacacgcccc gcagttcatta gacatcagt 360
cgcgtcagac gcaagccacaa gcagaattcg gccaaaaacagatcattatgc gtcacaaag 420
cgacaacaa cgaatggtgaa aagctgctcc gcacagtaac cgagaacagc atcgctatcg 480
gtaaaagag aacacagtgcgc gacggtgata caaagctgca ggtgtacacc aaaaagag 540
cctgtgcaac attgtgaaca attagttcag gatcagtatgac tctgacaattgc gtagcagcgtgc 600
gattggagc aagctggtgcgcc ggtgacgcaac aatgctgatg agaaccagct aagcaggtttg 660
tccctgggaccaaatctgctt acggacaacag gtacgctgct gagcgggttg gatgaagac 720
tggcaagagc gtagttctgca gggatatgaag aacgccaaag ggtctgagc acaagccttg ctctgcggtc 780
tgtagccatct tcaaccatgt gttccagtca atgtaacggt gcagttgtcg ccggctacaat 840
cgtagtcggcg ccagctcagacctgctgctc tccggttgaa cgaagaaaat cgcggccagag 900
caagcgtggtc agtcgctccct cttccgcagcc ctcaggatgtagcctgc 960
acgatgtaa

<210> SEQ ID NO 16
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: forward primer

<400> SEQUENCE: 16

gtcgaagttccctctattgga

<210> SEQ ID NO 17
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: reverse primer

<400> SEQUENCE: 17
cagagcgattgcaacgttc

<210> SEQ ID NO 18
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: forward primer

<400> SEQUENCE: 18
cgcgcagcctcgagcagcaactatttcgg

<210> SEQ ID NO 19
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: reverse primer

<400> SEQUENCE: 19
cccgcgtgacgccgcggtactattg

<210> SEQ ID NO 20
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: forward primer

<400> SEQUENCE: 20
cgcgcatcggctgcagaacacgacaacgg

<210> SEQ ID NO 21
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: reverse primer
<400> SEQUENCE: 21
cccgcctcgg ttaccagcgg tagctaatg tt 32

<210> SEQ ID NO 22
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: mutagenesis primer

<400> SEQUENCE: 22
ctctattgc gaagcntgcgct caccatagt tttctatgct g 41

<210> SEQ ID NO 23
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: mutagenesis primer

<400> SEQUENCE: 23
catctatgct aaacattgtg gcacagct gcgtcatagtg g 41

<210> SEQ ID NO 24
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: forward primer

<400> SEQUENCE: 24
cgggatccg ctatcagaca cacttatattc gg 32

<210> SEQ ID NO 25
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: reverse primer

<400> SEQUENCE: 25
cccgcctcgg cagcgcgtca agccttt 26

<210> SEQ ID NO 26
<211> LENGTH: 124
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<221> NAME/KEY: misc_feature
<223> OTHER INFORMATION: forward primer

<400> SEQUENCE: 26
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tggctatgc cttacgca taactcgaag cgcgtagcga caacgcgcac agcccttgacg 120
cgct 124

<210> SEQ ID NO 27
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
  <221> NAME/KEY: misc_feature
  <223> OTHER INFORMATION: reverse primer
<400> SEQUENCE: 27
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<210> SEQ ID NO 28
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
  <221> NAME/KEY: misc_feature
  <223> OTHER INFORMATION: knockout primer
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gctctagag aggctgtcga aacc

<210> SEQ ID NO 29
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<212> TYPE: DNA
<213> ORGANISM: Artificial
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  <223> OTHER INFORMATION: knockout primer
<400> SEQUENCE: 29
tcccccggc ggttgtcgtt tgctg

<210> SEQ ID NO 30
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
  <221> NAME/KEY: misc_feature
  <223> OTHER INFORMATION: knockout primer
<400> SEQUENCE: 30
tcccccggg cggtgctctca ataggc

<210> SEQ ID NO 31
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
  <221> NAME/KEY: misc_feature
  <223> OTHER INFORMATION: knockout primer
<400> SEQUENCE: 31
ccgctcgag cgcaacgcgt gcgtgac

<210> SEQ ID NO 32
<211> LENGTH: 1455
<212> TYPE: PRT
<213> ORGANISM: Neisseria species
<220> FEATURE:
  <221> NAME/KEY: MISC_FEATURE
  <223> OTHER INFORMATION: SEQ ID 650 from WO99/24578
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Lys Thr Gly Arg Ile Arg Phe Ser Pro Ala Tyr Leu Ala Ile Cys Leu
20       25       30
Ser Phe Gly Ile Leu Pro Gln Ala Trp Ala Gly His Thr Tyr Phe Gly
35       40       45
Ile Asn Tyr Gln Tyr Tyr Arg Asp Phe Ala Glu Asn Gly Lys Phe
50       55       60
Ala Val Gly Ala Lys Asp Ile Glu Val Tyr Asn Lys Gly Glu Leu
65       70       75       80
Val Gly Lys Ser Met Thr Lys Ala Pro Met Ile Asp Phe Ser Val Val
85       90       95
Ser Arg Asn Gly Val Ala Ala Leu Val Gly Asp Gln Tyr Ile Val Ser
100      105      110
Val Ala His Asn Gly Gly Tyr Asn Asn Val Asp Phe Gly Ala Glu Gly
115      120      125
Arg Asn Pro Asp Gln His Arg Phe Thr Tyr Lys Ile Val Lys Arg Asn
130      135      140
Asn Tyr Lys Ala Gly Thr Lys Gly His Pro Tyr Gly Asp Tyr His
145      150      155      160
Met Pro Arg Leu His Lys Phe Val Thr Asp Ala Glu Pro Val Glu Met
165      170      175
Thr Ser Tyr Met Asp Gly Arg Lys Tyr Ile Asp Gln Asn Asn Tyr Pro
180      185      190
Asp Arg Val Arg Ile Gly Ala Gly Arg Gln Tyr Trp Arg Ser Asp Glu
195      200      205
Asp Glu Pro Asn Asn Arg Glu Ser Ser Tyr His Ile Ala Ser Ala Tyr
210      215      220
Ser Thr Leu Val Gly Asn Thr Phe Ala Gln Asn Gly Ser Gly Gly
225      230      235      240
Gly Thr Val Asn Leu Gly Ser Glu Lys Ile Lys His Ser Pro Tyr Gly
245      250      255
Phe Leu Pro Thr Gly Ser Phe Gly Asp Ser Gly Ser Pro Met Phe
260      265      270
Ile Tyr Asp Ala Gln Lys Gln Lys Trp Leu Ile Asn Gly Val Leu Gln
275      280      285
Thr Gly Asn Pro Tyr Ile Gly Lys Ser Asn Gly Phe Glu Leu Val Arg
290      295      300
Lys Asp Thr Phe Tyr Asp Glu Ile Phe Ala Gly Asp Thr His Ser Val
305      310      315      320
Phe Tyr Glu Pro Arg Gln Asn Gly Lys Tyr Ser Phe Asp Asp Asn
325      330      335
Asn Gly Thr Gly Lys Ile Asn Ala Lys His Glu His Asn Ser Leu Pro
340      345      350
Asn Arg Leu Lys Thr Arg Thr Val Glu Leu Phe Asn Val Ser Leu Ser
355      360      365
Glu Thr Ala Arg Glu Pro Val Tyr His Ala Ala Gly Gly Val Asn Ser
370      375      380
Tyr Arg Pro Arg Leu Asn Asn Gly Asn Ile Ser Phe Ile Asp Glu
385      390      395      400
Gly Lys Gly Glu Leu Ile Leu Thr Ser Asn Ile Asn Glu Gly Ala Gly
405      410      415
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Gly Leu Tyr Phe Gln Gly Asp Phe Thr Val Ser Pro glu Asn Asn Glu
420 425 430
Thr Trp Gln Gly Ala Gly Val His Ile Ser Glu Asp Ser Thr Val Thr
435 440 445
Trp Lys Val Arg Gly Val Ala Asp Arg Leu Ser Lys Ile Gly Lys
450 455 460
Gly Thr Leu His Val Gln Ala Lys Gly Glu Asn Glu Gly Ser Ile Ser
465 470 475 480
Val Gly Asp Gly Thr Val Ile Leu Asp Gin Gin Ala Asp Asp Lys Gly
485 490 495
Lys Lys Gin Ala Phe Ser Glu Ile Gly Leu Val Ser Gly Arg Gly Thr
500 505 510
Val Gin Leu Asn Ala Asp Asn Gin Phe Asn Pro Asp Lys Leu Tyr Phe
515 520 525
Gly Phe Arg Gly Gly Arg Leu Asp Leu Asn Gly His Ser Leu Ser Phe
530 535 540
His Arg Ile Gin Asn Thr Asp Glu Gly Ala Met Ile Val Asn Gin Gin
545 550 555 560
Gln Asp Lys Glu Ser Thr Val Thr Ile Thr Gly Asn Asp Ile Ala
565 570 575
Thr Thr Gly Asn Asn Ser Leu Arg Ser Lys Lys Glu Ile Ala Tyr
580 585 590
Asn Gly Trp Phe Gly Glu Lys Asp Thr Thr Lys Thr Gin Gly Arg Leu
595 600 605
Asn Leu Val Tyr Gin Pro Ala Ala Glu Asp Arg Thr Leu Leu Ser
610 615 620
Gly Gly Thr Gin Leu Asn Gly Gin Ile Thr Gin Thr Gin Gly Lys Leu
625 630 635 640
Phe Phe Ser Gly Arg Pro Thr Pro His Ala Tyr Gin His Leu Asn Asp
645 650 655
His Trp Ser Gin Lys Glu Gly Ile Pro Arg Gly Glu Ile Val Trp Asp
660 665 670
Asn Asp Trp Ile Asn Arg Thr Phe Lys Ala Glu Asn Phe Gin Ile Lys
675 680 685
Gly Gly Gin Ala Trp Ser Arg Asn Val Ala Lys Val Lys Gly Asp Trp
690 695 700
His Leu Ser Asn His Ala Gin Ala Val Phe Gly Val Ala Pro His Gin
705 710 715 720
Ser His Thr Ile Cys Thr Arg Ser Asp Thr Thr Gly Leu Thr Asn Cys
725 730 735
Val Glu Lys Thr Ile Thr Asp Asp Lys Val Ile Ala Ser Leu Thr Lys
740 745 750
Thr Asp Ile Ser Gly Asn Val Asp Leu Ala Asp His Ala His Leu Asn
755 760 765
Leu Thr Gly Leu Ala Thr Leu Asn Gly Asn Leu Ser Ala Asn Gly Asp
770 775 780
Thr Arg Tyr Thr Val Ser His Asn Ala Thr Gin Asn Gly Asn Leu Ser
785 790 795 800
Leu Val Gly Asn Ala Gin Ala Thr Phe Asn Gin Ala Thr Leu Asn Gly
805 810 815
Asn Thr Ser Ala Ser Gly Asn Ala Ser Phe Asn Leu Ser Asp His Ala
Val Ctn Asn Gly Ser Leu Thr Leu Ser Gly Asn Ala Lys Ala Asn Val
835 840 845
Ser His Ser Ala Leu Asn Gly Asn Val Ser Leu Ala Asp Lys Ala Val
850 855 860
Phe His Phe Glu Ser Ser Arg Phe Thr Gly Gin Ile Ser Gly Gly Lys
865 870 875 880
Asp Thr Ala Leu His Leu Lys Asp Ser Glu Trp Thr Leu Pro Ser Gly
885 890 895
Thr Glu Leu Gly Asn Leu Asn Ala Thr Ile Thr Leu Asn
900 905 910
Ser Ala Tyr Arg His Asp Ala Ala Gly Ala Gin Thr Gly Ser Ala Thr
915 920 925
Asp Ala Pro Arg Arg Ser Arg Arg Ser Arg Arg Ser Leu Leu Ser
930 935 940
Val Thr Pro Pro Thr Ser Val Glu Ser Arg Phe Asn Thr Leu Thr Val
945 950 955 960
Asn Gly Lys Leu Asn Gly Gin Gly Thr Phe Arg Phe Met Ser Glu Leu
965 970 975
Phe Gly Tyr Arg Ser Asp Lys Leu Leu Ala Glu Ser Ser Glu Gly
980 985 990
Thr Tyr Thr Leu Ala Val Asn Ser Thr Gly Gin Glu Pro Ala Ser Leu
995 1000 1005
Glu Gin Leu Thr Trp Glu Gly Lys Asp Arg Asn Arg Leu Ser Glu
1010 1015 1020
Asn Leu Asn Phe Thr Leu Gin Asn Glu His Val Asp Ala Gly Ala
1025 1030 1035
Trp Arg Tyr Gin Leu Ile Arg Lys Asp Gly Glu Phe Arg Leu His
1040 1045 1050
Asn Pro Val Lys Glu Gin Glu Leu Ser Asp Lys Lys Leu Gly Lys Ala
1055 1060 1065
Glu Ala Lys Lys Gin Ala Glu Lys Asp Arg Ala Gin Ser Leu Asp
1070 1075 1080
Ala Leu Ile Ala Ala Gly Arg Asp Ala Val Glu Lys Thr Glu Ser
1095 1099
Val Ala Glu Pro Ala Arg Gin Ala Gly Gly Glu Asn Val Gly Ile
1100 1105 1110
Met Gin Ala Glu Glu Gly Lys Lys Arg Val Gin Ala Asp Lys Asp
1115 1120 1125
Thr Ala Leu Ala Lys Gin Arg Glu Ala Glu Thr Arg Pro Ala Thr
1130 1135 1140
Thr Ala Phe Pro Arg Ala Arg Arg Ala Arg Arg Asp Leu Pro Gin
1145 1150 1155
Leu Gin Pro Gin Pro Gin Pro Gin Pro Gin Pro Gin Arg Asp Leu Ile Ser
1160 1165 1170
Arg Tyr Ala Asn Ser Gly Leu Ser Glu Phe Ser Ala Thr Leu Asn
1175 1180 1185
Ser Val Phe Ala Val Gin Asp Glu Leu Asp Arg Val Phe Ala Glu
1190 1195 1200
Asp Arg Arg Asn Ala Val Trp Thr Ser Gly Ile Arg Asp Thr Lys
1205 1210 1215
His Tyr Arg Ser Gln Asp Phe Arg Ala Tyr Arg Gln Gln Thr Asp  
1 1220 1225 1230
Leu Arg Gln Ile Gly Met Gln Lys Asn Leu Gly Ser Gly Arg Val  
1235 1240 1245
Gly Ile Leu Phe Ser His Asn Arg Thr Glu Asn Thr Phe Asp Asp  
1250 1255 1260
Gly Ile Gly Asn Ser Ala Arg Leu Ala His Gly Ala Val Phe Gly  
1265 1270 1275
Gln Tyr Gly Ile Asp Arg Phe Tyr Ile Gly Ile Ser Ala Gly Ala  
1280 1285 1290
Gly Phe Ser Ser Gly Ser Leu Ser Asp Gly Ile Gly Gly Lys Ile  
1295 1300 1305
Arg Arg Arg Val Leu His Tyr Gly Ile Gln Ala Arg Tyr Arg Ala  
1310 1315 1320
Gly Phe Gly Gly Phe Gly Ile Glu Pro His Ile Gly Ala Thr Arg  
1325 1330 1335
Tyr Phe Val Gln Lys Ala Asp Tyr Arg Tyr Glu Asn Val Asn Ile  
1340 1345 1350
Ala Thr Pro Gly Leu Ala Phe Asn Arg Tyr Arg Ala Gly Ile Lys  
1355 1360 1365
Ala Asp Tyr Ser Phe Lys Pro Ala Gln His Ile Ser Ile Thr Pro  
1370 1375 1380
Tyr Leu Ser Leu Ser Tyr Thr Asp Ala Ala Ser Gly Lys Val Arg  
1385 1390 1395
Thr Arg Val Asn Thr Ala Val Leu Ala Gln Asp Phe Gly Lys Thr  
1400 1405 1410
Arg Ser Ala Glu Trp Gly Val Asn Ala Glu Ile Lys Gly Phe Thr  
1415 1420 1425
Leu Ser Leu His Ala Ala Ala Lys Gly Pro Gln Leu Glu Ala  
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Gln His Ser Ala Gly Ile Lys Leu Gly Tyr Arg Trp  
1445 1450 1455

<210> SEQ ID NO 33
<211> LENGTH: 956
<212> TYPE: PRT
<213> ORGANISM: Neisseria species
<220> FEATURE:
<221> NAME/KEY: MISC_FEATURE
<223> OTHER INFORMATION: App domain derivative

<400> SEQUENCE: 33
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Lys Thr Gly Arg Ile Arg Phe Ser Pro Ala Tyr Leu Ala Ile Cys Leu  
20 25 30
Ser Phe Gly Ile Leu Pro Gin Ala Trp Ala Gly His Thr Tyr Phe Gly  
35 40 45
Ile Asn Tyr Gln Tyr Tyr Arg Asp Phe Ala Glu Asn Lys Gly Lys Phe  
50 55 60
Ala Val Gly Ala Lys Asp Ile Glu Val Tyr Asn Lys Lys Gly Glu Leu  
65 70 75 80
Val Gly Lys Ser Met Thr Lys Ala Pro Met Ile Asp Phe Ser Val Val
95  90  95
Ser Arg Asn Gly Val Ala Ala Leu Val Gly Asp Gln Tyr Ile Val Ser
100 105 110
Val Ala His Asn Gly Tyr Asn Asn Val Asp Phe Gly Ala G1y
115 120 125
Arg Asn Pro Asp Gln His Arg Phe Thr Tyr Lys Ile Val Lys Arg Asn
130 135 140
Asn Tyr Lys Ala Gly Thr Lys Gly His Pro Tyr Gly Gly Asp Tyr His
145 150 155 160
Met Pro Arg Leu His Lys Phe Val Thr Asp Ala Glu Pro Val Glu Met
165 170 175
Thr Ser Tyr Met Asp Gly Arg Tyr Ile Asp Gln Asn Asn Tyr Pro
180 185 190
Asp Arg Val Arg Ile Gly Ala Gly Arg Gln Tyr Trp Arg Ser Asp Glu
195 200 205
Asp Glu Pro Arg Asn Asn Arg Glu Ser Ser Tyr His Ile Ala Ser Ala Tyr
210 215 220
Ser Trp Leu Val Gly Asn Thr Phe Ala Gln Asn Gly Ser Gly Gly
225 230 235 240
Gly Thr Val Asn Leu Gly Ser Glu Lys Ile Lys His Ser Pro Tyr Gly
245 250 255
Phe Leu Pro Thr Gly Ser Phe Gly Asp Ser Gly Ser Pro Met Phe
260 265 270
Ile Tyr Asp Ala Glu Lys Glu Gly Trp Leu Ile Asn Gly Val Leu Glu
275 280 285
Thr Gly Asn Pro Tyr Ile Gly Lys Ser Asn Gly Phe Gln Leu Val Arg
290 295 300
Lys Asp Trp Phe Tyr Asp Glu Ile Phe Ala Gly Asp Thr His Ser Val
305 310 315 320
Phe Tyr Glu Pro Arg Gln Asn G1y Lys Tyr Ser Phe Asn Asp Asp Asn
325 330 335
Asn Gly Thr Gly Lys Ile Asn Ala Lys His Glu His Asn Ser Leu Pro
340 345 350
Asn Arg Leu Lys Thr Arg Thr Val Gln Leu Phe Asn Val Ser Leu Ser
355 360 365
Glu Thr Ala Arg Glu Pro Val Tyr His Ala Ala Gly Gly Val Asn Ser
370 375 380
Tyr Arg Pro Arg Leu Asn Asn Gly Asn Ile Ser Phe Ile Asp Glu
385 390 395 400
Gly Lys Gly Glu Leu Ile Leu Thr Ser Asn Ile Asn Gln Gly Ala Gly
405 410 415
Gly Leu Tyr Phe Glu Gly Asp Phe Thr Val Ser Pro Glu Asn Asn Glu
420 425 430
Thr Trp Gln Gly Ala Gly Val His Ile Ser Glu Asp Ser Thr Val Thr
435 440 445
Trp Lys Val Asn Gly Val Ala Asp Arg Leu Ser Lys Ile Gly Lys
450 455 460
Gly Thr Leu His Val Gln Ala Lys Gly Glu Asn Glu Gly Ser Ile Ser
465 470 475 480
Val Gly Asp Gly Thr Val Ile Leu Asp Glu Gln Ala Asp Asp Lys Gly
485 490 495
Lys Lys Gln Ala Phe Ser Glu Ile Gly Leu Val Ser Gly Arg Gly Thr 500 505 510
Val Gln Leu Asn Ala Asp Asn Gln Phe Asn Pro Asp Lys Leu Tyr Phe 515 520 525
Gly Phe Arg Gly Gly Arg Leu Asp Leu Asn Gly His Ser Leu Ser Phe 530 535 540
His Arg Ile Gln Thr Asp Gly Ala Met Ile Val Asn His Asn 545 550 555 560
Gln Asp Lys Glu Ser Thr Val Thr Lys Gly Lys Asp Ile Ala 565 570 575
Thr Thr Gly Asn Asn Asn Ser Asp Ser Lys Lys Gly Ala Tyr 580 585 590
Asn Gly Trp Phe Gly Glu Lys Asp Thr Thr Lys Thr Asn Gly Arg Leu 595 600 605
Asn Leu Val Tyr Gln Pro Ala Ala Glu Asp Arg Thr Leu Leu Ser 610 615 620
Gly Gly Thr Asn Leu Asn Gly Asn Ile Thr Gin Thr Asn Gly Lys Leu 625 630 635 640
Phe Phe Ser Gly Arg Pro Thr Pro His Ala Tyr Asn His Leu Asn Asp 645 650 655
His Trp Ser Gin Lys Glu Gly Ile Pro Arg Gly Glu Ile Val Trp Asp 660 665 670
Asn Asp Trp Ile Asn Arg Thr Phe Lys Ala Glu Asp Phe Gin Ile Lys 675 680 685
Gly Gly Gln Ala Val Val Ser Arg Asn Val Ala Lys Val Lys Gly Asp 690 695 700
Trp His Leu Ser Asn His Ala Gin Ala Val Phe Gly Val Ala Pro His 705 710 715 720
Gln Ser His Thr Ile Cys Thr Arg Ser Asp Trp Thr Gly Leu Thr Asn 725 730 735
Cys Val Glu Lys Thr Ile Thr Asp Asp Lys Val Ile Ala Ser Leu Thr 740 745 750
Lys Thr Asp Ile Ser Gly Asn Val Asp Leu Ala Asp His Ala His Leu 755 760 765
Asn Leu Thr Gly Leu Ala Thr Leu Asn Gly Asn Leu Ser Ala Asn Gly 770 775 780 785
Asp Thr Arg Tyr Thr Val Ser His Asn Ala Thr Gin Asn Gly Asn Leu 785 790 795 800
Ser Leu Val Gly Asn Ala Gin Ala Thr Phe Asn Gin Ala Thr Leu Asn 805 810 815
Gly Asn Thr Ser Ala Ser Gly Asn Ala Ser Phe Asn Leu Ser Asp His 820 825 830
Ala Val Gin Asn Gly Ser Leu Thr Leu Ser Gly Asn Ala Lys Ala Ann 835 840 845
Val Ser His Ser Ala Leu Gin Ala Thr Val Ser Leu Ala Asp Lys Ala 850 855 860
Val Phe His Phe Glu Ser Ser Arg Phe Thr Gly Gin Ile Ser Gly Gly 865 870 875 880
Lys Asp Thr Ala Leu His Leu Lys Asp Ser Glu Thr Thr Leu Pro Ser 885 890 895
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SEQ ID NO: 34
LENGTH: 1178
TYPE: PRT
ORGANISM: Neisseria species
FEATURE:
NAME/KEY: MISC_FEATURE
OTHER INFORMATION: App domain derivative

SEQUENCE: 34

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Ile Asp Phe Ser Val Ser Gin Gin Val Gin Gin Gin Val Gin Gin Gin Val

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Gln Thr Asn Gly Lys Leu Phe Phe Ser Gly Arg Pro Thr Pro His Ala 595 600 605
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Gly Glu Ile Val Trp Asp Asn Asp Trp Ile Asn Arg Thr Phe Lys Ala 625 630 635 640
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 Ala Lys Val Lys Gly Asp Trp His Leu Ser Asn His Ala Gln Ala Val 660 665 670
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Gly Gin Ile Ser Gly Gly Lys Asp Thr Ala Leu His Leu Lys Asp Ser 835 840 845
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<223> OTHER INFORMATION: App domain derivative

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 35  40  45
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 65  70  75  80
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210  215  220

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Glu Ser Ser Glu Gly Thr Tyr Thr Leu Ala Val Asn Asn Thr Gin Gin
35  40  45

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Lys Pro Leu Ser Glu Asn Leu Asn Phe Thr Leu Gin Asn Glu His Val
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Arg Leu His Asn Pro Val Lys Gin Gin Leu Ser Asp Lys Leu Gin
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130  135  140

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145  150  155  160

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165  170  175

Leu Ala Lys Gin Arg Glu Ala Glu Thr Arg Pro Ala Thr Thr Ala Phe
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taataataa aataaataa ttgcccccaat gtattgata tatggctcct ttcatatata 180
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<210> SEQ ID NO 43
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<223> OTHER INFORMATION: N. meningitidis

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<223> OTHER INFORMATION: N. meningitidis

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Leu

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1. An isolated antibody that specifically binds to NMB1994, wherein NMB1994 comprises residues 24-343 of SEQ ID NO:2, or residues 24-350 of SEQ ID NO:3.

2. The antibody of claim 1, wherein said antibody is a polyclonal antibody.

3. The antibody of claim 1, wherein said antibody is a monoclonal antibody.

4. The antibody of claim 1, wherein said antibody specifically binds to said NMB1994 comprising residues 24-343 of SEQ ID NO:2.

5. The antibody of claim 1, wherein said antibody specifically binds to said NMB1994 comprising residues 24-350 of SEQ ID NO:3.

6. The antibody of claim 1, wherein said antibody is able to bind to a bacterium of a Neisseria meningitidis strain that expresses NadA.

7. The antibody of claim 1, wherein said antibody is able to induce complement-mediated killing of a Neisseria meningitidis strain that expresses NadA.

8. The antibody of claim 7, wherein said strain is selected from the group consisting of 2996, C11, F6124 and MC58.

9. The antibody of claim 1, wherein said antibody is able to reduce bacteremia in a subject exposed to a Neisseria meningitidis strain that expresses NadA.

10. A method, comprising:
    administering an isolated antibody that specifically binds to NMB1994 to a subject,
    wherein NMB1994 comprises residues 24-343 of SEQ ID NO:2, or residues 24-350 of SEQ ID NO:3.

11. The method of claim 10, wherein said subject has been exposed to a Neisseria meningitidis strain that expresses NadA.

12. The method of claim 11, wherein said administering reduces bacteremia.

13. The method of claim 10, wherein said antibody specifically binds to said NMB1994 comprising residues 24-343 of SEQ ID NO:2.

14. The method of claim 10, wherein said antibody specifically binds to said NMB1994 comprising residues 24-350 of SEQ ID NO:3.

15. A method, comprising:
    contacting a bacterium of a Neisseria meningitidis strain that expresses NadA with an isolated antibody that specifically binds to NMB1994, wherein NMB1994 comprises residues 24-343 of SEQ ID NO:2, or residues 24-350 of SEQ ID NO:3.

16. The method of claim 15, wherein said antibody binds to said bacterium.

17. The method of claim 16, wherein the ability of said bacterium to bind to an epithelial cell is blocked when bound to said antibody.

18. A method for preventing the attachment of a Neisseria meningitidis cell to an epithelial cell through NMB1994 mediated adhesion in a human subject by administering to the human subject an antibody that specifically binds to NMB1994, wherein NMB1994 comprises residues 24-343 of SEQ ID NO:2 or residues 24-350 of SEQ ID NO:3, and wherein the antibody inhibits NMB1994’s ability to bind to the epithelial cell.


* * * * *