HYBRID EXPRESSION OF NEISSERIAL PROTEINS

Two or more Neisserial proteins (e.g. A and B) are expressed as a single hybrid protein which can be represented simply by the formula NH₂-A-B-COOH.
HYBRID EXPRESSION OF NEISSERIAL PROTEINS

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

TECHNICAL FIELD

This invention is in the field of protein expression. In particular, it relates to the heterologous expression of proteins from *Neisseria* (*e.g.* *N.gonorrhoeae* or, preferably, *N.meningitidis*).

BACKGROUND ART

International patent applications WO99/24578, WO99/36544, WO99/57280 and WO00/22430 disclose proteins from *Neisseria meningitidis* and *Neisseria gonorrhoeae*. These proteins are typically described as being expressed in *E.coli* (*i.e.* heterologous expression) as either N-terminal GST-fusions or C-terminal His-tag fusions, although other expression systems, including expression in native *Neisseria*, are also disclosed.

It is an object of the present invention to provide alternative and improved approaches for the heterologous expression of these proteins. These approaches will typically affect the level of expression, the ease of purification, the cellular localisation of expression, and/or the immunological properties of the expressed protein.

DISCLOSURE OF THE INVENTION

In accordance with the invention, two or more (*e.g.* 3, 4, 5, 6 or more) proteins of the invention are expressed as a single hybrid protein. It is preferred that no non-Neisserial fusion partner (*e.g.* GST or poly-His) is used.

This offers two advantages. Firstly, a protein that may be unstable or poorly expressed on its own can be assisted by adding a suitable hybrid partner that overcomes the problem. Secondly, commercial manufacture is simplified – only one expression and purification need be employed in order to produce two separately-useful proteins.

Thus the invention provides a method for the simultaneous heterologous expression of two or more proteins of the invention, in which said two or more proteins of the invention are fused (*i.e.* they are translated as a single polypeptide chain).

The method will typically involve the steps of: obtaining a first nucleic acid encoding a first protein of the invention; obtaining a second nucleic acid encoding a second protein of the
invention; ligating the first and second nucleic acids. The resulting nucleic acid may be inserted into an expression vector, or may already be part of an expression vector.

Where just two proteins are joined, the hybrid protein can be represented simply by the formula NH₂-A—B-COOH. A and B can each be selected from any Neisserial proteins, and in particular those represented by SEQ#s 1-4326. The method is well suited to the expression of proteins orf1, orf4, orf25, orf40, Orf46/46.1, orf83, 233, 287, 292L, 564, 687, 741, 907, 919, 953, 961 and 983.

The 42 hybrids indicated by ‘X’ in the following table of form NH₂-A—B-COOH are preferred:

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>Orf46.1</th>
<th>287</th>
<th>741</th>
<th>919</th>
<th>953</th>
<th>961</th>
<th>983</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF46.1</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>287</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>741</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>919</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>953</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>961</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>983</td>
<td></td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

Preferred proteins to be expressed as hybrids are thus ORF46.1, 287, 741, 919, 953, 961 and 983. These may be used in their essentially full-length form, or poly-glycine deletions (ΔG) forms may be used (e.g. ΔG-287, ΔGTbp2, ΔG741, ΔG983 etc.), or truncated forms may be used (e.g. Δ1-287, Δ2-287 etc.), or domain-deleted versions may be used (e.g. 287B, 287C, 287BC, ORF46.1-433, ORF46.433-608, ORF46, 961 etc.) and so on.

Particularly preferred are: (a) a hybrid protein comprising 919 and 287; (b) a hybrid protein comprising 953 and 287; (c) a hybrid protein comprising 287 and ORF46.1; (d) a hybrid protein comprising ORF1 and ORF46.1; (e) a hybrid protein comprising 919 and ORF46.1; (f) a hybrid protein comprising ORF46.1 and 919; (g) a hybrid protein comprising ORF46.1, 287 and 919; (h) a hybrid protein comprising 919 and 519; and (i) a hybrid protein comprising ORF97 and 225.

Further embodiments are shown in the drawings and include ΔG287-919, ΔG287-953, ΔG287-961, ΔG983-ORF46.1, ΔG983-741, ΔG983-961, ΔG983-961C, ΔG741-961, ΔG741-961C, ΔG741-983, ΔG741-ORF46.1, ORF46.1-741, ORF46.1-961, ORF46.1-961C,
961-ORF46.1, 961-741, 961-983, 961C-ORF46.1, 961C-741, 961C-983, 961CL-ORF46.1, 961CL-741, and 961CL-983.

Where 287 is used, it is preferably at the C-terminal end of a hybrid; if it is to be used at the N-terminus, if is preferred to use a ΔG form of 287 is used (e.g. as the N-terminus of a hybrid with ORF46.1, 919, 953 or 961).

Where 287 is used, this is preferably from strain 2996 or from strain 394/98.

Where 961 is used, this is preferably at the N-terminus. Domain forms of 961 may be used.

Alignments of polymorphic forms of ORF46, 287, 919 and 953 are disclosed in WO00/66741. Any of these polymorphs can be used according to the present invention.

Preferably, the constituent proteins (A and B) in a hybrid protein according to the invention will be from the same strain.

The fused proteins in the hybrid may be joined directly, or may be joined via a linker peptide e.g. via a poly-glycine linker (i.e. Gₙ where n = 3, 4, 5, 6, 7, 8, 9, 10 or more) or via a short peptide sequence which facilitates cloning. It is evidently preferred not to join a ΔG protein to the C-terminus of a poly-glycine linker.

The fused proteins may lack native leader peptides or may include the leader peptide sequence of the N-terminal fusion partner.

Host

It is preferred to utilise a heterologous host. The heterologous host may be prokaryotic or eukaryotic. It is preferably E.coli, but other suitable hosts include Bacillus subtilis, Vibrio cholerae, Salmonella typhi, Salmonella typhimurium, Neisseria meningitidis, Neisseria gonorrhoeae, Neisseria lactamica, Neisseria cinerea, Mycobacteria (e.g. M.tuberculosis), yeast etc.

Vectors, hosts etc.

As well as the methods described above, the invention provides (a) nucleic acid and vectors useful in these methods (b) host cells containing said vectors (c) proteins expressed or expressable by the methods (d) compositions comprising these proteins, which may be suitable as vaccines, for instance, or as diagnostic reagents, or as immunogenic compositions (e) these compositions for use as medicaments (e.g. as vaccines) or as diagnostic reagents (f)
the use of these compositions in the manufacture of (1) a medicament for treating or preventing infection due to Neisseria bacteria (2) a diagnostic reagent for detecting the presence of Neisseria bacteria or of antibodies raised against Neisseria bacteria, and/or (3) a reagent which can raise antibodies against Neisseria bacteria and (g) a method of treating a patient, comprising administering to the patient a therapeutically effective amount of these compositions.

Sequences

The invention also provides a protein or a nucleic acid having any of the sequences set out in the following examples. It also provides proteins and nucleic acid having sequence identity to these. As described above, the degree of ‘sequence identity’ is preferably greater than 50% (eg. 60%, 70%, 80%, 90%, 95%, 99% or more).

Nomenclature herein

The 2166 protein sequences disclosed in WO99/24578, WO99/36544 and WO99/57280 are referred to herein by the following SEQ# numbers:

<table>
<thead>
<tr>
<th>Application</th>
<th>Protein sequences</th>
<th>SEQ# herein</th>
</tr>
</thead>
<tbody>
<tr>
<td>WO99/24578</td>
<td>Even SEQ IDs 2-892</td>
<td>SEQ#s 1-446</td>
</tr>
<tr>
<td>WO99/36544</td>
<td>Even SEQ IDs 2-90</td>
<td>SEQ#s 447-491</td>
</tr>
<tr>
<td>WO99/57280</td>
<td>Even SEQ IDs 2-3020</td>
<td>SEQ#s 492-2001</td>
</tr>
<tr>
<td></td>
<td>Even SEQ IDs 3040-3114</td>
<td>SEQ#s 2002-2039</td>
</tr>
<tr>
<td></td>
<td>SEQ IDs 3115-3241</td>
<td>SEQ#s 2040-2166</td>
</tr>
</tbody>
</table>

In addition to this SEQ# numbering, the naming conventions used in WO99/24578, WO99/36544 and WO99/57280 are also used (e.g. ‘ORF4’, ‘ORF40’, ‘ORF40-1’ etc. as used in WO99/24578 and WO99/36544; ‘m919’, ‘g919’ and ‘a919’ etc. as used in WO99/57280).

The 2160 proteins NMB0001 to NMB2160 from Tettelin et al. [Science (2000) 287:1809-1815] are referred to herein as SEQ#s 2167-4326 [see also WO00/66791].

The term ‘protein of the invention’ as used herein refers to a protein comprising:

(a) one of sequences SEQ#s 1-4326; or
(b) a sequence having sequence identity to one of SEQ#s 1-4326; or
(c) a fragment of one of SEQ#s 1-4326.
The degree of 'sequence identity' referred to in (b) is preferably greater than 50% (e.g. 60%, 70%, 80%, 90%, 95%, 99% or more). This includes mutants and allelic variants [e.g. see WO00/66741]. Identity is preferably determined by the Smith-Waterman homology search algorithm as implemented in the MPSRCH 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.

The 'fragment' referred to in (c) should comprise at least n consecutive amino acids from one of SEQ#s 1-4326 and, depending on the particular sequence, n 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 of SEQ#s 1-4326. Preferred fragments are those disclosed in WO00/71574 and WO01/04316.

Preferred proteins of the invention are found in N.meningitidis serogroup B.

Preferred proteins for use according to the invention are those of serogroup B N.meningitidis strain 2996 or strain 394/98 (a New Zealand strain). Unless otherwise stated, proteins mentioned herein are from N.meningitidis strain 2996. It will be appreciated, however, that the invention is not in general limited by strain. References to a particular protein (e.g. ‘287’, ‘919’ etc.) may be taken to include that protein from any strain.

It will be appreciated that references to “nucleic acid” includes DNA and RNA, and also their analogues, such as those containing modified backbones, and also peptide nucleic acids (PNA) etc.

**BRIEF DESCRIPTION OF DRAWINGS**

Figures 1 to 26 show hybrid proteins according to the invention.

**MODES FOR CARRYING OUT THE INVENTION**

**Example 1 – hybrids of ORF46**

The complete ORF46 protein from *N.meningitidis* (serogroup B, strain 2996) has the following sequence:

1. LQISRKLSILASLAVCLPMHANASDLANDSFTRQVLDRQHEPEDFKXYHL
2. PFSRGIHAERSHHGIGKIQSHQLNMLTQQAIAKINSYIVRFSDFHYHR
3. VHSPFDPNAS HDSDSDEAGSP VDGFLYR1H WDGEHHHPAD GYDGPOGGY
The leader peptide is underlined.

The sequences of ORF46 from other strains can be found in WO00/66741.

ORF46 has been fused at its C-terminus and N-terminus with 287, 919, and ORF1. The hybrid proteins were generally insoluble, but gave some good ELISA and bactericidal results (against the homologous 2996 strain):

<table>
<thead>
<tr>
<th>Protein</th>
<th>ELISA</th>
<th>Bactericidal Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orf1-Orf46.1-His</td>
<td>850</td>
<td>256</td>
</tr>
<tr>
<td>919-Orf46.1-His</td>
<td>12900</td>
<td>512</td>
</tr>
<tr>
<td>919-287-Orf46-His</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Orf46.1-287His</td>
<td>150</td>
<td>8192</td>
</tr>
<tr>
<td>Orf46.1-919His</td>
<td>2800</td>
<td>2048</td>
</tr>
<tr>
<td>Orf46.1-287-919His</td>
<td>3200</td>
<td>16384</td>
</tr>
</tbody>
</table>

For comparison, ‘triple’ hybrids of ORF46.1, 287 (either as a GST fusion, or in ΔG287 form) and 919 were constructed and tested against various strains (including the homologous 2996 strain) versus a simple mixture of the three antigens. FCA was used as adjuvant:

<table>
<thead>
<tr>
<th>Mixture</th>
<th>2996</th>
<th>BZ232</th>
<th>MC58</th>
<th>NGH38</th>
<th>F6124</th>
<th>2996</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orf46.1-287-919His</td>
<td>16384</td>
<td>256</td>
<td>512</td>
<td>1024</td>
<td>&gt;2048</td>
<td>&gt;2048</td>
</tr>
<tr>
<td>ΔG287-919-ORF46.1His</td>
<td>8192</td>
<td>64</td>
<td>4096</td>
<td>8192</td>
<td>8192</td>
<td>8192</td>
</tr>
<tr>
<td>ΔG287-ORF46.1-919His</td>
<td>4096</td>
<td>128</td>
<td>512</td>
<td>8192</td>
<td>16384</td>
<td>1024</td>
</tr>
</tbody>
</table>

Again, the hybrids show equivalent or superior immunological activity.

Hybrids of two proteins (strain 2996) were compared to the individual proteins against various heterologous strains:

<table>
<thead>
<tr>
<th>ORF46.1-His</th>
<th>1000</th>
<th>MC58</th>
<th>F6124 (MenA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ORF1-His</td>
<td>8</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td>ORF1—ORF46.1-His</td>
<td>1024</td>
<td>512</td>
<td>1024</td>
</tr>
</tbody>
</table>

Again, the hybrid shows equivalent or superior immunological activity.
Example 2 – hybrids of ΔG287

The deletion of the (Gly)₆ sequence in 287 was found to have a dramatic effect on protein expression. The protein lacking the N-terminal amino acids up to GGGGGG is called ‘ΔG287’. In strain MC58, its basic sequence (leader peptide underlined) is:

ΔG287, with or without His-tag (‘ΔG287-His’ and ‘ΔG287K’, respectively), are expressed at very good levels in comparison with the ‘287-His’ or ‘287 untagged’.

On the basis of gene variability data, variants of ΔG287-His were expressed in E.coli from a number of MenB strains, in particular from strains 2996, MC58, 1000, and BZ232. The results were also good – each of these gave high ELISA titres and also serum bactericidal titres of >8192. ΔG287K, expressed from pET-24b, gave excellent titres in ELISA and the serum bactericidal assay.

Deletion of poly-Gly sequences is also applicable to Tbp2 (NMB0460), 741 (NMB 1870) and 983 (NMB1969). When cloned in pET vector and expressed in E.coli without the sequence coding for their leader peptides and without poly-Gly (i.e. as “ΔG forms”), the same effect was seen – expression was good in the clones carrying the deletion of the poly-glycine stretch, and poor or absent if the glycines were present in the expressed protein.

ΔG287 was fused directly in-frame upstream of 919, 953, 961 (sequences shown below) and ORF46.1:
The bactericidal efficacy (homologous strain) of antibodies raised against the hybrid proteins was compared with antibodies raised against simple mixtures of the component antigens (using 287-GST) for 919 and ORF46.1:

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th>Bactericidal</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔG287-953-His</td>
<td>3834</td>
<td>65536</td>
</tr>
<tr>
<td>ΔG287-961-His</td>
<td>108627</td>
<td>65536</td>
</tr>
</tbody>
</table>

Data for bactericidal activity against heterologous MenB strains and against serotypes A and C were also obtained:

<table>
<thead>
<tr>
<th></th>
<th>919</th>
<th>ORF46.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix</td>
<td>Mixture</td>
<td>Hybrid</td>
</tr>
<tr>
<td>919</td>
<td>32000</td>
<td>128000</td>
</tr>
<tr>
<td>ORF46.1</td>
<td>128</td>
<td>-</td>
</tr>
</tbody>
</table>

The hybrid proteins with ΔG287 at the N-terminus are therefore immunologically superior to simple mixtures, with ΔG287-ORF46.1 being particularly effective, even against heterologous strains. ΔG287-ORF46.1K may be expressed in pET-24b.

The same hybrid proteins were made using New Zealand strain 394/98 rather than 2996:

```
ΔG287NZ-919
ATGCTTGACGCACGATGTCAGTGCGACACGTGCTCAAACCTGCGCACCTGTCAGCTCTTCTTTCTGACAAAGAGACAGAG
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Example 3 – hybrids of ΔG983

Protein 983 has the following sequence:

```
983  =>  ΔG983
5   1  MRTTPFTPTK  TFRTAAMALAA  VATTLSACLG  GGGGTSAPD  FNAGOTGIGS
51  2  NSRATTASKA  AVSAYGNKNE  MCKDRSMLCA  GRDADVYTRD  DAKINAPPF
101 3  LIHFDPFNPW  DAYXNLINLK  PATAGAYTG  QGWGIVQDVG  DSGVISFPP
151 4  LTHQGHEGNY  ENYNTXAYMK  RKEADPDGGG  KDIBASDPEDE  AVHEEAKPT
201 5  DIHRVKEIGHY  IDLWNYIHGG  RSVQGQPSGG  IAPDALTLMH  MTNDEKTNEM
251 6  MVAARNWAV  KLGORGVIV  NNSFGTTSRRA  GTADLFQIAN  SEEQYRQALL
301 7  DYSGQKDTDE  GRLMMQSDY  GNLSYHINRK  NMLFIFSTGN  DAQAPQNYTA
351 8  LLFRFYEKDAQ  KGIIITVAGVD  RSGEKFKREM  GYGEPTQTELIE  YGSGNSCIGTA
401 9  MWCILAEA YEVA SVFRFTNPI  QIAIGTFSSAP  IVTGTAAAL  LQKYQPSNNMDN
451 10 LARKTLTQAT  DIGAVGVDSK  FGHWLDLQD  AMQGAPSFPP  GBTADTKGOT
501 11 SDIAIFSRND  ISEQGGFLRKL  GQSLQRGHLN  NTTYKTIIE  GSGSLVGSNN
551 12 KSRMRRVMTG  ALINYGAAGG  GSNLSQDVAYV  LADTDQQSGAN  ETIVHIGSLQ
601 13 LDQKGTLYTR  LGKLLKDVGTT  AIIGKGLYMS  ARBGKAGYILN  STGRVPFLLS
651 14 AAIQKQDSFY  PTNIIITDQGL  LALSDVEKTM  AGSBGMGLTY  YVVRGNAART
701 15 OSAANNASAPA  GLKHAVEQGG  SNNLENMVLE  DASESSSATPE  TVETAAADRT
751 16 DMGIRPYQGA  TRFAAAAVQQH  ANAADVGRVRF  NLSLAATVYAD  STAHAAMDQG
801 17 RRLKAVESDLG  DHNGTGLRVI  AQTPQDQGWT  EGQVQVGBMR  GCTQVQGIA
851 18 KRTNTAAAM  TLQMRSTWSM  BESNANAKTS  ISLFGAIRKHG  AGDQIGLXKGL
901 19 PSYGRXNSI  SRXJGQAHED  EGSSVNTLMQ  LQALGQVGNVP  FAATGGLTVIE
951 20 GGRNYDLIKQ  DAFERKSQSL  GWSGNLTBQ  TIVGLAGKQ  SQPLHSDKAVL
1001 21 FATAGVERDL  NRHDVTTGQG  PTGTATACTG  TGAMMPMPHR  LVAGLGDADV
1051 22 FONGWNGLAR  YSYAGSSEQGQ  NSSGRVGVGUY  RF*
```

ΔG983 thus has the following basic sequence:

```
ΔG983 thus has the following basic sequence:

TSAPD  FNAGOTGIGS
30   NSRATTASKA  AVSAYGNKNE  MCKDRSMLCA  GRDADVYTRD  DAKINAPPF
35   LIFHFDPFNW  DAYXNLINLK  PATAGAYTG  QGWGIVQDVG  DSGVISFPP
40   LTHQGHEGNY  ENYNTXAYMK  RKEADPDGGG  KDIBASDPEDE  AVHEEAKPT
45   DIHRVKEIGHY  IDLWNYIHGG  RSVQGQPSGG  IAPDALTLMH  MTNDEKTNEM
50   MVAARNWAV  KLGORGVIV  NNSFGTTSRRA  GTADLFQIAN  SEEQYRQALL
55   DYSGQKDTDE  GRLMMQSDY  GNLSYHINRK  NMLFIFSTGN  DAQAPQNYTA
60   LLFRFYEKDAQ  KGIIITVAGVD  RSGEKFKREM  GYGEPTQTELIE  YGSGNSCIGTA
65   MWCILAEA YEVA SVFRFTNPI  QIAIGTFSSAP  IVTGTAAAL  LQKYQPSNNMDN
70   LARKTLTQAT  DIGAVGVDSK  FGHWLDLQD  AMQGAPSFPP  GBTADTKGOT
75   SDIAIFSRND  ISEQGGFLRKL  GQSLQRGHLN  NTTYKTIIE  GSGSLVGSNN
80   KSRMRRVMTG  ALINYGAAGG  GSNLSQDVAYV  LADTDQQSGAN  ETIVHIGSLQ
85   LDQKGTLYTR  LGKLLKDVGTT  AIIGKGLYMS  ARBGKAGYILN  STGRVPFLLS
90   AAIQKQDSFY  PTNIIITDQGL  LALSDVEKTM  AGSBGMGLTY  YVVRGNAART
95   OSAANNASAPA  GLKHAVEQGG  SNNLENMVLE  DASESSSATPE  TVETAAADRT
100  DMGIRPYQGA  TRFAAAAVQQH  ANAADVGRVRF  NLSLAATVYAD  STAHAAMDQG
105  RRLKAVESDLG  DHNGTGLRVI  AQTPQDQGWT  EGQVQVGBMR  GCTQVQGIA
110  KRTNTAAAM  TLQMRSTWSM  BESNANAKTS  ISLFGAIRKHG  AGDQIGLXKGL
115  PSYGRXNSI  SRXJGQAHED  EGSSVNTLMQ  LQALGQVGNVP  FAATGGLTVIE
120  GGRNYDLIKQ  DAFERKSQSL  GWSGNLTBQ  TIVGLAGKQ  SQPLHSDKAVL
125  FATAGVERDL  NRHDVTTGQG  PTGTATACTG  TGAMMPMPHR  LVAGLGDADV
130  FONGWNGLAR  YSYAGSSEQGQ  NSSGRVGVGUY  RF*
```

ΔG983 was expressed as a hybrid, with ORF46.1, 741, 961 or 961c at its C-terminus:

```
ΔG983 - ORF46.1
ATACCTTGGCCGCCGCACTTCAAGCGACGGCTACCCGATGCGATGCGAGAGAACAGGACAGAACAAACAGCGAAATCGAGCA
GCAGCGCACTTCAAGCGACGGCTACCCGATGCGATGCGAGAGAACAGGACAGAACAAACAGCGAAATCGAGCA
GTTAGCACAGGACAGGATACCAAAATCAATGCGCTCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGC...
Example 4 – hybrids of ΔG741

Protein 741 has the following sequence:

ΔG741 thus has the following basic sequence:

ΔG741 was fused directly in-frame upstream of proteins 961, 961c, 983 and ORF41.6:
Example 5 – hybrids of 287

Expression of 287 as full-length with a C-terminal His-tag, or without its leader peptide but with a C-terminal His-tag, gives fairly low expression levels. Better expression is achieved using a N-terminal GST-fusion. As an alternative to using GST as an N-terminal fusion partner, 287 was placed at the C-terminus of protein 919 (‘919-287’), of protein 953 (‘953-287’), and of proteins ORF46.1 (‘ORF46.1-287’). In both cases, the leader peptides were deleted, and the hybrids were direct in-frame fusions.

To generate the 953-287 hybrid, the leader peptides of the two proteins were omitted by designing the forward primer downstream from the leader of each sequence; the stop codon sequence was omitted in the 953 reverse primer but included in the 287 reverse primer. For the 953 gene, the 5’ and the 3’ primers used for amplification included a Ndel and a BamHI restriction sites respectively, whereas for the amplification of the 287 gene the 5’ and the 3’ primers included a BamHI and a XhoI restriction sites respectively. In this way a sequential directional cloning of the two genes in pET21b+, using Ndel-BamHI (to clone the first gene) and subsequently BamHI-XhoI (to clone the second gene) could be achieved.
The 919-287 hybrid was obtained by cloning the sequence coding for the mature portion of 287 into the XhoI site at the 3'-end of the 919-His clone in pET21b+. The primers used for amplification of the 287 gene were designed for introducing a SalI restriction site at the 5'- and a XhoI site at the 3'- of the PCR fragment. Since the cohesive ends produced by the SalI and XhoI restriction enzymes are compatible, the 287 PCR product digested with SalI-XhoI could be inserted in the pET21b-919 clone cleaved with XhoI.

The ORF46.1-287 hybrid was obtained similarly.

The bactericidal efficacy (homologous strain) of antibodies raised against the hybrid proteins was compared with antibodies raised against simple mixtures of the component antigens:

<table>
<thead>
<tr>
<th></th>
<th>Mixture with 287</th>
<th>Hybrid with 287</th>
</tr>
</thead>
<tbody>
<tr>
<td>919</td>
<td>32000</td>
<td>16000</td>
</tr>
<tr>
<td>953</td>
<td>8192</td>
<td>8192</td>
</tr>
<tr>
<td>ORF46.1</td>
<td>128</td>
<td>8192</td>
</tr>
</tbody>
</table>

Data for bactericidal activity against heterologous MenB strains and against serotypes A and C were also obtained for 919-287 and 953-287:

<table>
<thead>
<tr>
<th></th>
<th>919</th>
<th>953</th>
<th>ORF46.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td>Mixture</td>
<td>Hybrid</td>
<td>Mixture</td>
</tr>
<tr>
<td>MC58</td>
<td>512</td>
<td>1024</td>
<td>512</td>
</tr>
<tr>
<td>NGH38</td>
<td>1024</td>
<td>2048</td>
<td>2048</td>
</tr>
<tr>
<td>BZ232</td>
<td>512</td>
<td>128</td>
<td>1024</td>
</tr>
<tr>
<td>MenA (F6124)</td>
<td>&gt;2048</td>
<td>n.d.</td>
<td>&gt;2048</td>
</tr>
<tr>
<td>MenC (C11)</td>
<td>&gt;4096</td>
<td>&gt;8192</td>
<td>&gt;4096</td>
</tr>
<tr>
<td>MenC (BZ133)</td>
<td>&gt;4096</td>
<td>&gt;8192</td>
<td>&gt;4096</td>
</tr>
</tbody>
</table>

Hybrids of ORF46.1 and 919 were also constructed. Best results (four-fold higher titre) were achieved with 919 at the N-terminus.

Hybrids 919-519His, ORF97-225His and 225-ORF97His were also tested. These gave moderate ELISA titres and bactericidal antibody responses.

As hybrids of two proteins A & B may be either NH₂–A–B–COOH or NH₂–B–A–COOH, the “reverse” hybrids with 287 at the N-terminus were also made, but using ΔG287. A panel of strains was used, including homologous strain 2996. FCA was used as adjuvant:
Better bactericidal titres are generally seen with 287 at the N-terminus.

When fused to protein 961 [NH₂-ΔG287-961-COOH – sequence shown above], the resulting protein is insoluble and must be denatured and renatured for purification. Following renaturation, around 50% of the protein was found to remain insoluble. The soluble and insoluble proteins were compared, and much better bactericidal titres were obtained with the soluble protein (FCA as adjuvant):

<table>
<thead>
<tr>
<th>Strain</th>
<th>287 &amp; 919</th>
<th>287 &amp; 953</th>
<th>287 &amp; ORF46.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2996</td>
<td>ΔG287-919</td>
<td>919-287</td>
<td>ΔG287-953</td>
</tr>
<tr>
<td></td>
<td>128000</td>
<td>16000</td>
<td>65536</td>
</tr>
<tr>
<td></td>
<td>8192</td>
<td></td>
<td>16384</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>8192</td>
</tr>
<tr>
<td>BZ2322</td>
<td>256</td>
<td>128</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&lt;4</td>
</tr>
<tr>
<td>1000</td>
<td>2048</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>&lt;4</td>
</tr>
<tr>
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<td>1024</td>
<td>16384</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>NGH38</td>
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<td>2048</td>
<td>&gt;2048</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4096</td>
</tr>
<tr>
<td></td>
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<td>16384</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4096</td>
</tr>
<tr>
<td>394/98</td>
<td>4096</td>
<td>32</td>
<td>256</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>128</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>MenA (F6124)</td>
<td>32000</td>
<td>2048</td>
<td>&gt;2048</td>
</tr>
<tr>
<td>MenC (BZ133)</td>
<td>64000</td>
<td>&gt;8192</td>
<td>&gt;8192</td>
</tr>
</tbody>
</table>

Titres with the insoluble form were, however, improved by using alum adjuvant instead:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Soluble</th>
<th>BZ232</th>
<th>MC58</th>
<th>NGH38</th>
<th>F6124</th>
<th>BZ133</th>
</tr>
</thead>
<tbody>
<tr>
<td>2996</td>
<td>65536</td>
<td>128</td>
<td>4096</td>
<td>&gt;2048</td>
<td>&gt;2048</td>
<td>4096</td>
</tr>
<tr>
<td>BZ232</td>
<td>128</td>
<td>&lt;4</td>
<td>&lt;4</td>
<td>16</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

961c was also used in hybrid proteins (see above). As 961 and its domain variants direct efficient expression, they are ideally suited as the N-terminal portion of a hybrid protein.

**Example 23 – further hybrids**

Further hybrid proteins of the invention are shown in the drawings and have the sequences set out below. These are advantageous when compared to the individual proteins:

```
ORF46.1-741
AGGCCGAGATTCTGGCAAGCCATTCCTTTATCCGCCAGGTCTTCGACCAGACATTCTTGCAACCCGACCGGAAATACCGAC
CTCTTGCACTCTGGCAGGGGCTGGCCAGCAGCGGCGCTGGGTAACCGAACGTACCTGACACTGCAACCATTCCTGGGTC
AACCTGACATCAGAATCGAGGTACACCATACGTCATCTGCAACCCGACCGGCGTAATCGAGAGGCGGGCCAAGGCGCGGC
GTCCGCGAGGGATATATACATGCACTGACATAAAAAAGGGGCTGCGCCAAAATAATCGCGCTCAGACCTAGCAGCAACCGC
AGCCGAGCAACGCGCTGGCCAGCGGCGTTTCCACCAATGGCAGCGATAGTCAGCGAGAAGATAGCCGACCCGATTCAAA
CGCGGCAAGCTAAAGCCAGCCAGTTCTGAGAACATGGCCGAGCTGGGTAACCGAACGTACCTGACACTGCAACCATTCCTGGGTC
AACCTGACATCAGAATCGAGGTACACCATACGTCATCTGCAACCCGACCGGCGTAATCGAGAGGCGGGCCAAGGCGCGGC
GTCCGCGAGGGATATATACATGCACTGACATAAAAAAGGGGCTGCGCCAAAATAATCGCGCTCAGACCTAGCAGCAACCGC
AGCCGAGCAACGCGCTGGCCAGCGGCGTTTCCACCAATGGCAGCGATAGTCAGCGAGAAGATAGCCGACCCGATTCAAA
CGCGGCAAGCTAAAGCCAGCCAGTTCTGAGAACATGGCCGAGCTGGGTAACCGAACGTACCTGACACTGCAACCATTCCTGGGTC
AACCTGACATCAGAATCGAGGTACACCATACGTCATCTGCAACCCGACCGGCGTAATCGAGAGGCGGGCCAAGGCGCGGC
GTCCGCGAGGGATATATACATGCACTGACATAAAAAAGGGGCTGCGCCAAAATAATCGCGCTCAGACCTAGCAGCAACCGC
AGCCGAGCAACGCGCTGGCCAGCGGCGTTTCCACCAATGGCAGCGATAGTCAGCGAGAAGATAGCCGACCCGATTCAAA
```
It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention. For instance, the use of proteins from other strains is envisaged [*e.g.* see WO00/66741 for polymorphic sequences for ORF4, ORF40, ORF46, 225, 235, 287, 519, 726, 919 and 953].

**EXPERIMENTAL DETAILS**

**Cloning strategy and oligonucleotide design**

Genes coding for antigens of interest were amplified by PCR, using oligonucleotides designed on the basis of the genomic sequence of *N. meningitidis* B MC58. Genomic DNA from strain 2996 was always used as a template in PCR reactions, unless otherwise specified, and the amplified fragments were cloned in the expression vector pET21b+ (Novagen) to express the protein as C-terminal His-tagged product, or in pET-24b+(Novagen) to express the protein in ‘untagged’ form (*e.g.* ΔG 287K).

Where a protein was expressed without a fusion partner and with its own leader peptide (if present), amplification of the open reading frame (ATG to STOP codons) was performed.

Where a protein was expressed in ‘untagged’ form, the leader peptide was omitted by designing the 5'-end amplification primer downstream from the predicted leader sequence.

The melting temperature of the primers used in PCR depended on the number and type of hybridising nucleotides in the whole primer, and was determined using the formulae:

\[
T_{m1} = 4 \text{(G+C)} + 2 \text{(A+T)} \quad \text{(tail excluded)}
\]

\[
T_{m2} = 64.9 + 0.41 \times \text{(% GC)} - 600/N \quad \text{(whole primer)}
\]

The melting temperatures of the selected oligonucleotides were usually 65-70°C for the whole oligo and 50-60°C for the hybridising region alone.

Oligonucleotides were synthesised using a Perkin Elmer 394 DNA/RNA Synthesizer, eluted from the columns in 2.0ml NH₄OH, and deprotected by 5 hours incubation at 56°C. The oligos were precipitated by addition of 0.3M Na-Acetate and 2 volumes ethanol. The samples were centrifuged and the pellets resuspended in water.

<table>
<thead>
<tr>
<th></th>
<th>Sequences</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>fu (961)</td>
<td>Fwd CGCggATCC -GGAGgGggGTTgGTCg</td>
<td>BamHI</td>
</tr>
<tr>
<td>Sequence</td>
<td>Forward (Fwd)</td>
<td>Reverse (Rev)</td>
</tr>
<tr>
<td>----------</td>
<td>--------------</td>
<td>---------------</td>
</tr>
<tr>
<td>fu (961)-983-His</td>
<td>CGCAGATGCC-GGGAGGACGGCAGCTT</td>
<td>CCCCTGCGAG-CTATTTTGAGCAACAGATTC</td>
</tr>
<tr>
<td>fu (961)-983-His</td>
<td>CCCCTGCGAG-CTATTTTGAGCAACAGATTC</td>
<td>CCCCTGCGAG-CTATTTTGAGCAACAGATTC</td>
</tr>
<tr>
<td>fu (961c-L)-741(MCS8)</td>
<td>Fwd: CGCAGATGCC-GGGAGGACGGCAGCTT</td>
<td>Rev: CCCCTGCGAG-CTATTTTGAGCAACAGATTC</td>
</tr>
<tr>
<td>fu (961c-L)-983</td>
<td>Fwd: CGCAGATGCC-GGGAGGACGGCAGCTT</td>
<td>Rev: CCCCTGCGAG-CTATTTTGAGCAACAGATTC</td>
</tr>
<tr>
<td>fu (961c-L)-Orf46.1</td>
<td>Fwd: CGCAGATGCC-GGGAGGACGGCAGCTT</td>
<td>Rev: CCCCTGCGAG-CTATTTTGAGCAACAGATTC</td>
</tr>
<tr>
<td>fu-(ΔG287)-919-His</td>
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<td>Rev: CCCAAGCTCT-TTAAAAAGCTAGAC</td>
</tr>
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<td>Rev: CCCAAGCTCT-TTAAAAAGCTAGAC</td>
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</tr>
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<td>Rev: CCCAAGCTCT-TTAAAAAGCTAGAC</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>Rev: CCCAAGCTCT-TTAAAAAGCTAGAC</td>
</tr>
<tr>
<td>fu Orf1-(Orf46.1)-H1s</td>
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<td>XhoI</td>
</tr>
<tr>
<td>Rev</td>
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<td>XhoI</td>
</tr>
<tr>
<td>(∆G741)-961-His</td>
<td>Fwd1</td>
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</tr>
<tr>
<td>Fwd2</td>
<td>GCCGGCCTCGAG-GTGGGCGGAGGAGACCTGGAAGTCGCC</td>
<td>XhoI</td>
</tr>
<tr>
<td>Rev</td>
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<td>XhoI</td>
</tr>
<tr>
<td>(∆G741)-983-His</td>
<td>Fwd</td>
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</tr>
<tr>
<td>Rev</td>
<td>CCCGCTCGAGATCTGCTTCTTTTGTGGGCC</td>
<td>XhoI</td>
</tr>
<tr>
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</tr>
<tr>
<td>Fwd2</td>
<td>GCCGGCCTCGAG-GTGGGCGGAGGAGACCTGGAAGTCGCC</td>
<td>XhoI</td>
</tr>
<tr>
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<td>XhoI</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>Fwd1</td>
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<tr>
<td>Rev</td>
<td>CCCGCTCGAGATCTGCTTCTTTTGTGGGCC</td>
<td>XhoI</td>
</tr>
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</table>

* This primer was used as a Reverse primer for all the C terminal fusions of 287 to the His-tag.

§ Forward primers used in combination with the 287-His Reverse primer.

NB – All PCR reactions use strain 2996 unless otherwise specified (e.g. strain MC58)

In all constructs starting with an ATG not followed by a unique NheI site, the ATG codon is part of the NdeI site used for cloning. The constructs made using NheI as a cloning site at the 5' end (e.g. all those containing 287 at the N-terminus) have two additional codons (GCT AGC) fused to the coding sequence of the antigen.

**Preparation of chromosomal DNA templates**

*N. meningitidis* strains 2996, MC58, 394.98, 1000 and BZ232 (and others) were grown to exponential phase in 100ml of GC medium, harvested by centrifugation, and resuspended in 5ml buffer (20% w/v sucrose, 50mM Tris-HCl, 50mM EDTA, pH8). After 10 minutes incubation on ice, the bacteria were lysed by adding 10ml of lysis solution (50mM NaCl, 1% Na-Sarkosyl, 50µg/ml Proteinase K), and the suspension incubated at 37°C for 2 hours. Two
phenol extractions (equilibrated to pH 8) and one CHCl₃/isoamylalcohol (24:1) extraction were performed. DNA was precipitated by addition of 0.3M sodium acetate and 2 volumes of ethanol, and collected by centrifugation. The pellet was washed once with 70%(v/v) ethanol and redissolved in 4.0ml TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). The DNA concentration was measured by reading OD₂₆₀.

**PCR Amplification**

The standard PCR protocol was as follows: 200ng of genomic DNA from 2996, MC581000, or BZ232 strains or 10ng of plasmid DNA preparation of recombinant clones were used as template in the presence of 40µM of each oligonucleotide primer, 400-800 µM dNTPs solution, 1x PCR buffer (including 1.5mM MgCl₂), 2.5 units TaqI DNA polymerase (using Perkin-Elmer AmpliTaq, Boehringer Mannheim Expand™ Long Template).

After a preliminary 3 minute incubation of the whole mix at 95°C, each sample underwent a two-step amplification: the first 5 cycles were performed using the hybridisation temperature that excluded the restriction enzyme tail of the primer (Tₘ₁). This was followed by 30 cycles according to the hybridisation temperature calculated for the whole length oligos (Tₘ₂). Elongation times, performed at 68°C or 72°C, varied according to the length of the Orf to be amplified. In the case of Orf1 the elongation time, starting from 3 minutes, was increased by 15 seconds each cycle. The cycles were completed with a 10 minute extension step at 72°C.

The amplified DNA was either loaded directly on a 1% agarose gel. The DNA fragment corresponding to the band of correct size was purified from the gel using the Qiagen Gel Extraction Kit, following the manufacturer’s protocol.

**Digestion of PCR fragments and of the cloning vectors**

The purified DNA corresponding to the amplified fragment was digested with the appropriate restriction enzymes for cloning into pET-21b, pET22b+ or pET-24b+. Digested fragments were purified using the QIAquick PCR purification kit (following the manufacturer’s instructions) and eluted with either H₂O or 10mM Tris, pH 8.5. Plasmid vectors were digested with the appropriate restriction enzymes, loaded onto a 1.0% agarose gel and the band corresponding to the digested vector purified using the Qiagen QIAquick Gel Extraction Kit.
**Cloning**

The fragments corresponding to each gene, previously digested and purified, were ligated into pET21b+, pET22b+ or pET-24b+. A molar ratio of 3:1 fragment/vector was used with T4 DNA ligase in the ligation buffer supplied by the manufacturer. Recombinant plasmid was transformed into competent *E.coli* DH5 or HB101 by incubating the ligase reaction solution and bacteria for 40 minutes on ice, then at 37°C for 3 minutes. This was followed by the addition of 800μl LB broth and incubation at 37°C for 20 minutes. The cells were centrifuged at maximum speed in an Eppendorf microfuge, resuspended in approximately 200μl of the supernatant and plated onto LB ampicillin (100mg/ml) agar.

Screening for recombinant clones was performed by growing randomly selected colonies overnight at 37°C in 4.0ml of LB broth + 100μg/ml ampicillin. Cells were pelleted and plasmid DNA extracted using the Qiagen QIAprep Spin Miniprep Kit, following the manufacturer’s instructions. Approximately 1μg of each individual miniprep was digested with the appropriate restriction enzymes and the digest loaded onto a 1-1.5% agarose gel (depending on the expected insert size), in parallel with the molecular weight marker (1kb DNA Ladder, GIBCO). Positive clones were selected on the basis of the size of insert.

**Expression**

After cloning each gene into the expression vector, recombinant plasmids were transformed into *E.coli* strains suitable for expression of the recombinant protein. 1μl of each construct was used to transform *E.coli* BL21-DE3 as described above. Single recombinant colonies were inoculated into 2ml LB+Amp (100μg/ml), incubated at 37°C overnight, then diluted 1:30 in 20ml of LB+Amp (100μg/ml) in 100ml flasks, to give an OD<sub>600</sub> between 0.1 and 0.2. The flasks were incubated at 30°C or at 37°C in a gyratory water bath shaker until OD<sub>600</sub> indicated exponential growth suitable for induction of expression (0.4-0.8 OD). Protein expression was induced by addition of 1.0mM IPTG. After 3 hours incubation at 30°C or 37°C the OD<sub>600</sub> was measured and expression examined. 1.0ml of each sample was centrifuged in a microfuge, the pellet resuspended in PBS and analysed by SDS-PAGE and Coomassie Blue staining.

**Purification of His-tagged proteins**

Various forms of 287 were cloned from strains 2996 and MC58. They were constructed with a C-terminus His-tagged fusion and included a mature form (aa 18-427), constructs with
deletions (Δ1, Δ2, Δ3 and Δ4) and clones composed of either B or C domains. For each clone purified as a His-fusion, a single colony was streaked and grown overnight at 37°C on a LB/Amp (100 μg/ml) agar plate. An isolated colony from this plate was inoculated into 20 ml of LB/Amp (100 μg/ml) liquid medium and grown overnight at 37°C with shaking. The overnight culture was diluted 1:30 into 1.0 L LB/Amp (100 μg/ml) liquid medium and allowed to grow at the optimal temperature (30 or 37°C) until the OD_{550} reached 0.6-0.8. Expression of recombinant protein was induced by addition of IPTG (final concentration 1.0 mM) and the culture incubated for a further 3 hours. Bacteria were harvested by centrifugation at 8000 g for 15 min at 4°C. The bacterial pellet was resuspended in 7.5 ml of either (i) cold buffer A (300 mM NaCl, 50 mM phosphate buffer, 10 mM imidazole, pH 8.0) for soluble proteins or (ii) buffer B (10 mM Tris-HCl, 100 mM phosphate buffer, pH 8.8 and, optionally, 8 M urea) for insoluble proteins. Proteins purified in a soluble form included 287-His, Δ1, Δ2, Δ3 and Δ4287-His, Δ4287MC58-His, 287c-His and 287cMC58-His. Protein 287bMC58-His was insoluble and purified accordingly. Cells were disrupted by sonication on ice four times for 30 sec at 40 W using a Branson sonifier 450 and centrifuged at 13000 x g for 30 min at 4°C. For insoluble proteins, pellets were resuspended in 2.0 ml buffer C (6 M guanidine hydrochloride, 100 mM phosphate buffer, 10 mM Tris- HCl, pH 7.5 and treated with 10 passes of a Dounce homogenizer. The homogenate was centrifuged at 13000 g for 30 min and the supernatant retained. Supernatants for both soluble and insoluble preparations were mixed with 150 μl Ni^{2+}-resin (previously equilibrated with either buffer A or buffer B, as appropriate) and incubated at room temperature with gentle agitation for 30 min. The resin was Chelating Sepharose Fast Flow (Pharmacia), prepared according to the manufacturer's protocol. The batch-wise preparation was centrifuged at 700 g for 5 min at 4°C and the supernatant discarded. The resin was washed twice (batch-wise) with 10 ml buffer A or B for 10 min, resuspended in 1.0 ml buffer A or B and loaded onto a disposable column. The resin continued to be washed with either (i) buffer A at 4°C or (ii) buffer B at room temperature, until the OD_{280} of the flow-through reached 0.02-0.01. The resin was further washed with either (i) cold buffer C (300 mM NaCl, 50 mM phosphate buffer, 20 mM imidazole, pH 8.0) or (ii) buffer D (10 mM Tris-HCl, 100 mM phosphate buffer, pH 6.3 and, optionally, 8 M urea) until OD_{280} of the flow-through reached 0.02-0.01. The His-fusion protein was eluted by addition of 700 μl of either (i) cold elution buffer A (300 mM NaCl, 50 mM phosphate buffer, 250 mM imidazole, pH 8.0) or (ii) elution buffer B (10 mM Tris-HCl, 100 mM phosphate buffer, pH 4.5 and, optionally, 8 M urea) and fractions
collected until the OD$_{280}$ indicated all the recombinant protein was obtained. 20µl aliquots of each elution fraction were analysed by SDS-PAGE. Protein concentrations were estimated using the Bradford assay.

**Renaturation of denatured His-fusion proteins.**

Denaturation was required to solubilize 287bMC8, so a renaturation step was employed prior to immunisation. Glycerol was added to the denatured fractions obtained above to give a final concentration of 10% v/v. The proteins were diluted to 200 µg/ml using dialysis buffer I (10% v/v glycerol, 0.5M arginine, 50 mM phosphate buffer, 5.0 mM reduced glutathione, 0.5 mM oxidised glutathione, 2.0M urea, pH 8.8) and dialysed against the same buffer for 12-14 hours at 4°C. Further dialysis was performed with buffer II (10% v/v glycerol, 0.5M arginine, 50mM phosphate buffer, 5.0mM reduced glutathione, 0.5mM oxidised glutathione, pH 8.8) for 12-14 hours at 4°C. Protein concentration was estimated using the formula:

$$\text{Protein (mg/ml)} = (1.55 \times \text{OD}_{280}) - (0.76 \times \text{OD}_{260})$$

**Immunization**

Balb/C mice were immunized with antigens on days 0, 21 and 35 and sera analyzed at day 49.

**Sera analysis – ELISA**

The acapsulated MenB M7 and the capsulated strains were plated on chocolate agar plates and incubated overnight at 37°C with 5% CO$_2$. Bacterial colonies were collected from the agar plates using a sterile dracon swab and inoculated into Mueller-Hinton Broth (Difco) containing 0.25% glucose. Bacterial growth was monitored every 30 minutes by following OD$_{620}$. The bacteria were let to grow until the OD reached the value of 0.4-0.5. The culture was centrifuged for 10 minutes at 4000rpm. The supernatant was discarded and bacteria were washed twice with PBS, resuspended in PBS containing 0.025% formaldehyde, and incubated for 1 hour at 37°C and then overnight at 4°C with stirring. 100µl bacterial cells were added to each well of a 96 well Greiner plate and incubated overnight at 4°C. The wells were then washed three times with PBT washing buffer (0.1% Tween-20 in PBS). 200µl of saturation buffer (2.7% polyvinylpyrrolidone 10 in water) was added to each well and the plates incubated for 2 hours at 37°C. Wells were washed three times with PBT. 200µl of diluted sera (Dilution buffer: 1% BSA, 0.1% Tween-20, 0.1% NaN$_3$ in PBS) were added to each well and the plates incubated for 2 hours at 37°C. Wells were washed three times with PBT. 100µl of HRP-conjugated rabbit anti-mouse (Dako) serum diluted 1:2000 in dilution buffer were added to each well and the plates were incubated for 90 minutes at 37°C. Wells
were washed three times with PBT buffer. 100μl of substrate buffer for HRP (25ml of citrate buffer pH5, 10mg of O-phenyldiamine and 10μl of H₂O₂) were added to each well and the plates were left at room temperature for 20 minutes. 100μl 12.5% H₂SO₄ was added to each well and OD₄₉₀ was followed. The ELISA titers were calculated arbitrarily as the dilution of sera which gave an OD₄₉₀ value of 0.4 above the level of preimmune sera. The ELISA was considered positive when the dilution of sera with OD₄₉₀ of 0.4 was higher than 1:400.

**Sera analysis – FACS Scan bacteria binding assay**

The acapsulated MenB M7 strain was plated on chocolate agar plates and incubated overnight at 37°C with 5% CO₂. Bacterial colonies were collected from the agar plates using a sterile dracon swab and inoculated into 4 tubes containing 8ml each Mueller-Hinton Broth (Difco) containing 0.25% glucose. Bacterial growth was monitored every 30 minutes by following OD₆₂₀. The bacteria were let to grow until the OD reached the value of 0.35-0.5. The culture was centrifuged for 10 minutes at 4000rpm. The supernatant was discarded and the pellet was resuspended in blocking buffer (1% BSA in PBS, 0.4% NaN₃) and centrifuged for 5 minutes at 4000rpm. Cells were resuspended in blocking buffer to reach OD₆₂₀ of 0.05. 100μl bacterial cells were added to each well of a Costar 96 well plate. 100μl of diluted (1:100, 1:200, 1:400) sera (in blocking buffer) were added to each well and plates incubated for 2 hours at 4°C. Cells were centrifuged for 5 minutes at 4000rpm, the supernatant aspirated and cells washed by addition of 200μl/well of blocking buffer in each well. 100μl of R-Phicoerytrin conjugated F(ab)₂ goat anti-mouse, diluted 1:100, was added to each well and plates incubated for 1 hour at 4°C. Cells were spun down by centrifugation at 4000rpm for 5 minutes and washed by addition of 200μl/well of blocking buffer. The supernatant was aspirated and cells resuspended in 200μl/well of PBS, 0.25% formaldehyde. Samples were transferred to FACScan tubes and read. The condition for FACScan (Laser Power 15mW) setting were: FL2 on; FSC-H threshold:92; FSC PMT Voltage: E 01; SSC PMT: 474; Amp. Gains 6.1; FL-2 PMT: 586; compensation values: 0.

**Sera analysis – bactericidal assay**

*N. meningitidis* strain 2996 was grown overnight at 37°C on chocolate agar plates (starting from a frozen stock) with 5% CO₂. Colonies were collected and used to inoculate 7ml Mueller-Hinton broth, containing 0.25% glucose to reach an OD₆₂₀ of 0.05-0.08. The culture was incubated for approximately 1.5 hours at 37 degrees with shacking until the OD₆₂₀ reached the value of 0.23-0.24. Bacteria were diluted in 50mM Phosphate buffer pH 7.2 containing 10mM MgCl₂, 10mM CaCl₂ and 0.5% (w/v) BSA (assay buffer) at the working dilution of 10⁵ CFU/ml. The total volume of the final reaction mixture was 50 μl with 25 μl
of serial two fold dilution of test serum, 12.5 μl of bacteria at the working dilution, 12.5 μl of baby rabbit complement (final concentration 25%).

Controls included bacteria incubated with complement serum, immune sera incubated with bacteria and with complement inactivated by heating at 56°C for 30'. Immediately after the addition of the baby rabbit complement, 10μl of the controls were plated on Mueller-Hinton agar plates using the tilt method (time 0). The 96-wells plate was incubated for 1 hour at 37°C with rotation. 7μl of each sample were plated on Mueller-Hinton agar plates as spots, whereas 10μl of the controls were plated on Mueller-Hinton agar plates using the tilt method (time 1). Agar plates were incubated for 18 hours at 37 degrees and the colonies corresponding to time 0 and time 1 were counted.

Sera analysis – western blots

Purified proteins (500ng/lane), outer membrane vesicles (5μg) and total cell extracts (25μg) derived from MenB strain 2996 were loaded onto a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The transfer was performed for 2 hours at 150mA at 4°C, using transfer buffer (0.3% Tris base, 1.44% glycine, 20% (v/v) methanol). The membrane was saturated by overnight incubation at 4°C in saturation buffer (10% skimmed milk, 0.1% Triton X100 in PBS). The membrane was washed twice with washing buffer (3% skimmed milk, 0.1% Triton X100 in PBS) and incubated for 2 hours at 37°C with mice sera diluted 1:200 in washing buffer. The membrane was washed twice and incubated for 90 minutes with a 1:2000 dilution of horseradish peroxidase labelled anti-mouse Ig. The membrane was washed twice with 0.1% Triton X100 in PBS and developed with the Opti-4CN Substrate Kit (Bio-Rad). The reaction was stopped by adding water.

The OMVs were prepared as follows: N. meningitidis strain 2996 was grown overnight at 37 degrees with 5% CO2 on 5 GC plates, harvested with a loop and resuspended in 10 ml of 20mM Tris-HCl pH 7.5, 2 mM EDTA. Heat inactivation was performed at 56°C for 45 minutes and the bacteria disrupted by sonication for 5 minutes on ice (50% duty cycle, 50% output , Branson sonifier 3 mm microtip). Unbroken cells were removed by centrifugation at 5000g for 10 minutes, the supernatant containing the total cell envelope fraction recovered and further centrifuged overnight at 50000g at the temperature of 4°C. The pellet containing the membranes was resuspended in 2% sarkosyl, 20mM Tris-HCl pH 7.5, 2 mM EDTA and incubated at room temperature for 20 minutes to solubilise the inner membranes. The suspension was centrifuged at 10000g for 10 minutes to remove aggregates, the supernatant was further centrifuged at 50000g for 3 hours. The pellet, containing the outer membranes
was washed in PBS and resuspended in the same buffer. Protein concentration was measured by the D.C. Bio-Rad Protein assay (Modified Lowry method), using BSA as a standard.

Total cell extracts were prepared as follows: *N. meningitidis* strain 2996 was grown overnight on a GC plate, harvested with a loop and resuspended in 1ml of 20mM Tris-HCl. Heat inactivation was performed at 56°C for 30 minutes.
CLAIMS

1. A method for the simultaneous heterologous expression of two or more proteins of the invention, in which (a) two or more proteins of the invention are fused.

2. The method of claim 23, in which the two or more proteins are: (a) 919 and 287; (b) 953 and 287; (c) 287 and ORF46.1; (d) ORF1 and ORF46.1; (e) 919 and ORF46.1; (f) ORF46.1, 287 and 919; (g) 919 and 519; and (h) ORF97 and 225.

3. The method of claim 24, in which 287 is at the C-terminal end of protein (a), (b) or (c).

4. The method of any preceding claim, in which the expression is in an *E.coli* host.

5. A protein expressed by the method of any preceding claim.

6. A hybrid protein of formula NH$_2$-A—B-COOH, wherein A and B are different Neisserial proteins.

7. The protein of claim 6, wherein A and B are each selected from orf1, orf4, orf25, orf40, orf46, orf83, 233, 287, 2921, 564, 687, 741, 907, 919, 953, 961 and 983.

8. The protein of claim 7, wherein A and B are each selected from ORF46.1, 287, 741, 919, 953, 961 and 983.

9. The protein of claim 8, wherein at least one of said ORF46.1, 287, 741, 919, 953, 961 and 983 is used in essentially full-length form.

10. The protein of claim 8 or claim 9, wherein at least one of said ORF46.1, 287, 741, 919, 953, 961 and 983 has a deletion.

11. The protein of claim 10, wherein A and/or B has a poly-glycine deletion ('ΔG').

12. The protein of claim 11, wherein A and/or B is ΔG-287, ΔGTbp2, ΔG741, or ΔG983.

13. The protein of claim 10, wherein A and/or B is a truncated protein.

14. The protein of claim 13, wherein A and/or B is Δ1-287, Δ2-287, Δ3-287 or Δ4-287.

15. The protein of claim 10, wherein a domain of A and/or B is deleted.
16. The protein of claim 15, wherein A and/or B is 287B, 287C, 287BC, ORF461-433, ORF46433-608, ORF46, or 961c.

17. The protein of claim 6, wherein A and B are: (a) 919 and 287; (b) 953 and 287; (c) 287 and ORF46.1; (d) ORF1 and ORF46.1; (e) 919 and ORF46.1; (f) ORF46.1 and 919; (g) 919 and 519; or (h) ORF97 and 225.


19. The protein of claim 8, wherein A or B is 287.

20. The protein of claim 19, wherein B is 287.

21. The protein of claim 19, wherein A is ΔG-287.

22. The protein of claim 21, wherein B is ORF46, 919, 953 or 961.

23. The protein of any one of claims 19 to 22, wherein 287 is from strain 2996 or 394/98.

24. The protein of claim 8, wherein A is 961.

25. The protein of any one of claims 6 to 24, wherein A and B are from the same strain.

26. The protein of any one of claims 6 to 24, wherein A and B are joined directly.

27. The protein of any one of claims 6 to 24, wherein A and B are joined via a linker peptide.

28. The protein of claim 27, wherein the linker peptide is a poly-glycine linker, with the proviso that B is not a ΔG protein.
FIGURE 17 — ORF46.1—961c

FIGURE 18 — 961-ORF46.1

FIGURE 19 — 961-741

FIGURE 20 — 961-983

FIGURE 21 — 961c-ORF46.1

FIGURE 22 — 961c-741