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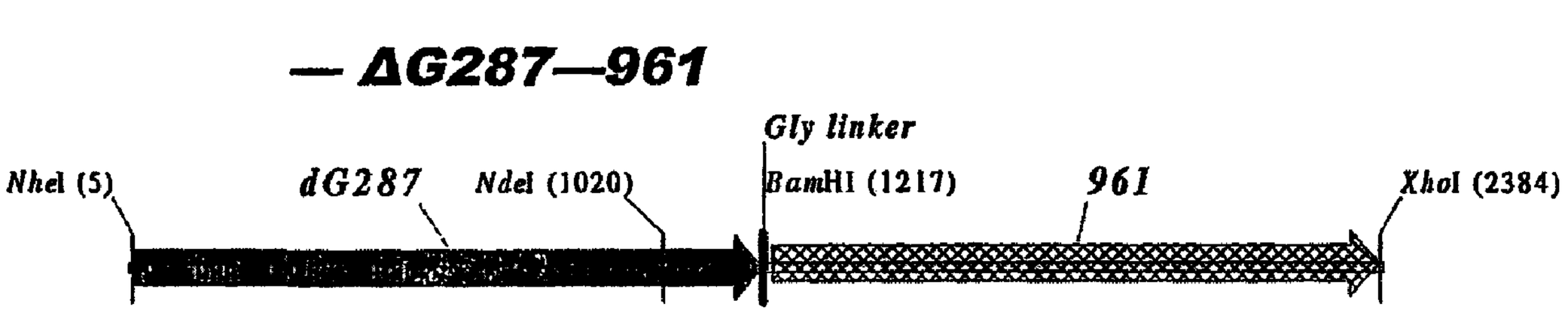

(57) Abrégé/Abstract:
A hybrid protein of formula NH_2-A-B-COOH, wherein A comprises the Neisserial protein ΔG287 and B comprises the Neisserial protein 961, and wherein the amino acid sequence of the hybrid protein is as disclosed in SEQ ID NO: 8 or is a sequence having greater than 70% sequence identity thereto.

ABSTRACT

A hybrid protein of formula NH_2-A-B-COOH, wherein A comprises the Neisserial protein ΔG287 and B comprises the Neisserial protein 961, and wherein the amino acid sequence of the hybrid protein is as disclosed in SEQ ID NO: 8 or is a sequence having greater than 70% sequence identity thereto.

HYBRID EXPRESSION OF NEISSERIAL PROTEINS

This application is a divisional application of co-pending application Serial No. 2,400,562, filed February 28, 2001.

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 NH_2-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 NH_2-A—B-COOH are preferred:

↓A B→	ORF46.1	287	741	919	953	961	983
ORF46.1		X	X	X	X	X	X
287	X		X	X	X	X	X
741	X	X		X	X	X	X
919	X	X	X		X	X	X
953	X	X	X	X		X	X
961	X	X	X	X	X		X
983	X	X	X	X	X	X	

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, 961c *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.* G_n 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, Salmonenna typhimurium, Neisseria meningitidis, Neisseria gonorrhoeae, Neisseria lactamica, Neisseria cinerea, Mycobateria* (*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 Neisserial bacteria (2) a diagnostic reagent for detecting the presence of Neisserial bacteria or of antibodies raised against Neisserial bacteria, and/or (3) a reagent which can raise antibodies against Neisserial 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:

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

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% (*eg.* 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 (*eg.* 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  LGISRKISLI LSILAVCLPM HAHASDLAND SFIRQVLDRQ HFEPDGKYHL
  51  FGSRGELAER SGHIGLGKIQ SHQLGNLMIQ QAAIKGNIGY IVRFSDHGHE
 101  VHSPFDNHAS HSDSDEAGSP VDGFSLYRIH WDGYEHHPAD GYDGPQGGGY
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```
151  PAPKGARDIY SYDIKGVAQN IRLNLTDNRS TGQRLADRFH NAGSMLTQGV
201  GDGFKRATRY SPELDRSGNA AEAFNGTADI VKNIIGAAGE IVGAGDAVQG
251  ISEGSNIAVM HGLGLLSTEN KMARINDLAD MAQLKDYAAA AIRDWAVQNP
301  NAAQGIEAVS NIFMAAIPIK GIGAVRGKYG LGGITAHPIK RSQMGAIALP
351  KGKSAVSDNF ADAAYAKYPS PYHSRNIRSN LEQRYGKENI TSSTVPPSNG
401  KNVKLADQRH PKTGVPFDGK GFPNFEKHVK YDTKLDIQEL SGGGIPKAKP
451  VSDAKPRWEV DRKLNKLTTR EQVEKNVQEI RNGNKNSNFS QHAQLEREIN
501  KLKSADEINF ADGMGKFTDS MNDKAFSRLV KSVKENGFTN PVVEYVEING
551  KAYIVRGNNR VFAAEYLGRI HELKFKKVDF PVPNTSWKNP TDVLNESGNV
601  KRPRYRSK*
```

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):

Protein	ELISA	Bactericidal Ab
Orf1-Orf46.1-His	850	256
919-Orf46.1-His	12900	512
919-287-Orf46-His	n.d.	n.d.
Orf46.1-287His	150	8192
Orf46.1-919His	2800	2048
Orf46.1-287-919His	3200	16384

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:

	2996	BZ232	MC58	NGH38	F6124	BZ133
Mixture	8192	256	512	1024	>2048	>2048
ORF46.1-287-919his	16384	256	4096	8192	8192	8192
ΔG287-919-ORF46.1his	8192	64	4096	8192	8192	16384
ΔG287-ORF46.1-919his	4096	128	256	8192	512	1024

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:

	1000	MC58	F6124 (MenA)
ORF46.1-His	<4	4096	<4
ORF1-His	8	256	128
ORF1—ORF46.1-His	1024	512	1024

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:

SPDVKS ADTLSKPAAP VVSEKETEAK EDAPQAGSQG QGAPSAQGSQ DMAAVSEENT
GNGGAVTADN PKNEDEVAQN DMPQNAAGTD SSTPNHTPDP NMLAGNMENQ ATDAGESSQP
ANQPDMANAA DGMQGDDPSA GGQNAGNTAA QGANQAGNNQ AAGSSDPIPA SNPAPANGGS
NFGRVDLANG VLIDGPSQNI TLTHCKGDSC SGNNFLDEEV QLKSEFEKLS DADKISNYKK
DGKNDKFVGL VADSVQMKGI NQYIIFYKPK PTSFARFRRS ARSRRSLPAE MPLIPVNQAD
TLIVDGEAVS LTGHSGNIFA PEGNYRYLTY GAEKLPGGSY ALRVQGEPAK GEMLAGAAVY
NGEVLHFHTE NGRPYPTRGR FAAKVDFGSK SVDGIIDSGD DLHMGTQKFK AAIDGNGFKG
TWTENGSGDV SGKFYGPAGE EVAGKYSYRP TDAEKGGFGV FAGKKEQD*

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:

<u>**ΔG287-919**</u>

ATGGCTAGCCCCGATGTTAAATCGGCGGACACGCTGTCAAAACCGGCCGCTCCTGTTGTTGCTGAAAAAGAGACAGAG
GTAAAAGAAGATGCGCCACAGGCAGGTTCTCAAGGACAGGGCGCGCCATCCACACAAGGCAGCCAAGATATGGCGGCA
GTTTCGGCAGAAAATACAGGCAATGGCGGTGCGGCAACAACGGACAAACCCAAAAATGAAGACGAGGGACCGCAAAAT
GATATGCCGCAAAATTCCGCCGAATCCGCAAATCAAACAGGGAACAACCAACCCGCCGATTCTTCAGATTCCGCCCCC
GCGTCAAACCCTGCACCTGCGAATGGCGGTAGCAATTTTGGAAGGGTTGATTTGGCTAATGGCGTTTTGATTGATGGG
CCGTCGCAAAATATAACGTTGACCCACTGTAAAGGCGATTCTTGTAATGGTGATAATTTATTGGATGAAGAAGCACCG
TCAAAATCAGAATTTGAAAATTTAAATGAGTCTGAACGAATTGAGAAATATAAGAAAGATGGGAAAAGCGATAAATTT
ACTAATTTGGTTGCGACAGCAGTTCAAGCTAATGGAACTAACAAATATGTCATCATTTATAAAGACAAGTCCGCTTCA
TCTTCATCTGCGCGATTCAGGCGTTCTGCACGGTCGAGGAGGTCGCTTCCTGCCGAGATGCCGCTAATCCCCGTCAAT
CAGGCGGATACGCTGATTGTCGATGGGGAAGCGGTCAGCCTGACGGGGCATTCCGGCAATATCTTCGCGCCCGAAGGG
AATTACCGGTATCTGACTTACGGGGCGGAAAAATTGCCCGGCGGATCGTATGCCCTCCGTGTGCAAGGCGAACCGGCA
AAAGGCGAAATGCTTGCTGGCACGGCCGTGTACAACGGCGAAGTGCTGCATTTTCATACGGAAAACGGCCGTCCGTAC
CCGACTAGAGGCAGGTTTGCCGCAAAAGTCGATTTCGGCAGCAAATCTGTGGACGGCATTATCGACAGCGGCGATGAT
TTGCATATGGGTACGCAAAAATTCAAAGCCGCCATCGATGGAAACGGCTTTAAGGGGACTTGGACGGAAAATGGCGGC
GGGGATGTTTCCGGAAGGTTTTACGGCCCGGCCGGCGAGGAAGTGGCGGGAAAATACAGCTATCGCCCGACAGATGCG

GAAAAGGGCGGATTCGGCGTGTTTGCCGGCAAAAAAGAGCAGGATGGATCCGGAGGAGGAGGATGCCAAAGCAAGAGC
ATCCAAACCTTTCCGCAACCCGACACATCCGTCATCAACGGCCCGGACCGGCCGGTCGGCATCCCCGACCCCGCCGGA
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GCCAAAAGCCTGCAATCCTTCCGCCTCGGCTGCGCCAATTTGAAAAACCGCCAAGGCTGGCAGGATGTGTGCGCCCAA
GCCTTTCAAACCCCCGTCCATTCCTTTCAGGCAAAACAGTTTTTTGAACGCTATTTCACGCCGTGGCAGGTTGCAGGC
AACGGAAGCCTTGCCGGTACGGTTACCGGCTATTACGAGCCGGTGCTGAAGGGCGACGACAGGCGGACGGCACAAGCC
CGCTTCCCGATTTACGGTATTCCCGACGATTTTATCTCCGTCCCCCTGCCTGCCGGTTTGCGGAGCGGAAAAGCCCTT
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TTCCCCATCACCGCGCGCACAACGGCAATCAAAGGCAGGTTTGAAGGAAGCCGCTTCCTCCCCTACCACACGCGCAAC
CAAATCAACGGCGGCGCGCTTGACGGCAAAGCCCCGATACTCGGTTACGCCGAAGACCCCGTCGAACTTTTTTTTATG
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CCCTACGTTTCCATCGGACGCTATATGGCGGACAAAGGCTACCTCAAGCTCGGGCAGACCTCGATGCAGGGCATCAAA
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GCCGGAAGCAGCAATGACGGTCCCGTCGGCGCACTGGGCACGCCGTTGATGGGGGAATATGCCGGCGCAGTCGACCGG
CACTACATTACCTTGGGCGCGCCCTTATTTGTCGCCACCGCCCATCCGGTTACCCGCAAAGCCCTCAACCGCCTGATT
ATGGCGCAGGATACCGGCAGCGCGATTAAAGGCGCGGTGCGCGTGGATTATTTTTGGGGATACGGCGACGAAGCCGGC
GAACTTGCCGGCAAACAGAAAACCACGGGTTACGTCTGGCAGCTCCTACCCAACGGTATGAAGCCCGAATACCGCCCG
TAACTCGAG

MASPDVKSAD TLSKPAAPVV AEKETEVKED APQAGSQGQG APSTQGSQDM
AAVSAENTGN GGAATTDKPK NEDEGPQNDM PQNSAESANQ TGNNQPADSS
DSAPASNPAP ANGGSNFGRV DLANGVLIDG PSQNITLTHC KGDSCNGDNL
LDEEAPSKSE FENLNESERI EKYKKDGKSD KFTNLVATAV QANGTNKYVI
IYKDKSASSS SARFRRSARS RRSLPAEMPL IPVNQADTLI VDGEAVSLTG
HSGNIFAPEG NYRYLTYGAE KLPGGSYALR VQGEPAKGEM LAGTAVYNGE
VLHFHTENGR PYPTRGRFAA KVDFGSKSVD GIIDSGDDLH MGTQKFKAAI
DGNGFKGTWT ENGGGDVSGR FYGPAGEEVA GKYSYRPTDA EKGGFGVFAG
KKEQDGSGGG GCQSKSIQTF PQPDTSVING PDRPVGIPDP AGTTVGGGGA
VYTVVPHLSL PHWAAQDFAK SLQSFRLGCA NLKNRQGWQD VCAQAFQTPV
HSFQAKQFFE RYFTPWQVAG NGSLAGTVTG YYEPVLKGDD RRTAQARFPI
YGIPDDFISV PLPAGLRSGK ALVRIRQTGK NSGTIDNTGG THTADLSRFP
ITARTTAIKG RFEGSRFLPY HTRNQINGGA LDGKAPILGY AEDPVELFFM
HIQGSGRLKT PSGKYIRIGY ADKNEHPYVS IGRYMADKGY LKLGQTSMQG
IKAYMRQNPQ RLAEVLGQNP SYIFFRELAG SSNDGPVGAL GTPLMGEYAG
AVDRHYITLG APLFVATAHP VTRKALNRLI MAQDTGSAIK GAVRVDYFWG
YGDEAGELAG KQKTTGYVWQ LLPNGMKPEY RP*

<u>**ΔG287–953**</u>

ATGGCTAGCCCCGATGTTAAATCGGCGGACACGCTGTCAAAACCGGCCGCTCCTGTTGTTGCTGAAAAAGAGACAGAG
GTAAAAGAAGATGCGCCACAGGCAGGTTCTCAAGGACAGGGCGCGCCATCCACACAAGGCAGCCAAGATATGGCGGCA
GTTTCGGCAGAAAATACAGGCAATGGCGGTGCGGCAACAACGGACAAACCCAAAAATGAAGACGAGGGACCGCAAAAT
GATATGCCGCAAAATTCCGCCGAATCCGCAAATCAAACAGGGAACAACCAACCCGCCGATTCTTCAGATTCCGCCCCC
GCGTCAAACCCTGCACCTGCGAATGGCGGTAGCAATTTTGGAAGGGTTGATTTGGCTAATGGCGTTTTGATTGATGGG
CCGTCGCAAAATATAACGTTGACCCACTGTAAAGGCGATTCTTGTAATGGTGATAATTTATTGGATGAAGAAGCACCG
TCAAAATCAGAATTTGAAAATTTAAATGAGTCTGAACGAATTGAGAAATATAAGAAAGATGGGAAAAGCGATAAATTT
ACTAATTTGGTTGCGACAGCAGTTCAAGCTAATGGAACTAACAAATATGTCATCATTTATAAAGACAAGTCCGCTTCA
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CAGGCGGATACGCTGATTGTCGATGGGGAAGCGGTCAGCCTGACGGGGCATTCCGGCAATATCTTCGCGCCCGAAGGG
AATTACCGGTATCTGACTTACGGGGCGGAAAAATTGCCCGGCGGATCGTATGCCCTCCGTGTGCAAGGCGAACCGGCA
AAAGGCGAAATGCTTGCTGGCACGGCCGTGTACAACGGCGAAGTGCTGCATTTTCATACGGAAAACGGCCGTCCGTAC
CCGACTAGAGGCAGGTTTGCCGCAAAAGTCGATTTCGGCAGCAAATCTGTGGACGGCATTATCGACAGCGGCGATGAT
TTGCATATGGGTACGCAAAAATTCAAAGCCGCCATCGATGGAAACGGCTTTAAGGGGACTTGGACGGAAAATGGCGGC
GGGGATGTTTCCGGAAGGTTTTACGGCCCGGCCGGCGAGGAAGTGGCGGGAAAATACAGCTATCGCCCGACAGATGCG
GAAAAGGGCGGATTCGGCGTGTTTGCCGGCAAAAAAGAGCAGGATGGATCCGGAGGAGGAGGAGCCACCTACAAAGTG
GACGAATATCACGCCAACGCCCGTTTCGCCATCGACCATTTCAACACCAGCACCAACGTCGGCGGTTTTTACGGTCTG
ACCGGTTCCGTCGAGTTCGACCAAGCAAAACGCGACGGTAAAATCGACATCACCATCCCCGTTGCCAACCTGCAAAGC
GGTTCGCAACACTTTACCGACCACCTGAAATCAGCCGACATCTTCGATGCCGCCCAATATCCGGACATCCGCTTTGTT
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GTCAAACTCAAAGCCGAAAAATTCAACTGCTACCAAAGCCCGATGGCGAAAACCGAAGTTTGCGGCGGCGACTTCAGC
ACCACCATCGACCGCACCAAATGGGGCGTGGACTACCTCGTTAACGTTGGTATGACCAAAAGCGTCCGCATCGACATC
CAAATCGAGGCAGCCAAACAATAACTCGAG

MASPDVKSAD TLSKPAAPVV AEKETEVKED APQAGSQGQG APSTQGSQDM
AAVSAENTGN GGAATTDKPK NEDEGPQNDM PQNSAESANQ TGNNQPADSS
DSAPASNPAP ANGGSNFGRV DLANGVLIDG PSQNITLTHC KGDSCNGDNL
LDEEAPSKSE FENLNESERI EKYKKDGKSD KFTNLVATAV QANGTNKYVI

201 IYKDKSASSS SARFRRSARS RRSLPAEMPL IPVNQADTLI VDGEAVSLTG
251 HSGNIFAPEG NYRYLTYGAE KLPGGSYALR VQGEPAKGEM LAGTAVYNGE
301 VLHFHTENGR PYPTRGRFAA KVDFGSKSVD GIIDSGDDLH MGTQKFKAAI
351 DGNGFKGTWT ENGGGDVSGR FYGPAGEEVA GKYSYRPTDA EKGGFGVFAG
401 KKEQDGSGGG GATYKVDEYH ANARFAIDHF NTSTNVGGFY GLTGSVEFDQ
451 AKRDGKIDIT IPVANLQSGS QHFTDHLKSA DIFDAAQYPD IRFVSTKFNF
501 NGKKLVSVDG NLTMHGKTAP VKLKAEKFNC YQSPMAKTEV CGGDFSTTID
551 RTKWGVDYLV NVGMTKSVRI DIQIEAAKQ*

ΔG287-961

ATGGCTAGCCCCGATGTTAAATCGGCGGACACGCTGTCAAAACCGGCCGCTCCTGTTGTTGCTGAAAAAGAGACAGAG
GTAAAAGAAGATGCGCCACAGGCAGGTTCTCAAGGACAGGGCGCGCCATCCACACAAGGCAGCCAAGATATGGCGGCA
GTTTCGGCAGAAAATACAGGCAATGGCGGTGCGGCAACAACGGACAAACCCAAAAATGAAGACGAGGGACCGCAAAAT
GATATGCCGCAAAATTCCGCCGAATCCGCAAATCAAACAGGGAACAACCAACCCGCCGATTCTTCAGATTCCGCCCCC
GCGTCAAACCCTGCACCTGCGAATGGCGGTAGCAATTTTGGAAGGGTTGATTTGGCTAATGGCGTTTTGATTGATGGG
CCGTCGCAAAATATAACGTTGACCCACTGTAAAGGCGATTCTTGTAATGGTGATAATTTATTGGATGAAGAAGCACCG
TCAAAATCAGAATTTGAAAATTTAAATGAGTCTGAACGAATTGAGAAATATAAGAAAGATGGGAAAAGCGATAAATTT
ACTAATTTGGTTGCGACAGCAGTTCAAGCTAATGGAACTAACAAATATGTCATCATTTATAAAGACAAGTCCGCTTCA
TCTTCATCTGCGCGATTCAGGCGTTCTGCACGGTCGAGGAGGTCGCTTCCTGCCGAGATGCCGCTAATCCCCGTCAAT
CAGGCGGATACGCTGATTGTCGATGGGGAAGCGGTCAGCCTGACGGGGCATTCCGGCAATATCTTCGCGCCCGAAGGG
AATTACCGGTATCTGACTTACGGGGCGGAAAAATTGCCCGGCGGATCGTATGCCCTCCGTGTGCAAGGCGAACCGGCA
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GAGACCATCTACGACATTGATGAAGACGGCACAATTACCAAAAAAGACGCAACTGCAGCCGATGTTGAAGCCGACGAC
TTTAAAGGTCTGGGTCTGAAAAAAGTCGTGACTAACCTGACCAAAACCGTCAATGAAAACAAACAAAACGTCGATGCC
AAAGTAAAAGCTGCAGAATCTGAAATAGAAAAGTTAACAACCAAGTTAGCAGACACTGATGCCGCTTTAGCAGATACT
GATGCCGCTCTGGATGCAACCACCAACGCCTTGAATAAATTGGGAGAAAATATAACGACATTTGCTGAAGAGACTAAG
ACAAATATCGTAAAAATTGATGAAAAATTAGAAGCCGTGGCTGATACCGTCGACAAGCATGCCGAAGCATTCAACGAT
ATCGCCGATTCATTGGATGAAACCAACACTAAGGCAGACGAAGCCGTCAAAACCGCCAATGAAGCCAAACAGACGGCC
GAAGAAACCAAACAAAACGTCGATGCCAAAGTAAAAGCTGCAGAAACTGCAGCAGGCAAAGCCGAAGCTGCCGCTGGC
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AAAGATAATATTGCTAAAAAAGCAAACAGTGCCGACGTGTACACCAGAGAAGAGTCTGACAGCAAATTTGTCAGAATT
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CGCCTGAACGGTTTGGATAAAACAGTGTCAGACCTGCGCAAAGAAACCCGCCAAGGCCTTGCAGAACAAGCCGCGCTC
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GTCGCCATCGGTACCGGCTTCCGCTTTACCGAAAACTTTGCCGCCAAAGCAGGCGTGGCAGTCGGCACTTCGTCCGGT
TCTTCCGCAGCCTACCATGTCGGCGTCAATTACGAGTGGTAACTCGAG

1 MASPDVKSAD TLSKPAAPVV AEKETEVKED APQAGSQGQG APSTQGSQDM
51 AAVSAENTGN GGAATTDKPK NEDEGPQNDM PQNSAESANQ TGNNQPADSS
101 DSAPASNPAP ANGGSNFGRV DLANGVLIDG PSQNITLTHC KGDSCNGDNL
151 LDEEAPSKSE FENLNESERI EKYKKDGKSD KFTNLVATAV QANGTNKYVI
201 IYKDKSASSS SARFRRSARS RRSLPAEMPL IPVNQADTLI VDGEAVSLTG
251 HSGNIFAPEG NYRYLTYGAE KLPGGSYALR VQGEPAKGEM LAGTAVYNGE
301 VLHFHTENGR PYPTRGRFAA KVDFGSKSVD GIIDSGDDLH MGTQKFKAAI
351 DGNGFKGTWT ENGGGDVSGR FYGPAGEEVA GKYSYRPTDA EKGGFGVFAG
401 KKEQDGSGGG GATNDDDVKK AATVAIAAAY NNGQEINGFK AGETIYDIDE
451 DGTITKKDAT AADVEADDFK GLGLKKVVTN LTKTVNENKQ NVDAKVKAAE
501 SEIEKLTTKL ADTDAALADT DAALDATTNA LNKLGENITT FAEETKTNIV
551 KIDEKLEAVA DTVDKHAEAF NDIADSLDET NTKADEAVKT ANEAKQTAEE
601 TKQNVDAKVK AAETAAGKAE AAAGTANTAA DKAEAVAAKV TDIKADIATN
651 KDNIAKKANS ADVYTREESD SKFVRIDGLN ATTEKLDTRL ASAEKSIADH
701 DTRLNGLDKT VSDLRKETRQ GLAEQAALSG LFQPYNVGRF NVTAAVGGYK
751 SESAVAIGTG FRFTENFAAK AGVAVGTSSG SSAAYHVGVN YEW*

	ELISA	Bactericidal
ΔG287-953-His	3834	65536
ΔG287-961-His	108627	65536

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:

	Mixture with 287	Hybrid with ΔG287
919	32000	128000
ORF46.1	128	16000

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

	919		ORF46.1	
Strain	*Mixture*	*Hybrid*	*Mixture*	*Hybrid*
NGH38	1024	32000	-	16384
MC58	512	8192	-	512
BZ232	512	512	-	-
MenA (F6124)	512	32000	-	8192
MenC (C11)	>2048	>2048	-	-
MenC (BZ133)	>4096	64000	-	8192

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

ATGGCTAGCCCCGATGTCAAGTCGGCGGACACGCTGTCAAAACCTGCCGCCCCTGTTGTTTCTGAAAAAGAGACAGAG
GCAAAGGAAGATGCGCCACAGGCAGGTTCTCAAGGACAGGGCGCGCCATCCGCACAAGGCGGTCAAGATATGGCGGCG
GTTTCGGAAGAAAATACAGGCAATGGCGGTGCGGCAGCAACGGACAAACCCAAAAATGAAGACGAGGGGGCGCAAAAT
GATATGCCGCAAAATGCCGCCGATACAGATAGTTTGACACCGAATCACACCCCGGCTTCGAATATGCCGGCCGGAAAT
ATGGAAAACCAAGCACCGGATGCCGGGGAATCGGAGCAGCCGGCAAACCAACCGGATATGGCAAATACGGCGGACGGA
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AATCAAACCGCCGGTTCTCAAAATCCTGCCTCTTCAACCAATCCTAGCGCCACGAATAGCGGTGGTGATTTTGGAAGG
ACGAACGTGGGCAATTCTGTTGTGATTGACGGGCCGTCGCAAAATATAACGTTGACCCACTGTAAAGGCGATTCTTGT
AGTGGCAATAATTTCTTGGATGAAGAAGTACAGCTAAAATCAGAATTTGAAAAATTAAGTGATGCAGACAAAATAAGT
AATTACAAGAAAGATGGGAAGAATGACGGGAAGAATGATAAATTTGTCGGTTTGGTTGCCGATAGTGTGCAGATGAAG
GGAATCAATCAATATATTATCTTTTATAAACCTAAACCCACTTCATTTGCGCGATTTAGGCGTTCTGCACGGTCGAGG
CGGTCGCTTCCGGCCGAGATGCCGCTGATTCCCGTCAATCAGGCGGATACGCTGATTGTCGATGGGGAAGCGGTCAGC
CTGACGGGGCATTCCGGCAATATCTTCGCGCCCGAAGGGAATTACCGGTATCTGACTTACGGGGCGGAAAAATTGCCC
GGCGGATCGTATGCCCTCCGTGTTCAAGGCGAACCTTCAAAAGGCGAAATGCTCGCGGGCACGGCAGTGTACAACGGC
GAAGTGCTGCATTTTCATACGGAAAACGGCCGTCCGTCCCCGTCCAGAGGCAGGTTTGCCGCAAAAGTCGATTTCGGC
AGCAAATCTGTGGACGGCATTATCGACAGCGGCGATGGTTTGCATATGGGTACGCAAAAATTCAAAGCCGCCATCGAT
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TACCTCAAGCTCGGGCAGACCTCGATGCAGGGCATCAAAGCCTATATGCGGCAAAATCCGCAACGCCTCGCCGAAGTT
TTGGGTCAAAACCCCAGCTATATCTTTTTCCGCGAGCTTGCCGGAAGCAGCAATGACGGTCCCGTCGGCGCACTGGGC
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GCCCATCCGGTTACCCGCAAAGCCCTCAACCGCCTGATTATGGCGCAGGATACCGGCAGCGCGATTAAAGGCGCGGTG
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MASPDVKSAD TLSKPAAPVV SEKETEAKED APQAGSQGQG APSAQGGQDM
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PAGNMENQAP DAGESEQPAN QPDMANTADG MQGDDPSAGG ENAGNTAAQG
TNQAENNQTA GSQNPASSTN PSATNSGGDF GRTNVGNSVV IDGPSQNITL
THCKGDSCSG NNFLDEEVQL KSEFEKLSDA DKISNYKKDG KNDGKNDKFV
GLVADSVQMK GINQYIIFYK PKPTSFARFR RSARSRRSLP AEMPLIPVNQ
ADTLIVDGEA VSLTGHSGNI FAPEGNYRYL TYGAEKLPGG SYALRVQGEP
SKGEMLAGTA VYNGEVLHFH TENGRPSPSR GRFAAKVDFG SKSVDGIIDS
GDGLHMGTQK FKAAIDGNGF KGTWTENGGG DVSGKFYGPA GEEVAGKYSY
RPTDAEKGGF GVFAGKKEQD GSGGGGCQSK SIQTFPQPDT SVINGPDRPV
GIPDPAGTTV GGGGAVYTVV PHLSLPHWAA QDFAKSLQSF RLGCANLKNR
QGWQDVCAQA FQTPVHSFQA KQFFERYFTP WQVAGNGSLA GTVTGYYEPV
LKGDDRRTAQ ARFPIYGIPD DFISVPLPAG LRSGKALVRI RQTGKNSGTI
DNTGGTHTAD LSRFPITART TAIKGRFEGS RFLPYHTRNQ INGGALDGKA
PILGYAEDPV ELFFMHIQGS GRLKTPSGKY IRIGYADKNE HPYVSIGRYM
ADKGYLKLGQ TSMQGIKAYM RQNPQRLAEV LGQNPSYIFF RELAGSSNDG
PVGALGTPLM GEYAGAVDRH YITLGAPLFV ATAHPVTRKA LNRLIMAQDT
GSAIKGAVRV DYFWGYGDEA GELAGKQKTT GYVWQLLPNG MKPEYRP*

<u>ΔG287NZ-953</u>

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GTTTCGGAAGAAAATACAGGCAATGGCGGTGCGGCAGCAACGGACAAACCCAAAAATGAAGACGAGGGGGCGCAAAAT
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GAAGTGGCGGGAAAATACAGCTATCGCCCAACAGATGCGGAAAAGGGCGGATTCGGCGTGTTTGCCGGCAAAAAAGAG
CAGGATGGATCCGGAGGAGGAGGAGCCACCTACAAAGTGGACGAATATCACGCCAACGCCCCGTTTCGCCATCGACCAT
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AAAATCGACATCACCATCCCCGTTGCCAACCTGCAAAGCGGTTCGCAACACTTTACCGACCACCTGAAATCAGCCGAC
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1 MASPDVKSAD TLSKPAAPVV SEKETEAKED APQAGSQGQG APSAQGGQDM

```
  51    AAVSEENTGN GGAAATDKPK NEDEGAQNDM PQNAADTDSL TPNHTPASNM
 101    PAGNMENQAP DAGESEQPAN QPDMANTADG MQGDDPSAGG ENAGNTAAQG
 151    TNQAENNQTA GSQNPASSTN PSATNSGGDF GRTNVGNSVV IDGPSQNITL
 201    THCKGDSCSG NNFLDEEVQL KSEFEKLSDA DKISNYKKDG KNDGKNDKFV
 251    GLVADSVQMK GINQYIIFYK PKPTSFARFR RSARSRRSLP AEMPLIPVNQ
 301    ADTLIVDGEA VSLTGHSGNI FAPEGNYRYL TYGAEKLPGG SYALRVQGEP
 351    SKGEMLAGTA VYNGEVLHFH TENGRPSPSR GRFAAKVDFG SKSVDGIIDS
 401    GDGLHMGTQK FKAAIDGNGF KGTWTENGGG DVSGKFYGPA GEEVAGKYSY
 451    RPTDAEKGGF GVFAGKKEQD GSGGGGATYK VDEYHANARF AIDHFNTSTN
 501    VGGFYGLTGS VEFDQAKRDG KIDITIPVAN LQSGSQHFTD HLKSADIFDA
 551    AQYPDIRFVS TKFNFNGKKL VSVDGNLTMH GKTAPVKLKA EKFNCYQSPM
 601    AKTEVCGGDF STTIDRTKWG VDYLVNVGMT KSVRIDIQIE AAKQ*
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ΔG287NZ-961

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ATGGCTAGCCCCGATGTCAAGTCGGCGGACACGCTGTCAAAACCTGCCGCCCCCTGTTGTTTCTGAAAAAGAGACAGAG
GCAAAGGAAGATGCGCCACAGGCAGGTTCTCAAGGACAGGGCGCGCCATCCGCACAAGGCGGTCAAGATATGGCGGCG
GTTTCGGAAGAAAATACAGGCAATGGCGGTGCGGCAGCAACGGACAAACCCAAAAATGAAGACGAGGGGGCGCAAAAT
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AATCAAACCGCCGGTTCTCAAAATCCTGCCTCTTCAACCAATCCTAGCGCCACGAATAGCGGTGGTGATTTTGGAAGG
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CGGTCGCTTCCGGCCGAGATGCCGCTGATTCCCGTCAATCAGGCGGATACGCTGATTGTCGATGGGGAAGCGGTCAGC
CTGACGGGGCATTCCGGCAATATCTTCGCGCCCGAAGGGAATTACCGGTATCTGACTTACGGGGCGGAAAAATTGCCC
GGCGGATCGTATGCCCTCCGTGTTCAAGGCGAACCTTCAAAAGGCGAAATGCTCGCGGGCACGGCAGTGTACAACGGC
GAAGTGCTGCATTTTCATACGGAAAACGGCCGTCCGTCCCCGTCCAGAGGCAGGTTTGCCGCAAAAGTCGATTTCGGC
AGCAAATCTGTGGACGGCATTATCGACAGCGGCGATGGTTTGCATATGGGTACGCAAAAATTCAAAGCCGCCATCGAT
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GAAGTGGCGGGAAAATACAGCTATCGCCCAACAGATGCGGAAAAGGGCGGATTCGGCGTGTTTGCCGGCAAAAAAGAG
CAGGATGGATCCGGAGGAGGAGGAGCCACAAACGACGACGATGTTAAAAAAGCTGCCACTGTGGCCATTGCTGCTGCC
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GAAGCCGTCAAAACCGCCAATGAAGCCAAACAGACGGCCGAAGAAACCAAACAAAACGTCGATGCCAAAGTAAAAGCT
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GTAACGGCTGCAGTCGGCGGCTACAAATCCGAATCGGCAGTCGCCATCGGTACCGGCTTCCGCTTTACCGAAAACTTT
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TAAAAGCTT
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   1    MASPDVKSAD TLSKPAAPVV SEKETEAKED APQAGSQGQG APSAQGGQDM
  51    AAVSEENTGN GGAAATDKPK NEDEGAQNDM PQNAADTDSL TPNHTPASNM
 101    PAGNMENQAP DAGESEQPAN QPDMANTADG MQGDDPSAGG ENAGNTAAQG
 151    TNQAENNQTA GSQNPASSTN PSATNSGGDF GRTNVGNSVV IDGPSQNITL
 201    THCKGDSCSG NNFLDEEVQL KSEFEKLSDA DKISNYKKDG KNDGKNDKFV
 251    GLVADSVQMK GINQYIIFYK PKPTSFARFR RSARSRRSLP AEMPLIPVNQ
 301    ADTLIVDGEA VSLTGHSGNI FAPEGNYRYL TYGAEKLPGG SYALRVQGEP
 351    SKGEMLAGTA VYNGEVLHFH TENGRPSPSR GRFAAKVDFG SKSVDGIIDS
 401    GDGLHMGTQK FKAAIDGNGF KGTWTENGGG DVSGKFYGPA GEEVAGKYSY
 451    RPTDAEKGGF GVFAGKKEQD GSGGGGATND DDVKKAATVA IAAAYNNGQE
 501    INGFKAGETI YDIDEDGTIT KKDATAADVE ADDFKGLGLK KVVTNLTKTV
 551    NENKQNVDAK VKAAESEIEK LTTKLADTDA ALADTDAALD ATTNALNKLG
 601    ENITTFAEET KTNIVKIDEK LEAVADTVDK HAEAFNDIAD SLDETNTKAD
 651    EAVKTANEAK QTAEETKQNV DAKVKAAETA AGKAEAAAGT ANTAADKAEA
 701    VAAKVTDIKA DIATNKDNIA KKANSADVYT REESDSKFVR IDGLNATTEK
 751    LDTRLASAEK SIADHDTRLN GLDKTVSDLR KETRQGLAEQ AALSGLFQPY
 801    NVGRFNVTAA VGGYKSESAV AIGTGFRFTE NFAAKAGVAV GTSSGSSAAY
 851    HVGVNYEW*
```

Example 3 – hybrids of ΔG983

Protein 983 has the following sequence:

```
983                                                    ➡ ΔG983
  1 MRTTPTFPTK TFKPTAMALA VATTLSACLG GGGGGTSAPD FNAGGTGIGS
 51 NSRATTAKSA AVSYAGIKNE MCKDRSMLCA GRDDVAVTDR DAKINAPPPN
101 LHTGDFPNPN DAYKNLINLK PAIEAGYTGR GVEVGIVDTG ESVGSISFPE
151 LYGRKEHGYN ENYKNYTAYM RKEAPEDGGG KDIEASFDDE AVIETEAKPT
201 DIRHVKEIGH IDLVSHIIGG RSVDGRPAGG IAPDATLHIM NTNDETKNEM
251 MVAAIRNAWV KLGERGVRIV NNSFGTTSRA GTADLFQIAN SEEQYRQALL
301 DYSGGDKTDE GIRLMQQSDY GNLSYHIRNK NMLFIFSTGN DAQAQPNTYA
351 LLPFYEKDAQ KGIITVAGVD RSGEKFKREM YGEPGTEPLE YGSNHCGITA
401 MWCLSAPYEA SVRFTRTNPI QIAGTSFSAP IVTGTAALLL QKYPWMSNDN
451 LRTTLLTTAQ DIGAVGVDSK FGWGLLDAGK AMNGPASFPF GDFTADTKGT
501 SDIAYSFRND ISGTGGLIKK GGSQLQLHGN NTYTGKTIIE GGSLVLYGNN
551 KSDMRVETKG ALIYNGAASG GSLNSDGIVY LADTDQSGAN ETVHIKGSLQ
601 LDGKGTLYTR LGKLLKVDGT AIIGGKLYMS ARGKGAGYLN STGRRVPFLS
651 AAKIGQDYSF FTNIETDGGL LASLDSVEKT AGSEGDTLSY YVRRGNAART
701 ASAAAHSAPA GLKHAVEQGG SNLENLMVEL DASESSATPE TVETAAADRT
751 DMPGIRPYGA TFRAAAAVQH ANAADGVRIF NSLAATVYAD STAAHADMQG
801 RRLKAVSDGL DHNGTGLRVI AQTQQDGGTW EQGGVEGKMR GSTQTVGIAA
851 KTGENTTAAA TLGMGRSTWS ENSANAKTDS ISLFAGIRHD AGDIGYLKGL
901 FSYGRYKNSI SRSTGADEHA EGSVNGTLMQ LGALGGVNVP FAATGDLTVE
951 GGLRYDLLKQ DAFAEKGSAL GWSGNSLTEG TLVGLAGLKL SQPLSDKAVL
1001 FATAGVERDL NGRDYTVTGG FTGATAATGK TGARNMPHTR LVAGLGADVE
1051 FGNGWNGLAR YSYAGSKQYG NHSGRVGVGY RF*
```

ΔG983 thus has the following basic sequence:

```
                                                TSAPD FNAGGTGIGS
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LHTGDFPNPN DAYKNLINLK PAIEAGYTGR GVEVGIVDTG ESVGSISFPE
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 FGNGWNGLAR YSYAGSKQYG NHSGRVGVGY RF*
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ΔG983 was expressed as a hybrid, with ORF46.1, 741, 961 or 961c at its C-terminus:

ΔG983-ORF46.1

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<u>AG983-741</u>

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ΔG983-961

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GCAGCCGACAAGGCCGAAGCTGTCGCTGCAAAAGTTACCGACATCAAAGCTGATATCGCTACGAACAAAGATAATATT
GCTAAAAAAGCAAACAGTGCCGACGTGTACACCAGAGAAGAGTCTGACAGCAAATTTGTCAGAATTGATGGTCTGAAC
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CAACCTTACAACGTGGGTCGGTTCAATGTAACGGCTGCAGTCGGCGGCTACAAATCCGAATCGGCAGTCGCCATCGGT
ACCGGCTTCCGCTTTACCGAAAACTTTGCCGCCAAAGCAGGCGTGGCAGTCGGCACTTCGTCCGGTTCTTCCGCAGCC
TACCATGTCGGCGTCAATTACGAGTGGCTCGAGCACCACCACCACCACCACTGA

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   1    MTSAPDFNAG GTGIGSNSRA TTAKSAAVSY AGIKNEMCKD RSMLCAGRDD
  51    VAVTDRDAKI NAPPPNLHTG DFPNPNDAYK NLINLKPAIE AGYTGRGVEV
 101    GIVDTGESVG SISFPELYGR KEHGYNENYK NYTAYMRKEA PEDGGGKDIE
 151    ASFDDEAVIE TEAKPTDIRH VKEIGHIDLV SHIIGGRSVD GRPAGGIAPD
 201    ATLHIMNTND ETKNEMMVAA IRNAWVKLGE RGVRIVNNSF GTTSRAGTAD
 251    LFQIANSEEQ YRQALLDYSG GDKTDEGIRL MQQSDYGNLS YHIRNKNMLF
 301    IFSTGNDAQA QPNTYALLPF YEKDAQKGII TVAGVDRSGE KFKREMYGEP
 351    GTEPLEYGSN HCGITAMWCL SAPYEASVRF TRTNPIQIAG TSFSAPIVTG
 401    TAALLLQKYP WMSNDNLRTT LLTTAQDIGA VGVDSKFGWG LLDAGKAMNG
 451    PASFPFGDFT ADTKGTSDIA YSFRNDISGT GGLIKKGGSQ LQLHGNNTYT
 501    GKTIIEGGSL VLYGNNKSDM RVETKGALIY NGAASGGSLN SDGIVYLADT
 551    DQSGANETVH IKGSLQLDGK GTLYTRLGKL LKVDGTAIIG GKLYMSARGK
 601    GAGYLNSTGR RVPFLSAAKI GQDYSFFTNI ETDGGLLASL DSVEKTAGSE
 651    GDTLSYYVRR GNAARTASAA AHSAPAGLKH AVEQGGSNLE NLMVELDASE
 701    SSATPETVET AAADRTDMPG IRPYGATFRA AAAVQHANAA DGVRIFNSLA
 751    ATVYADSTAA HADMQGRRLK AVSDGLDHNG TGLRVIAQTQ QDGGTWEQGG
 801    VEGKMRGSTQ TVGIAAKTGE NTTAAATLGM GRSTWSENSA NAKTDSISLF
 851    AGIRHDAGDI GYLKGLFSYG RYKNSISRST GADEHAEGSV NGTLMQLGAL
 901    GGVNVPFAAT GDLTVEGGLR YDLLKQDAFA EKGSALGWSG NSLTEGTLVG
 951    LAGLKLSQPL SDKAVLFATA GVERDLNGRD YTVTGGFTGA TAATGKTGAR
1001    NMPHTRLVAG LGADVEFGNG WNGLARYSYA GSKQYGNHSG RVGVGYRFLE
1051    GGGGTGSATN DDDVKKAATV AIAAAYNNGQ EINGFKAGET IYDIDEDGTI
1101    TKKDATAADV EADDFKGLGL KKVVTNLTKT VNENKQNVDA KVKAAESEIE
1151    KLTTKLADTD AALADTDAAL DATTNALNKL GENITTFAEE TKTNIVKIDE
1201    KLEAVADTVD KHAEAFNDIA DSLDETNTKA DEAVKTANEA KQTAEETKQN
1251    VDAKVKAAET AAGKAEAAAG TANTAADKAE AVAAKVTDIK ADIATNKDNI
1301    AKKANSADVY TREESDSKFV RIDGLNATTE KLDTRLASAE KSIADHDTRL
1351    NGLDKTVSDL RKETRQGLAE QAALSGLFQP YNVGRFNVTA AVGGYKSESA
1401    VAIGTGFRFT ENFAAKAGVA VGTSSGSSAA YHVGVNYEWL EHHHHHH*
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ΔG983-961c

ATGACTTCTGCGCCCGACTTCAATGCAGGCGGTACCGGTATCGGCAGCAACAGCAGAGCAACAACAGCGAAATCAGCA
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AATGATGAAACCAAGAACGAAATGATGGTTGCAGCCATCCGCAATGCATGGGTCAAGCTGGGCGAACGTGGCGTGCGC
ATCGTCAATAACAGTTTTGGAACAACATCGAGGGCAGGCACTGCCGACCTTTTCCAAATAGCCAATTCGGAGGAGCAG
TACCGCCAAGCGTTGCTCGACTATTCCGGCGGTGATAAAACAGACGAGGGTATCCGCCTGATGCAACAGAGCGATTAC
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AACACATATGCCCTATTGCCATTTTATGAAAAAGACGCTCAAAAAGGCATTATCACAGTCGCAGGCGTAGACCGCAGT
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ACTGCCATGTGGTGCCTGTCGGCACCCTATGAAGCAAGCGTCCGTTTCACCCGTACAAACCCGATTCAAATTGCCGGA
ACATCCTTTTCCGCACCCATCGTAACCGGCACGGCGGCTCTGCTGCTGCAGAAATACCCGTGGATGAGCAACGACAAC
CTGCGTACCACGTTGCTGACGACGGCTCAGGACATCGGTGCAGTCGGCGTGGACAGCAAGTTCGGCTGGGGACTGCTG
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GCAGAATCTGAAATAGAAAAGTTAACAACCAAGTTAGCAGACACTGATGCCGCTTTAGCAGATACTGATGCCGCTCTG
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TTGGATAAAACAGTGTCAGACCTGCGCAAAGAAACCCGCCAAGGCCTTGCAGAACAAGCCGCGCTCTCCGGTCTGTTC
CAACCTTACAACGTGGGTCTCGAGCACCACCACCACCACCACTGA

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  1    MTSAPDFNAG GTGIGSNSRA TTAKSAAVSY AGIKNEMCKD RSMLCAGRDD
 51    VAVTDRDAKI NAPPPNLHTG DFPNPNDAYK NLINLKPAIE AGYTGRGVEV
101    GIVDTGESVG SISFPELYGR KEHGYNENYK NYTAYMRKEA PEDGGGKDIE
151    ASFDDEAVIE TEAKPTDIRH VKEIGHIDLV SHIIGGRSVD GRPAGGIAPD
201    ATLHIMNTND ETKNEMMVAA IRNAWVKLGE RGVRIVNNSF GTTSRAGTAD
251    LFQIANSEEQ YRQALLDYSG GDKTDEGIRL MQQSDYGNLS YHIRNKNMLF
301    IFSTGNDAQA QPNTYALLPF YEKDAQKGII TVAGVDRSGE KFKREMYGEP
351    GTEPLEYGSN HCGITAMWCL SAPYEASVRF TRTNPIQIAG TSFSAPIVTG
401    TAALLLQKYP WMSNDNLRTT LLTTAQDIGA VGVDSKFGWG LLDAGKAMNG
451    PASFPFGDFT ADTKGTSDIA YSFRNDISGT GGLIKKGGSQ LQLHGNNTYT
501    GKTIIEGGSL VLYGNNKSDM RVETKGALIY NGAASGGSLN SDGIVYLADT
551    DQSGANETVH IKGSLQLDGK GTLYTRLGKL LKVDGTAIIG GKLYMSARGK
601    GAGYLNSTGR RVPFLSAAKI GQDYSFFTNI ETDGGLLASL DSVEKTAGSE
651    GDTLSYYVRR GNAARTASAA AHSAPAGLKH AVEQGGSNLE NLMVELDASE
701    SSATPETVET AAADRTDMPG IRPYGATFRA AAAVQHANAA DGVRIFNSLA
751    ATVYADSTAA HADMQGRRLK AVSDGLDHNG TGLRVIAQTQ QDGGTWEQGG
801    VEGKMRGSTQ TVGIAAKTGE NTTAAATLGM GRSTWSENSA NAKTDSISLF
851    AGIRHDAGDI GYLKGLFSYG RYKNSISRST GADEHAEGSV NGTLMQLGAL
901    GGVNVPFAAT GDLTVEGGLR YDLLKQDAFA EKGSALGWSG NSLTEGTLVG
951    LAGLKLSQPL SDKAVLFATA GVERDLNGRD YTVTGGFTGA TAATGKTGAR
1001   NMPHTRLVAG LGADVEFGNG WNGLARYSYA GSKQYGNHSG RVGVGYRFLE
1051   GGGGTGSATN DDDVKKAATV AIAAAYNNGQ EINGFKAGET IYDIDEDGTI
1101   TKKDATAADV EADDFKGLGL KKVVTNLTKT VNENKQNVDA KVKAAESEIE
1151   KLTTKLADTD AALADTDAAL DATTNALNKL GENITTFAEE TKTNIVKIDE
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1201    KLEAVADTVD KHAEAFNDIA DSLDETNTKA DEAVKTANEA KQTAEETKQN
1251    VDAKVKAAET AAGKAEAAAG TANTAADKAE AVAAKVTDIK ADIATNKDNI
1301    AKKANSADVY TREESDSKFV RIDGLNATTE KLDTRLASAE KSIADHDTRL
1351    NGLDKTVSDL RKETRQGLAE QAALSGLFQP YNVGLEHHHH HH*
```

Example 4 – hybrids of ΔG741

Protein 741 has the following sequence:

```
  1  VNRTAFCCLS LTTALILTAC SSGGGGVAAD IGAGLADALT APLDHKDKGL
 51  QSLTLDQSVR KNEKLKLAAQ GAEKTYGNGD SLNTGKLKND KVSRFDFIRQ
101  IEVDGQLITL ESGEFQVYKQ SHSALTAFQT EQIQDSEHSG KMVAKRQFRI
151  GDIAGEHTSF DKLPEGGRAT YRGTAFGSDD AGGKLTYTID FAAKQGNGKI
201  EHLKSPELNV DLAAADIKPD GKRHAVISGS VLYNQAEKGS YSLGIFGGKA
251  QEVAGSAEVK TVNGIRHIGL AAKQ*
```

ΔG741 thus has the following basic sequence:

```
                                VAAD IGAGLADALT APLDHKDKGL
QSLTLDQSVR KNEKLKLAAQ GAEKTYGNGD SLNTGKLKND KVSRFDFIRQ
IEVDGQLITL ESGEFQVYKQ SHSALTAFQT EQIQDSEHSG KMVAKRQFRI
GDIAGEHTSF DKLPEGGRAT YRGTAFGSDD AGGKLTYTID FAAKQGNGKI
EHLKSPELNV DLAAADIKPD GKRHAVISGS VLYNQAEKGS YSLGIFGGKA
QEVAGSAEVK TVNGIRHIGL AAKQ*
```

ΔG741 was fused directly in-frame upstream of proteins 961, 961c, 983 and ORF46.1:

ΔG741-961

```
ATGGTCGCCGCCGACATCGGTGCGGGGCTTGCCGATGCACTAACCGCACCGCTCGACCATAAAGACAAAGGTTTGCAG
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AACGGTGACAGCCTCAATACGGGCAAATTGAAGAACGACAAGGTCAGCCGTTTCGACTTTATCCGCCAAATCGAAGTG
GACGGGCAGCTCATTACCTTGGAGAGTGGAGAGTTCCAAGTATACAAACAAAGCCATTCCGCCTTAACCGCCTTTCAG
ACCGAGCAAATACAAGATTCGGAGCATTCCGGGAAGATGGTTGCGAAACGCCAGTTCAGAATCGGCGACATAGCGGGC
GAACATACATCTTTTGACAAGCTTCCCGAAGGCGGCAGGGCGACATATCGCGGGACGGCGTTCGGTTCAGACGATGCC
GGCGGAAAACTGACCTACACCATAGATTTCGCCGCCAAGCAGGGAAACGGCAAAATCGAACATTTGAAATCGCCAGAA
CTCAATGTCGACCTGGCCGCCGCCGATATCAAGCCGGATGGAAAACGCCATGCCGTCATCAGCGGTTCCGTCCTTTAC
AACCAAGCCGAGAAAGGCAGTTACTCCCTCGGTATCTTTGGCGGAAAAGCCCAGGAAGTTGCCGGCAGCGCGGAAGTG
AAAACCGTAAACGGCATACGCCATATCGGCCTTGCCGCCAAGCAACTCGAGGGTGGCGGAGGCACTGGATCCGCCACA
AACGACGACGATGTTAAAAAAGCTGCCACTGTGGCCATTGCTGCTGCCTACAACAATGGCCAAGAAATCAACGGTTTC
AAAGCTGGAGAGACCATCTACGACATTGATGAAGACGGCACAATTACCAAAAAAGACGCAACTGCAGCCGATGTTGAA
GCCGACGACTTTAAAGGTCTGGGTCTGAAAAAAGTCGTGACTAACCTGACCAAAACCGTCAATGAAAACAAACAAAAC
GTCGATGCCAAAGTAAAAGCTGCAGAATCTGAAATAGAAAAGTTAACAACCAAGTTAGCAGACACTGATGCCGCTTTA
GCAGATACTGATGCCGCTCTGGATGCAACCACCAACGCCTTGAATAAATTGGGAGAAAATATAACGACATTTGCTGAA
GAGACTAAGACAAATATCGTAAAAATTGATGAAAAATTAGAAGCCGTGGCTGATACCGTCGACAAGCATGCCGAAGCA
TTCAACGATATCGCCGATTCATTGGATGAAACCAACACTAAGGCAGACGAAGCCGTCAAAACCGCCAATGAAGCCAAA
CAGACGGCCGAAGAAACCAAACAAAACGTCGATGCCAAAGTAAAAGCTGCAGAAACTGCAGCAGGCAAAGCCGAAGCT
GCCGCTGGCACAGCTAATACTGCAGCCGACAAGGCCGAAGCTGTCGCTGCAAAAGTTACCGACATCAAAGCTGATATC
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CACGATACTCGCCTGAACGGTTTGGATAAAACAGTGTCAGACCTGCGCAAAGAAACCCGCCAAGGCCTTGCAGAACAA
GCCGCGCTCTCCGGTCTGTTCCAACCTTACAACGTGGGTCGGTTCAATGTAACGGCTGCAGTCGGCGGCTACAAATCC
GAATCGGCAGTCGCCATCGGTACCGGCTTCCGCTTTACCGAAAACTTTGCCGCCAAAGCAGGCGTGGCAGTCGGCACT
TCGTCCGGTTCTTCCGCAGCCTACCATGTCGGCGTCAATTACGAGTGGCTCGAGCACCACCACCACCACCACTGA
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```
  1  MVAADIGAGL ADALTAPLDH KDKGLQSLTL DQSVRKNEKL KLAAQGAEKT
 51  YGNGDSLNTG KLKNDKVSRF DFIRQIEVDG QLITLESGEF QVYKQSHSAL
101  TAFQTEQIQD SEHSGKMVAK RQFRIGDIAG EHTSFDKLPE GGRATYRGTA
151  FGSDDAGGKL TYTIDFAAKQ GNGKIEHLKS PELNVDLAAA DIKPDGKRHA
201  VISGSVLYNQ AEKGSYSLGI FGGKAQEVAG SAEVKTVNGI RHIGLAAKQL
251  EGGGGTGSAT NDDDVKKAAT VAIAAAYNNG QEINGFKAGE TIYDIDEDGT
301  ITKKDATAAD VEADDFKGLG LKKVVTNLTK TVNENKQNVD AKVKAAESEI
351  EKLTTKLADT DAALADTDAA LDATTNALNK LGENITTFAE ETKTNIVKID
401  EKLEAVADTV DKHAEAFNDI ADSLDETNTK ADEAVKTANE AKQTAEETKQ
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451 NVDAKVKAAE TAAGKAEAAA GTANTAADKA EAVAAKVTDI KADIATNKDN
501 IAKKANSADV YTREESDSKF VRIDGLNATT EKLDTRLASA EKSIADHDTR
551 LNGLDKTVSD LRKETRQGLA EQAALSGLFQ PYNVGRFNVT AAVGGYKSES
601 AVAIGTGFRF TENFAAKAGV AVGTSSGSSA AYHVGVNYEW LEHHHHHH*

ΔG741-961c

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AACGGTGACAGCCTCAATACGGGCAAATTGAAGAACGACAAGGTCAGCCGTTTCGACTTTATCCGCCAAATCGAAGTG
GACGGGCAGCTCATTACCTTGGAGAGTGGAGAGTTCCAAGTATACAAACAAAGCCATTCCGCCTTAACCGCCTTTCAG
ACCGAGCAAATACAAGATTCGGAGCATTCCGGGAAGATGGTTGCGAAACGCCAGTTCAGAATCGGCGACATAGCGGGC
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FGSDDAGGKL TYTIDFAAKQ GNGKIEHLKS PELNVDLAAA DIKPDGKRHA
VISGSVLYNQ AEKGSYSLGI FGGKAQEVAG SAEVKTVNGI RHIGLAAKQL
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EKLEAVADTV DKHAEAFNDI ADSLDETNTK ADEAVKTANE AKQTAEETKQ
NVDAKVKAAE TAAGKAEAAA GTANTAADKA EAVAAKVTDI KADIATNKDN
IAKKANSADV YTREESDSKF VRIDGLNATT EKLDTRLASA EKSIADHDTR
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ΔG741-983

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TGCCTGTCGGCACCCTATGAAGCAAGCGTCCGTTTCACCCGTACAAACCCGATTCAAATTGCCGGAACATCCTTTTCC
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GCCATGAACGGACCCGCGTCCTTTCCGTTCGGCGACTTTACCGCCGATACGAAAGGTACATCCGATATTGCCTACTCC
TTCCGTAACGACATTTCAGGCACGGGCGGCCTGATCAAAAAAGGCGGCAGCCAACTGCAACTGCACGGCAACAACACC
TATACGGGCAAAACCATTATCGAAGGCGGTTCGCTGGTGTTGTACGGCAACAACAAATCGGATATGCGCGTCGAAACC
AAAGGTGCGCTGATTTATAACGGGGCGGCATCCGGCGGCAGCCTGAACAGCGACGGCATTGTCTATCTGGCAGATACC
GACCAATCCGGCGCAAACGAAACCGTACACATCAAAGGCAGTCTGCAGCTGGACGGCAAAGGTACGCTGTACACACGT
TTGGGCAAACTGCTGAAAGTGGACGGTACGGCGATTATCGGCGGCAAGCTGTACATGTCGGCACGCGGCAAGGGGCA
GGCTATCTCAACAGTACCGGACGACGTGTTCCCTTCCTGAGTGCCGCCAAAATCGGGCAGGATTATTCTTTCTTCACA
AACATCGAAACCGACGGCGGCCTGCTGGCTTCCCTCGACAGCGTCGAAAAAACAGCGGGCAGTGAAGGCGACACGCTG
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GCCGTAGAACAGGGCGGCAGCAATCTGGAAAACCTGATGGTCGAACTGGATGCCTCCGAATCATCCGCAACACCCGAG
ACGGTTGAAACTGCGGCAGCCGACCGCACAGATATGCCGGGCATCCGCCCCTACGGCGCAACTTTCCGCGCAGCGGCA
GCCGTACAGCATGCGAATGCCGCCGACGGTGTACGCATCTTCAACAGTCTCGCCGCTACCGTCTATGCCGACAGTACC
GCCGCCCATGCCGATATGCAGGGACGCCGCCTGAAAGCCGTATCGGACGGGTTGGACCACAACGGCACGGGTCTGCGC
GTCATCGCGCAAACCCAACAGGACGGTGGAACGTGGGAACAGGGCGGTGTTGAAGGCAAAATGCGCGGCAGTACCCAA
ACCGTCGGCATTGCCGCGAAAACCGGCGAAAATACGACAGCAGCCGCCACACTGGGCATGGGACGCAGCACATGGAGC
GAAAACAGTGCAAATGCAAAAACCGACAGCATTAGTCTGTTTGCAGGCATACGGCACGATGCGGGCGATATCGGCTAT
CTCAAAGGCCTGTTCTCCTACGGACGCTACAAAAACAGCATCAGCCGCAGCACCGGTGCGGACGAACATGCGGAAGGC
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GTCGAAGGCGGTCTGCGCTACGACCTGCTCAAACAGGATGCATTCGCCGAAAAAGGCAGTGCTTTGGGCTGGAGCGGC
AACAGCCTCACTGAAGGCACGCTGGTCGGACTCGCGGGTCTGAAGCTGTCGCAACCCTTGAGCGATAAAGCCGTCCTG
TTTGCAACGGCGGGCGTGGAACGCGACCTGAACGGACGCGACTACACGGTAACGGGCGGCTTTACCGGCGCGACTGCA
GCAACCGGCAAGACGGGGGCACGCAATATGCCGCACACCCGTCTGGTTGCCGGCCTGGGCGCGGATGTCGAATTCGGC
AACGGCTGGAACGGCTTGGCACGTTACAGCTACGCCGGTTCCAAACAGTACGGCAACCACAGCGGACGAGTCGGCGTA
GGCTACCGGTTCCTCGAGCACCACCACCACCACCACTGA

1 MVAADIGAGL ADALTAPLDH KDKGLQSLTL DQSVRKNEKL KLAAQGAEKT
51 YGNGDSLNTG KLKNDKVSRF DFIRQIEVDG QLITLESGEF QVYKQSHSAL
101 TAFQTEQIQD SEHSGKMVAK RQFRIGDIAG EHTSFDKLPE GGRATYRGTA
151 FGSDDAGGKL TYTIDFAAKQ GNGKIEHLKS PELNVDLAAA DIKPDGKRHA
201 VISGSVLYNQ AEKGSYSLGI FGGKAQEVAG SAEVKTVNGI RHIGLAAKQL
251 EGSGGGGTSA PDFNAGGTGI GSNSRATTAK SAAVSYAGIK NEMCKDRSML
301 CAGRDDVAVT DRDAKINAPP PNLHTGDFPN PNDAYKNLIN LKPAIEAGYT
351 GRGVEVGIVD TGESVGSISF PELYGRKEHG YNENYKNYTA YMRKEAPEDG
401 GGKDIEASFD DEAVIETEAK PTDIRHVKEI GHIDLVSHII GGRSVDGRPA
451 GGIAPDATLH IMNTNDETKN EMMVAAIRNA WVKLGERGVR IVNNSFGTTS
501 RAGTADLFQI ANSEEQYRQA LLDYSGGDKT DEGIRLMQQS DYGNLSYHIR
551 NKNMLFIFST GNDAQAQPNT YALLPFYEKD AQKGIITVAG VDRSGEKFKR
601 EMYGEPGTEP LEYGSNHCGI TAMWCLSAPY EASVRFTRTN PIQIAGTSFS
651 APIVTGTAAL LLQKYPWMSN DNLRTTLLTT AQDIGAVGVD SKFGWGLLDA
701 GKAMNGPASF PFGDFTADTK GTSDIAYSFR NDISGTGGLI KKGGSQLQLH
751 GNNTYTGKTI IEGGSLVLYG NNKSDMRVET KGALIYNGAA SGGSLNSDGI
801 VYLADTDQSG ANETVHIKGS LQLDGKGTLY TRLGKLLKVD GTAIIGGKLY
851 MSARGKGAGY LNSTGRRVPF LSAAKIGQDY SFFTNIETDG GLLASLDSVE
901 KTAGSEGDTL SYYVRRGNAA RTASAAAHSA PAGLKHAVEQ GGSNLENLMV
951 ELDASESSAT PETVETAAAD RTDMPGIRPY GATFRAAAAV QHANAADGVR
1001 IFNSLAATVY ADSTAAHADM QGRRLKAVSD GLDHNGTGLR VIAQTQQDGG
1051 TWEQGGVEGK MRGSTQTVGI AAKTGENTTA AATLGMGRST WSENSANAKT
1101 DSISLFAGIR HDAGDIGYLK GLFSYGRYKN SISRSTGADE HAEGSVNGTL
1151 MQLGALGGVN VPFAATGDLT VEGGLRYDLL KQDAFAEKGS ALGWSGNSLT
1201 EGTLVGLAGL KLSQPLSDKA VLFATAGVER DLNGRDYTVT GGFTGATAAT
1251 GKTGARNMPH TRLVAGLGAD VEFGNGWNGL ARYSYAGSKQ YGNHSGRVGV
1301 GYRFLEHHHH HH*

ΔG741–ORF46.1

ATGGTCGCCGCCGACATCGGTGCGGGGCTTGCCGATGCACTAACCGCACCGCTCGACCATAAAGACAAAGGTTTGCAG
TCTTTGACGCTGGATCAGTCCGTCAGGAAAAACGAGAAACTGAAGCTGGCGGCACAAGGTGCGGAAAAAACTTATGGA
AACGGTGACAGCCTCAATACGGGCAAATTGAAGAACGACAAGGTCAGCCGTTTCGACTTTATCCGCCAAATCGAAGTG
GACGGGCAGCTCATTACCTTGGAGAGTGGAGAGTTCCAAGTATACAAACAAAGCCATTCCGCCTTAACCGCCTTTCAG
ACCGAGCAAATACAAGATTCGGAGCATTCCGGGAAGATGGTTGCGAAACGCCAGTTCAGAATCGGCGACATAGCGGGC
GAACATACATCTTTTGACAAGCTTCCCGAAGGCGGCAGGGCGACATATCGCGGGACGGCGTTCGGTTCAGACGATGCC

GGCGGAAAACTGACCTACACCATAGATTTCGCCGCCAAGCAGGGAAACGGCAAAATCGAACATTTGAAATCGCCAGAA
CTCAATGTCGACCTGGCCGCCGCCGATATCAAGCCGGATGGAAAACGCCATGCCGTCATCAGCGGTTCCGTCCTTTAC
AACCAAGCCGAGAAAGGCAGTTACTCCCTCGGTATCTTTGGCGGAAAAGCCCAGGAAGTTGCCGGCAGCGCGGAAGTG
AAAACCGTAAACGGCATACGCCATATCGGCCTTGCCGCCAAGCAACTCGACGGTGGCGGAGGCACTGGATCCTCAGAT
TTGGCAAACGATTCTTTTATCCGGCAGGTTCTCGACCGTCAGCATTTCGAACCCGACGGGAAATACCACCTATTCGGC
AGCAGGGGGGAACTTGCCGAGCGCAGCGGCCATATCGGATTGGGAAAAATACAAAGCCATCAGTTGGGCAACCTGATG
ATTCAACAGGCGGCCATTAAAGGAAATATCGGCTACATTGTCCGCTTTTCCGATCACGGGCACGAAGTCCATTCCCCC
TTCGACAACCATGCCTCACATTCCGATTCTGATGAAGCCGGTAGTCCCGTTGACGGATTTAGCCTTTACCGCATCCAT
TGGGACGGATACGAACACCATCCCGCCGACGGCTATGACGGGCCACAGGGCGGCGGCTATCCCGCTCCCAAAGGCGCG
AGGGATATATACAGCTACGACATAAAAGGCGTTGCCCAAAATATCCGCCTCAACCTGACCGACAACCGCAGCACCGGA
CAACGGCTTGCCGACCGTTTCCACAATGCCGGTAGTATGCTGACGCAAGGAGTAGGCGACGGATTCAAACGCGCCACC
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ACCTCCTCAACCGTGCCGCCGTCAAACGGCAAAAATGTCAAACTGGCAGACCAACGCCACCCGAAGACAGGCGTACCG
TTTGACGGTAAAGGGTTTCCGAATTTTGAGAAGCACGTGAAATATGATACGCTCGAGCACCACCACCACCACCACTGA

MVAADIGAGL ADALTAPLDH KDKGLQSLTL DQSVRKNEKL KLAAQGAEKT
YGNGDSLNTG KLKNDKVSRF DFIRQIEVDG QLITLESGEF QVYKQSHSAL
TAFQTEQIQD SEHSGKMVAK RQFRIGDIAG EHTSFDKLPE GGRATYRGTA
FGSDDAGGKL TYTIDFAAKQ GNGKIEHLKS PELNVDLAAA DIKPDGKRHA
VISGSVLYNQ AEKGSYSLGI FGGKAQEVAG SAEVKTVNGI RHIGLAAKQL
DGGGGTGSSD LANDSFIRQV LDRQHFEPDG KYHLFGSRGE LAERSGHIGL
GKIQSHQLGN LMIQQAAIKG NIGYIVRFSD HGHEVHSPFD NHASHSDSDE
AGSPVDGFSL YRIHWDGYEH HPADGYDGPQ GGGYPAPKGA RDIYSYDIKG
VAQNIRLNLT DNRSTGQRLA DRFHNAGSML TQGVGDGFKR ATRYSPELDR
SGNAAEAFNG TADIVKNIIG AAGEIVGAGD AVQGISEGSN IAVMHGLGLL
STENKMARIN DLADMAQLKD YAAAAIRDWA VQNPNAAQGI EAVSNIFMAA
IPIKGIGAVR GKYGLGGITA HPIKRSQMGA IALPKGKSAV SDNFADAAYA
KYPSPYHSRN IRSNLEQRYG KENITSSTVP PSNGKNVKLA DQRHPKTGVP
FDGKGFPNFE KHVKYDTLEH HHHHH*

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 *Nde*I and a *Bam*HI restriction sites respectively, whereas for the amplification of the 287 gene the 5' and the 3' primers included a *Bam*HI and a *Xho*I restriction sites respectively. In this way a sequential directional cloning of the two genes in pET21b+, using *Nde*I-*Bam*HI (to clone the first gene) and subsequently *Bam*HI-*Xho*I (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 *Xho*I 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 *Sal*I restriction site at the 5'- and a *Xho*I site at the 3'- of the PCR fragment. Since the cohesive ends produced by the *Sal*I and *Xho*I restriction enzymes are compatible, the 287 PCR product digested with *Sal*I-*Xho*I could be inserted in the pET21b-919 clone cleaved with *Xho*I.

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:

	Mixture with 287	**Hybrid with 287**
919	32000	16000
953	8192	8192
ORF46.1	128	8192

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

	919		**953**		**ORF46.1**	
Strain	*Mixture*	*Hybrid*	*Mixture*	*Hybrid*	*Mixture*	*Hybrid*
MC58	512	1024	512	1024	-	1024
NGH38	1024	2048	2048	4096	-	4096
BZ232	512	128	1024	16	-	-
MenA (F6124)	512	2048	2048	32	-	1024
MenC (C11)	>2048	n.d.	>2048	n.d.	-	n.d.
MenC (BZ133)	>4096	>8192	>4096	<16	-	2048

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 fitres and bactericidal antibody responses.

As hybrids of two proteins A & B may be either NH_2–A–B–COOH or NH_2–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:

	287 & 919		287 & 953		287 & ORF46.1	
Strain	*ΔG287-919*	*919-287*	*ΔG287-953*	*953-287*	*ΔG287-46.1*	*46.1-287*
2996	128000	16000	65536	8192	16384	8192
BZ232	256	128	128	<4	<4	<4
1000	2048	<4	<4	<4	<4	<4
MC58	8192	1024	16384	1024	512	128
NGH38	32000	2048	>2048	4096	16384	4096
394/98	4096	32	256	128	128	16
MenA (F6124)	32000	2048	>2048	32	8192	1024
MenC (BZ133)	64000	>8192	>8192	<16	8192	2048

Better bactericidal titres are generally seen with 287 at the N-terminus.

When fused to protein 961 [NH_2-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):

	2996	BZ232	MC58	NGH38	F6124	BZ133
Soluble	65536	128	4096	>2048	>2048	4096
Insoluble	8192	<4	<4	16	n.d.	n.d.

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

Insoluble	32768	128	4096	>2048	>2048	2048

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

ATGTCAGATTTGGCAAACGATTCTTTTATCCGGCAGGTTCTCGACCGTCAGCATTTCGAACCCGACGGGAAATACCAC
CTATTCGGCAGCAGGGGGGAACTTGCCGAGCGCAGCGGCCATATCGGATTGGGAAAAATACAAAGCCATCAGTTGGGC
AACCTGATGATTCAACAGGCGGCCATTAAAGGAAATATCGGCTACATTGTCCGCTTTTCCGATCACGGGCACGAAGTC
CATTCCCCCTTCGACAACCATGCCTCACATTCCGATTCTGATGAAGCCGGTAGTCCCGTTGACGGATTTAGCCTTTAC
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AGCACCGGACAACGGCTTGCCGACCGTTTCCACAATGCCGGTAGTATGCTGACGCAAGGAGTAGGCGACGGATTCAAA
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AGCAATATCTTTATGGCAGCCATCCCCATCAAAGGGATTGGAGCTGTTCGGGGAAAATACGGCTTGGGCGGCATCACG
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GATGCGGCATACGCCAAATACCCGTCCCCTTACCATTCCCGAAATATCCGTTCAAACTTGGAGCAGCGTTACGGCAAA
GAAAACATCACCTCCTCAACCGTGCCGCCGTCAAACGGCAAAAATGTCAAACTGGCAGACCAACGCCACCCGAAGACA
GGCGTACCGTTTGACGGTAAAGGGTTTCCGAATTTTGAGAAGCACGTGAAATATGATACGGGATCCGGAGGGGGTGGT
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GGTGACAGCCTCAATACGGGCAAATTGAAGAACGACAAGGTCAGCCGTTTCGACTTTATCCGCCAAATCGAAGTGGAC
GGGCAGCTCATTACCTTGGAGAGTGGAGAGTTCCAAGTATACAAACAAAGCCATTCCGCCTTAACCGCCTTTCAGACC
GAGCAAATACAAGATTCGGAGCATTCCGGGAAGATGGTTGCGAAACGCCAGTTCAGAATCGGCGACATAGCGGGCGAA
CATACATCTTTTGACAAGCTTCCCGAAGGCGGCAGGGCGACATATCGCGGGACGGCGTTCGGTTCAGACGATGCCGGC
GGAAAACTGACCTACACCATAGATTTCGCCGCCAAGCAGGGAAACGGCAAAATCGAACATTTGAAATCGCCAGAACTC
AATGTCGACCTGGCCGCCGCCGATATCAAGCCGGATGGAAAACGCCATGCCGTCATCAGCGGTTCCGTCCTTTACAAC
CAAGCCGAGAAAGGCAGTTACTCCCTCGGTATCTTTGGCGGAAAAGCCCAGGAAGTTGCCGGCAGCGCGGAAGTGAAA
ACCGTAAACGGCATACGCCATATCGGCCTTGCCGCCAAGCAACTCGAGCACCACCACCACCACCACTGA

1	MSDLANDSFI RQVLDRQHFE PDGKYHLFGS RGELAERSGH IGLGKIQSHQ
51	LGNLMIQQAA IKGNIGYIVR FSDHGHEVHS PFDNHASHSD SDEAGSPVDG
101	FSLYRIHWDG YEHHPADGYD GPQGGGYPAP KGARDIYSYD IKGVAQNIRL
151	NLTDNRSTGQ RLADRFHNAG SMLTQGVGDG FKRATRYSPE LDRSGNAAEA
201	FNGTADIVKN IIGAAGEIVG AGDAVQGISE GSNIAVMHGL GLLSTENKMA
251	RINDLADMAQ LKDYAAAAIR DWAVQNPNAA QGIEAVSNIF MAAIPIKGIG
301	AVRGKYGLGG ITAHPIKRSQ MGAIALPKGK SAVSDNFADA AYAKYPSPYH
351	SRNIRSNLEQ RYGKENITSS TVPPSNGKNV KLADQRHPKT GVPFDGKGFP
401	NFEKHVKYDT GSGGGGVAAD IGAGLADALT APLDHKDKGL QSLTLDQSVR
451	KNEKLKLAAQ GAEKTYGNGD SLNTGKLKND KVSRFDFIRQ IEVDGQLITL
501	ESGEFQVYKQ SHSALTAFQT EQIQDSEHSG KMVAKRQFRI GDIAGEHTSF
551	DKLPEGGRAT YRGTAFGSDD AGGKLTYTID FAAKQGNGKI EHLKSPELNV
601	DLAAADIKPD GKRHAVISGS VLYNQAEKGS YSLGIFGGKA QEVAGSAEVK
651	TVNGIRHIGL AAKQLEHHHH HH*

ORF46.1-961

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CAAAACGTCGATGCCAAAGTAAAAGCTGCAGAATCTGAAATAGAAAAGTTAACAACCAAGTTAGCAGACACTGATGCC
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TGA

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   1    MSDLANDSFI RQVLDRQHFE PDGKYHLFGS RGELAERSGH IGLGKIQSHQ
  51    LGNLMIQQAA IKGNIGYIVR FSDHGHEVHS PFDNHASHSD SDEAGSPVDG
 101    FSLYRIHWDG YEHHPADGYD GPQGGGYPAP KGARDIYSYD IKGVAQNIRL
 151    NLTDNRSTGQ RLADRFHNAG SMLTQGVGDG FKRATRYSPE LDRSGNAAEA
 201    FNGTADIVKN IIGAAGEIVG AGDAVQGISE GSNIAVMHGL GLLSTENKMA
 251    RINDLADMAQ LKDYAAAAIR DWAVQNPNAA QGIEAVSNIF MAAIPIKGIG
 301    AVRGKYGLGG ITAHPIKRSQ MGAIALPKGK SAVSDNFADA AYAKYPSPYH
 351    SRNIRSNLEQ RYGKENITSS TVPPSNGKNV KLADQRHPKT GVPFDGKGFP
 401    NFEKHVKYDT GSGGGGATND DDVKKAATVA IAAAYNNGQE INGFKAGETI
 451    YDIDEDGTIT KKDATAADVE ADDFKGLGLK KVVTNLTKTV NENKQNVDAK
 501    VKAAESEIEK LTTKLADTDA ALADTDAALD ATTNALNKLG ENITTFAEET
 551    KTNIVKIDEK LEAVADTVDK HAEAFNDIAD SLDETNTKAD EAVKTANEAK
 601    QTAEETKQNV DAKVKAAETA AGKAEAAAGT ANTAADKAEA VAAKVTDIKA
 651    DIATNKDNIA KKANSADVYT REESDSKFVR IDGLNATTEK LDTRLASAEK
 701    SIADHDTRLN GLDKTVSDLR KETRQGLAEQ AALSGLFQPY NVGRFNVTAA
 751    VGGYKSESAV AIGTGFRFTE NFAAKAGVAV GTSSGSSAAY HVGVNYEWLE
 801    HHHHHH*
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ORF46.1-961c

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961-741

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961-983

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751	H*				

961c-741

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101	ADTDAALADT	DAALDATTNA	LNKLGENITT	FAEETKTNIV	KIDEKLEAVA
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961c-983

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961cL-741

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401 GAEKTYGNGD SLNTGKLKND KVSRFDFIRQ IEVDGQLITL ESGEFQVYKQ
451 SHSALTAFQT EQIQDSEHSG KMVAKRQFRI GDIAGEHTSF DKLPEGGRAT
501 YRGTAFGSDD AGGKLTYTID FAAKQGNGKI EHLKSPELNV DLAAADIKPD
551 GKRHAVISGS VLYNQAEKGS YSLGIFGGKA QEVAGSAEVK TVNGIRHIGL
601 AAKQLEHHHH HH*

961cL-983

ATGAAACACTTTCCATCCAAAGTACTGACCACAGCCATCCTTGCCACTTTCTGTAGCGGCGCACTGGCAGCCACAAAC
GACGACGATGTTAAAAAAGCTGCCACTGTGGCCATTGCTGCTGCCTACAACAATGGCCAAGAAATCAACGGTTTCAAA
GCTGGAGAGACCATCTACGACATTGATGAAGACGGCACAATTACCAAAAAAGACGCAACTGCAGCCGATGTTGAAGCC
GACGACTTTAAAGGTCTGGGTCTGAAAAAAGTCGTGACTAACCTGACCAAAACCGTCAATGAAAACAAACAAAACGTC
GATGCCAAAGTAAAAGCTGCAGAATCTGAAATAGAAAAGTTAACAACCAAGTTAGCAGACACTGATGCCGCTTTAGCA
GATACTGATGCCGCTCTGGATGCAACCACCAACGCCTTGAATAAATTGGGAGAAAATATAACGACATTTGCTGAAGAG
ACTAAGACAAATATCGTAAAAATTGATGAAAAATTAGAAGCCGTGGCTGATACCGTCGACAAGCATGCCGAAGCATTC
AACGATATCGCCGATTCATTGGATGAAACCAACACTAAGGCAGACGAAGCCGTCAAAACCGCCAATGAAGCCAAACAG
ACGGCCGAAGAAACCAAACAAAACGTCGATGCCAAAGTAAAAGCTGCAGAAACTGCAGCAGGCAAAGCCGAAGCTGCC
GCTGGCACAGCTAATACTGCAGCCGACAAGGCCGAAGCTGTCGCTGCAAAAGTTACCGACATCAAAGCTGATATCGCT
ACGAACAAAGATAATATTGCTAAAAAAGCAAACAGTGCCGACGTGTACACCAGAGAAGAGTCTGACAGCAAATTTGTC
AGAATTGATGGTCTGAACGCTACTACCGAAAAATTGGACACACGCTTGGCTTCTGCTGAAAAATCCATTGCCGATCAC
GATACTCGCCTGAACGGTTTGGATAAAACAGTGTCAGACCTGCGCAAAGAAACCCGCCAAGGCCTTGCAGAACAAGCC
GCGCTCTCCGGTCTGTTCCAACCTTACAACGTGGGTGGATCCGGCGGAGGCGGCACTTCTGCGCCCGACTTCAATGCA
GGCGGTACCGGTATCGGCAGCAACAGCAGAGCAACAACAGCGAAATCAGCAGCAGTATCTTACGCCGGTATCAAGAAC
GAAATGTGCAAAGACAGAAGCATGCTCTGTGCCGGTCGGGATGACGTTGCGGTTACAGACAGGGATGCCAAAATCAAT
GCCCCCCCCCCCGAATCTGCATACCGGAGACTTTCCAAACCCAAATGACGCATACAAGAATTTGATCAACCTCAAACCT
GCAATTGAAGCAGGCTATACAGGACGCGGGGTAGAGGTAGGTATCGTCGACACAGGCGAATCCGTCGGCAGCATATCC

TTTCCCGAACTGTATGGCAGAAAAGAACACGGCTATAACGAAAATTACAAAAACTATACGGCGTATATGCGGAAGGAA
GCGCCTGAAGACGGAGGCGGTAAAGACATTGAAGCTTCTTTCGACGATGAGGCCGTTATAGAGACTGAAGCAAAGCCG
ACGGATATCCGCCACGTAAAAGAAATCGGACACATCGATTTGGTCTCCCATATTATTGGCGGGCGTTCCGTGGACGGC
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GGAGAACCGGGTACAGAACCGCTTGAGTATGGCTCCAACCATTGCGGAATTACTGCCATGTGGTGCCTGTCGGCACCC
TATGAAGCAAGCGTCCGTTTCACCCGTACAAACCCGATTCAAATTGCCGGAACATCCTTTTCCGCACCCATCGTAACC
GGCACGGCGGCTCTGCTGCTGCAGAAATACCCGTGGATGAGCAACGACAACCTGCGTACCACGTTGCTGACGACGGCT
CAGGACATCGGTGCAGTCGGCGTGGACAGCAAGTTCGGCTGGGGACTGCTGGATGCGGGTAAGGCCATGAACGGACCC
GCGTCCTTTCCGTTCGGCGACTTTACCGCCGATACGAAAGGTACATCCGATATTGCCTACTCCTTCCGTAACGACATT
TCAGGCACGGGCGGCCTGATCAAAAAAGGCGGCAGCCAACTGCAACTGCACGGCAACAACACCTATACGGGCAAAACC
ATTATCGAAGGCGGTTCGCTGGTGTTGTACGGCAACAACAAATCGGATATGCGCGTCGAAACCAAAGGTGCGCTGATT
TATAACGGGGCGGCATCCGGCGGCAGCCTGAACAGCGACGGCATTGTCTATCTGGCAGATACCGACCAATCCGGCGCA
AACGAAACCGTACACATCAAAGGCAGTCTGCAGCTGGACGGCAAAGGTACGCTGTACACACGTTTGGGCAAACTGCTG
AAAGTGGACGGTACGGCGATTATCGGCGGCAAGCTGTACATGTCGGCACGCGGCAAGGGGGCAGGCTATCTCAACAGT
ACCGGACGACGTGTTCCCTTCCTGAGTGCCGCCAAAATCGGGCAGGATTATTCTTTCTTCACAAACATCGAAACCGAC
GGCGGCCTGCTGGCTTCCCTCGACAGCGTCGAAAAAACAGCGGGCAGTGAAGGCGACACGCTGTCCTATTATGTCCGT
CGCGGCAATGCGGCACGGACTGCTTCGGCAGCGGCACATTCCGCGCCCGCCGGTCTGAAACACGCCGTAGAACAGGGC
GGCAGCAATCTGGAAAACCTGATGGTCGAACTGGATGCCTCCGAATCATCCGCAACACCCGAGACGGTTGAAACTGCG
GCAGCCGACCGCACAGATATGCCGGGCATCCGCCCCTACGGCGCAACTTTCCGCGCAGCGGCAGCCGTACAGCATGCG
AATGCCGCCGACGGTGTACGCATCTTCAACAGTCTCGCCGCTACCGTCTATGCCGACAGTACCGCCGCCCATGCCGAT
ATGCAGGGACGCCGCCTGAAAGCCGTATCGGACGGGTTGGACCACAACGGCACGGGTCTGCGCGTCATCGCGCAAACC
CAACAGGACGGTGGAACGTGGGAACAGGGCGGTGTTGAAGGCAAAATGCGCGGCAGTACCCAAACCGTCGGCATTGCC
GCGAAAACCGGCGAAAATACGACAGCAGCCGCCACACTGGGCATGGGACGCAGCACATGGAGCGAAAACAGTGCAAAT
GCAAAAACCGACAGCATTAGTCTGTTTGCAGGCATACGGCACGATGCGGGCGATATCGGCTATCTCAAAGGCCTGTTC
TCCTACGGACGCTACAAAAACAGCATCAGCCGCAGCACCGGTGCGGACGAACATGCGGAAGGCAGCGTCAACGGCACG
CTGATGCAGCTGGGCGCACTGGGCGGTGTCAACGTTCCGTTTGCCGCAACGGGAGATTTGACGGTCGAAGGCGGTCTG
CGCTACGACCTGCTCAAACAGGATGCATTCGCCGAAAAAGGCAGTGCTTTGGGCTGGAGCGGCAACAGCCTCACTGAA
GGCACGCTGGTCGGACTCGCGGGTCTGAAGCTGTCGCAACCCTTGAGCGATAAAGCCGTCCTGTTTGCAACGGCGGGC
GTGGAACGCGACCTGAACGGACGCGACTACACGGTAACGGGCGGCTTTACCGGCGCGACTGCAGCAACCGGCAAGACG
GGGGCACGCAATATGCCGCACACCCGTCTGGTTGCCGGCCTGGGCGCGGATGTCGAATTCGGCAACGGCTGGAACGGC
TTGGCACGTTACAGCTACGCCGGTTCCAAACAGTACGGCAACCACAGCGGACGAGTCGGCGTAGGCTACCGGTTCTGA
CTCGAG

1	MKHFPSKVLT	TAILATFCSG	ALAATNDDDV	KKAATVAIAA	AYNNGQEING
51	FKAGETIYDI	DEDGTITKKD	ATAADVEADD	FKGLGLKKVV	TNLTKTVNEN
101	KQNVDAKVKA	AESEIEKLTT	KLADTDAALA	DTDAALDATT	NALNKLGENI
151	TTFAEETKTN	IVKIDEKLEA	VADTVDKHAE	AFNDIADSLD	ETNTKADEAV
201	KTANEAKQTA	EETKQNVDAK	VKAAETAAGK	AEAAAGTANT	AADKAEAVAA
251	KVTDIKADIA	TNKDNIAKKA	NSADVYTREE	SDSKFVRIDG	LNATTEKLDT
301	RLASAEKSIA	DHDTRLNGLD	KTVSDLRKET	RQGLAEQAAL	SGLFQPYNVG
351	GSGGGGTSAP	DFNAGGTGIG	SNSRATTAKS	AAVSYAGIKN	EMCKDRSMLC
401	AGRDDVAVTD	RDAKINAPPP	NLHTGDFPNP	NDAYKNLINL	KPAIEAGYTG
451	RGVEVGIVDT	GESVGSISFP	ELYGRKEHGY	NENYKNYTAY	MRKEAPEDGG
501	GKDIEASFDD	EAVIETEAKP	TDIRHVKEIG	HIDLVSHIIG	GRSVDGRPAG
551	GIAPDATLHI	MNTNDETKNE	MMVAAIRNAW	VKLGERGVRI	VNNSFGTTSR
601	AGTADLFQIA	NSEEQYRQAL	LDYSGGDKTD	EGIRLMQQSD	YGNLSYHIRN
651	KNMLFIFSTG	NDAQAQPNTY	ALLPFYEKDA	QKGIITVAGV	DRSGEKFKRE
701	MYGEPGTEPL	EYGSNHCGIT	AMWCLSAPYE	ASVRFTRTNP	IQIAGTSFSA
751	PIVTGTAALL	LQKYPWMSND	NLRTTLLTTA	QDIGAVGVDS	KFGWGLLDAG
801	KAMNGPASFP	FGDFTADTKG	TSDIAYSFRN	DISGTGGLIK	KGGSQLQLHG
851	NNTYTGKTII	EGGSLVLYGN	NKSDMRVETK	GALIYNGAAS	GGSLNSDGIV
901	YLADTDQSGA	NETVHIKGSL	QLDGKGTLYT	RLGKLLKVDG	TAIIGGKLYM
951	SARGKGAGYL	NSTGRRVPFL	SAAKIGQDYS	FFTNIETDGG	LLASLDSVEK
1001	TAGSEGDTLS	YYVRRGNAAR	TASAAAHSAP	AGLKHAVEQG	GSNLENLMVE
1051	LDASESSATP	ETVETAAADR	TDMPGIRPYG	ATFRAAAAVQ	HANAADGVRI
1101	FNSLAATVYA	DSTAAHADMQ	GRRLKAVSDG	LDHNGTGLRV	IAQTQQDGGT
1151	WEQGGVEGKM	RGSTQTVGIA	AKTGENTTAA	ATLGMGRSTW	SENSANAKTD
1201	SISLFAGIRH	DAGDIGYLKG	LFSYGRYKNS	ISRSTGADEH	AEGSVNGTLM
1251	QLGALGGVNV	PFAATGDLTV	EGGLRYDLLK	QDAFAEKGSA	LGWSGNSLTE
1301	GTLVGLAGLK	LSQPLSDKAV	LFATAGVERD	LNGRDYTVTG	GFTGATAATG
1351	KTGARNMPHT	RLVAGLGADV	EFGNGWNGLA	RYSYAGSKQY	GNHSGRVGVG
1401	YRF*				

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:

$T_{m1} = 4\,(G+C) + 2\,(A+T)$ (tail excluded)

$T_{m2} = 64.9 + 0.41\,(\%\ GC) - 600/N$ (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 NH_4OH, 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.

		Sequences	Restriction site
fu (961)-	Fwd	CGCGGATCC -GGAGGGGGTGGTGTCG	BamHI

741(MC58)-His			
	Rev	CCCGCTCGAG-TTGCTTGGCGGCAAGGC	XhoI
fu (961)-983-His	Fwd	CGCGGATCC - GGCGGAGGCGGCACTT	BamHI
	Rev	CCCGCTCGAG-GAACCGGTAGCCTACG	XhoI
fu (961)- Orf46.1-His	Fwd	CGCGGATCCGGTGGTGGTGGT-TCAGATTTGGCAAACGATTC	BamHI
	Rev	CCCGCTCGAG-CGTATCATATTTCACGTGC	XhoI
fu (961 c-L)-741(MC58)	Fwd	CGCGGATCC -GGAGGGGGTGGTGTCG	BamHI
	Rev	CCCGCTCGAG-TTATTGCTTGGCGGCAAG	XhoI
fu (961c-L)-983	Fwd	CGCGGATCC - GGCGGAGGCGGCACTT	BamHI
	Rev	CCCGCTCGAG-TCAGAACCGGTAGCCTAC	XhoI
fu (961c-L)-Orf46.1	Fwd	CGCGGATCCGGTGGTGGTGGT-TCAGATTTGGCAAACGATTC	BamHI
	Rev	CCCGCTCGAG-TTACGTATCATATTTCACGTGC	XhoI
fu-(ΔG287)-919-His	Fwd	CGCGGATCCGGTGGTGGTGGT-CAAAGCAAGAGCATCCAAACC	BamHI
	Rev	CCCAAGCTT-TTCGGGCGGTATTCGGGCTTC	HindIII
fu-(ΔG287)-953-His	Fwd	CGCGGATCCGGTGGTGGTGGT-GCCACCTACAAAGTGGAC	BamHI
	Rev	GCCCAAGCTT-TTGTTTGGCTGCCTCGAT	HindIII
fu-(ΔG287)-961-His	Fwd	CGCGGATCCGGTGGTGGTGGT-ACAAGCGACGACG	BamHI
	Rev	GCCCAAGCTT-CCACTCGTAATTGACGCC	HindIII
fu-(ΔG287)-Orf46.1-His	Fwd	CGCGGATCCGGTGGTGGTGGT-TCAGATTTGGCAAACGATTC	BamHI
	Rev	CCCAAGCTT-CGTATCATATTTCACGTGC	HindIII
fu-(ΔG287-919)-Orf46.1-His	Fwd	CCCAAGCTTGGTGGTGGTGGTGGT-TCAGATTTGGCAAACGATTC	HindIII
	Rev	CCCGCTCGAG-CGTATCATATTTCACGTGC	XhoI
fu-(ΔG287-Orf46.1)-919-His	Fwd	CCCAAGCTTGGTGGTGGTGGTGGT-CAAAGCAAGAGCATCCAAACC	HindIII
	Rev	CCCGCTCGAG-CGGGCGGTATTCGGGCTT	XhoI
fu ΔG287(394.98)-...	Fwd	CGCGGATCCGCTAGC-CCCGATGTTAAATCGGC	NheI
	Rev	CGGGGATCC-ATCCTGCTCTTTTTTGCCGG	BamHI
fu Orf1-(Orf46.1)-His	Fwd	CGCGGATCCGCTAGC-GGACACACTTATTTCGGCATC	NheI
	Rev	CGCGGATCC-CCAGCGGTAGCCTAATTTGAT	
fu (Orf1)-Orf46.1-His	Fwd	CGCGGATCCGGTGGTGGTGGT-TCAGATTTGGCAAACGATTC	BamHI
	Rev	CCCAAGCTT-CGTATCATATTTCACGTGC	HindIII
fu (919)-Orf46.1-His	Fwd1	GCGGCGTCGACGGTGGCGGAGGCACTGGATCCTCAG	SalI
	Fwd2	GGAGGCACTGGATCCTCAGATTTGGCAAACGATTC	
	Rev	CCCGCTCGAG-CGTATCATATTTCACGTGC	XhoI
Fu (orf46)-287-His	Fwd	CGGGGATCCGGGGGCGGCGGTGGCG	BamHI
	Rev	CCCAAGCTTATCCTGCTCTTTTTGCCGGC	HindIII
Fu (orf46)-919-His	Fwd	CGCGGATCCGGTGGTGGTGGTCAAAGCAAGAGCATCCAAACC	BamHI
	Rev	CCCAAGCTTCGGGCGGTATTCGGGCTTC	HindIII

Fu (orf46-919)-287-His	Fwd	CCCCAAGCTTGGGGGCGGCGGTGGCG	HindIII
	Rev	CCCGCTCGAGATCCTGCTCTTTTTTGCCGGC	XhoI
Fu (orf46-287)-919-His	Fwd	CCCAAGCTTGGTGGTGGTGGTGGTCAAAGCAAGAGCATCCAAACC	HindIII
	Rev	CCCGCTCGAGCGGGCGGTATTCGGGCTT	XhoI
(ΔG741)-961c-His	Fwd1 Fwd2	GGAGGCACTGGATCCGCAGCCACAAACGACGACGA GCGGCCTCGAG-GGTGGCGGAGGCACTGGATCCGCAG	XhoI
	Rev	CCCGCTCGAG-ACCCAGCTTGTAAGGTTG	XhoI
(ΔG741)-961-His	Fwd1 Fwd2	GGAGGCACTGGATCCGCAGCCACAAACGACGACGA GCGGCCTCGAG-GGTGGCGGAGGCACTGGATCCGCAG	XhoI
	Rev	CCCGCTCGAG-CCACTCGTAATTGACGCC	XhoI
(ΔG741)-983-His	Fwd	GCGGCCTCGAG-GGATCCGGCGGAGGCGGCACTTCTGCG	XhoI
	Rev	CCCGCTCGAG-GAACCGGTAGCCTACG	XhoI
(ΔG741)-orf46.1-His	Fwd1 Fwd2	GGAGGCACTGGATCCTCAGATTTGGCAAACGATTC GCGGCGTCGACGGTGGCGGAGGCACTGGATCCTCAGA	SalI
	Rev	CCCGCTCGAG-CGTATCATATTTCACGTGC	XhoI
(ΔG983)-741(MC58) -His	Fwd	GCGGCCTCGAG-GGATCCGGAGGGGGTGGTGTCGCC	XhoI
	Rev	CCCGCTCGAG-TTGCTTGGCGGCAAG	XhoI
(ΔG983)-961c-His	Fwd1 Fwd2	GGAGGCACTGGATCCGCAGCCACAAACGACGACGA GCGGCCTCGAG-GGTGGCGGAGGCACTGGATCCGCAG	XhoI
	Rev	CCCGCTCGAG-ACCCAGCTTGTAAGGTTG	XhoI
(ΔG983)-961-His	Fwd1 Fwd2	GGAGGCACTGGATCCGCAGCCACAAACGACGACGA GCGGCCTCGAG-GGTGGCGGAGGCACTGGATCCGCAG	XhoI
	Rev	CCCGCTCGAG-CCACTCGTAATTGACGCC	XhoI
(ΔG983)- Orf46.1-His	Fwd1 Fwd2	GGAGGCACTGGATCCTCAGATTTGGCAAACGATTC GCGGCGTCGACGGTGGCGGAGGCACTGGATCCTCAGA	SalI
	Rev	CCCGCTCGAG-CGTATCATATTTCACGTGC	XhoI

***** 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 *Nhe*I site, the ATG codon is part of the *Nde*I site used for cloning. The constructs made using *Nhe*I 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 $CHCl_3$/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 OD_{260}.

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 oligonucletide primer, 400-800 µM dNTPs solution, 1x PCR buffer (including 1.5mM $MgCl_2$), 2.5 units *TaqI* DNA polymerase (using Perkin-Elmer AmpliTaQ, Boerhingher 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 (T_{m1}). This was followed by 30 cycles according to the hybridisation temperature calculated for the whole length oligos (T_{m2}). 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 H_2O 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_{600} 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_{600} 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_{600} 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, Δ 2, 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 20ml 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 1.0mM) 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 (10mM Tris-HCl, 100 mM phosphate buffer, pH 8.8 and, optionally, 8M 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 40W using a Branson sonifier 450 and centrifuged at 13000xg 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 150µl 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 10ml 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 (300mM NaCl, 50mM phosphate buffer, 20mM imidazole, pH 8.0) or (ii) buffer D (10mM Tris-HCl, 100mM phosphate buffer, pH 6.3 and, optionally, 8M urea) until OD_{280} of the flow-through reached 0.02-0.01. The His-fusion protein was eluted by addition of 700µl of either (i) cold elution buffer A (300 mM NaCl, 50mM 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, 8M urea) and fractions

*Trade-mark

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:

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

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

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were washed three times with PBT buffer. 100µl of substrate buffer for HRP (25ml of citrate buffer pH5, 10mg of O-phenildiamine and 10µl of H_2O_2) were added to each well and the plates were left at room temperature for 20 minutes. 100µl 12.5% H_2SO_4 was added to each well and OD_{490} was followed. The ELISA titers were calculated abitrarely as the dilution of sera which gave an OD_{490} value of 0.4 above the level of preimmune sera. The ELISA was considered positive when the dilution of sera with OD_{490} 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% CO_2. 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 OD_{620}. 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% NaN_3) and centrifuged for 5 minutes at 4000rpm. Cells were resuspended in blocking buffer to reach OD_{620} 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% CO_2. Colonies were collected and used to inoculate 7ml Mueller-Hinton broth, containing 0.25% glucose to reach an OD_{620} of 0.05-0.08. The culture was incubated for approximately 1.5 hours at 37 degrees with shacking until the OD_{620} reached the value of 0.23-0.24. Bacteria were diluted in 50mM Phosphate buffer pH 7.2 containing 10mM $MgCl_2$, 10mM $CaCl_2$ and 0.5% (w/v) BSA (assay buffer) at the working dilution of 10^5 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% CO_2 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 5000*g* for 10 minutes, the supernatant containing the total cell envelope fraction recovered and further centrifuged overnight at 50000*g* 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 10000*g* for 10 minutes to remove aggregates, the supernatant was further centrifuged at 50000*g* for 3 hours. The pellet, containing the outer membranes

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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 hybrid protein of formula NH_2-A-B-COOH, wherein A comprises the Neisserial protein ΔG287 and B comprises the Neisserial protein 961, and wherein the amino acid sequence of the hybrid protein is as disclosed in SEQ ID NO: 8 or is a sequence having greater than 70% sequence identity thereto.

2. The protein of claim 1, wherein ΔG287 is from strain 2996 or 394/98.

3. The protein of claim 1, wherein 961 is from strain 2996 or 394/98.

4. The protein of claim 1, wherein A and B are from the same strain.

5. The hybrid protein of claim 1, comprising the amino acid sequence recited in SEQ ID NO: 8.

FIGURE 1 — ΔG287—919

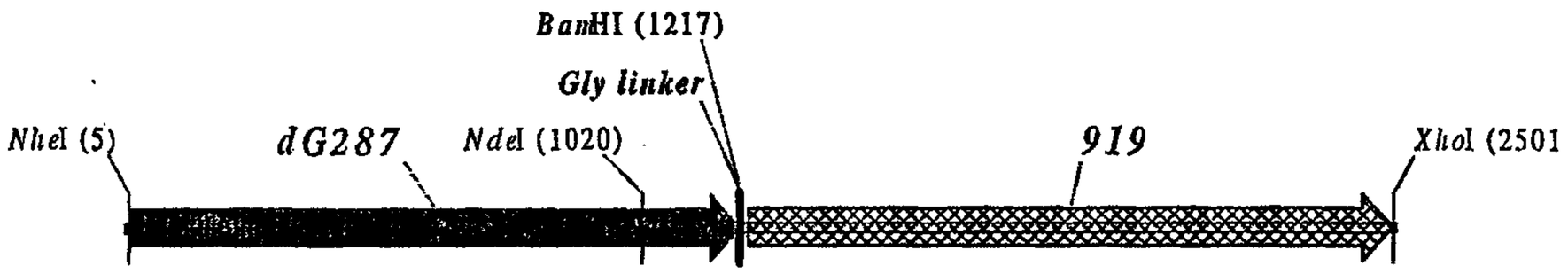


FIGURE 2 — ΔG287—953

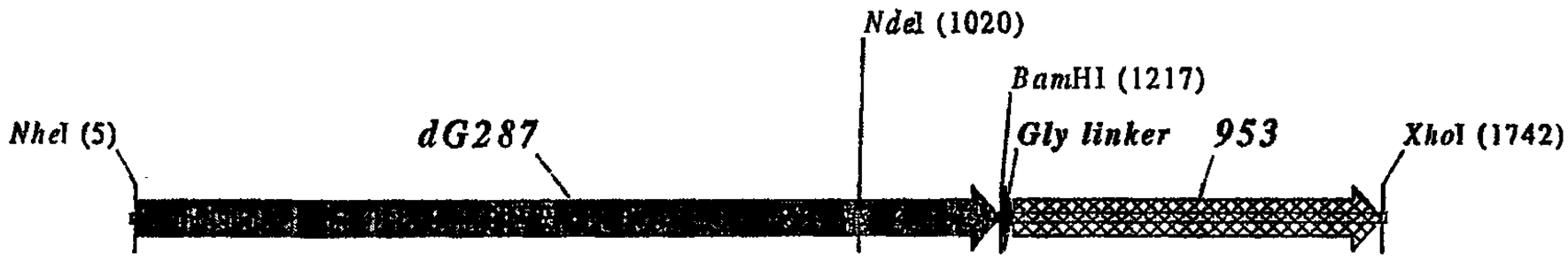


FIGURE 3 — ΔG287—961

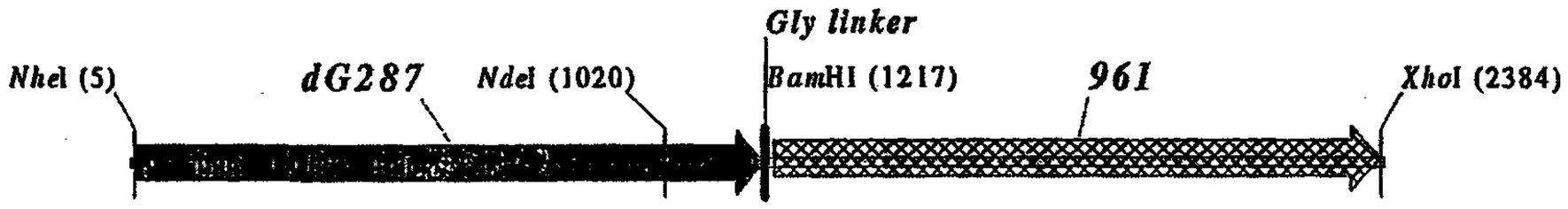


FIGURE 4 — ΔG287NZ—919

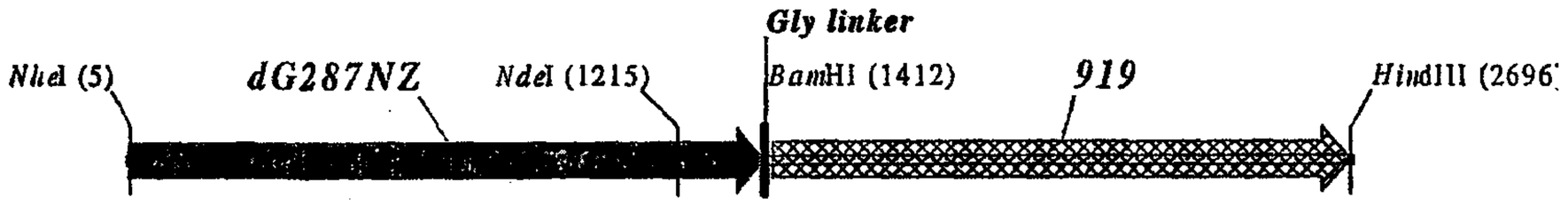


FIGURE 5 — ΔG287NZ—953

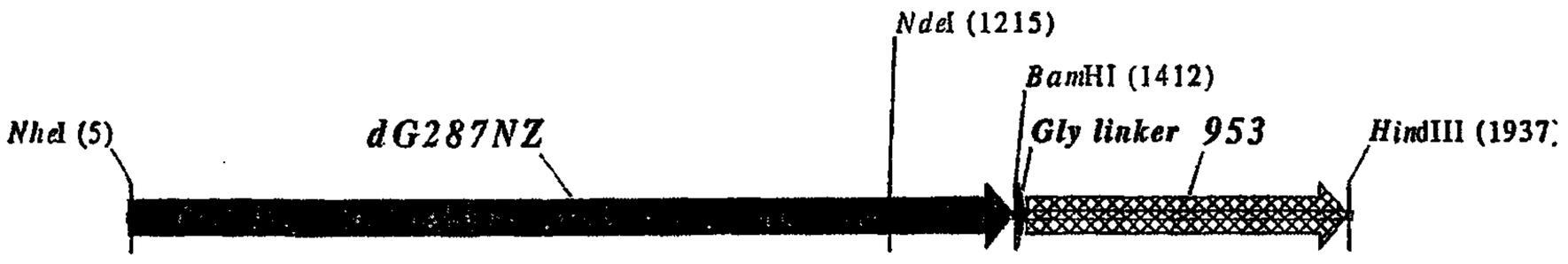


FIGURE 6 — ΔG287NZ—961

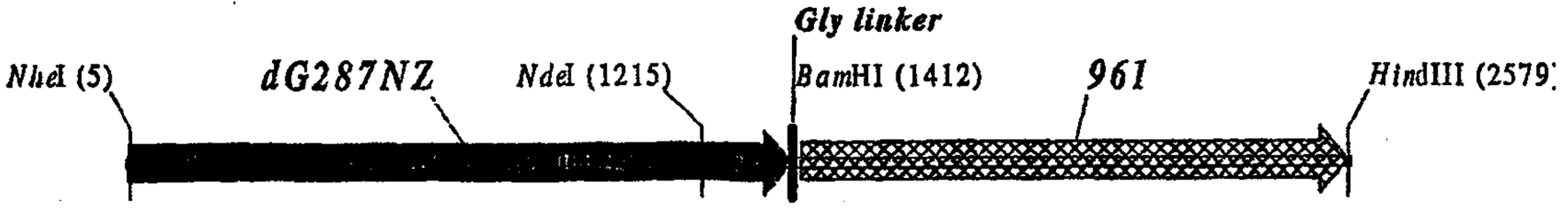


FIGURE 7 — ΔG983-ORF46.1

FIGURE 8 — ΔG983-741

FIGURE 9 — ΔG983-961

FIGURE 10 — ΔG983-961c

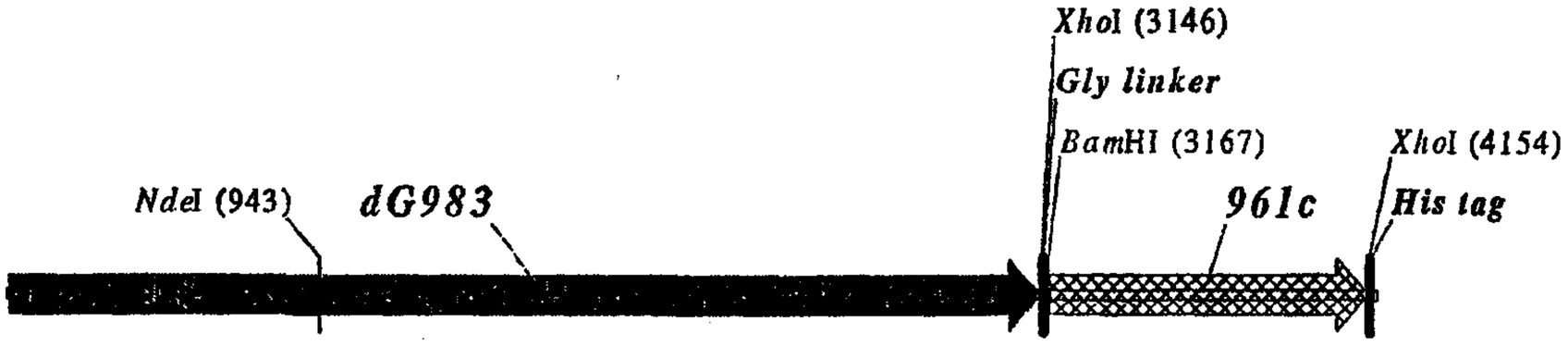


FIGURE 11 — ΔG741-961

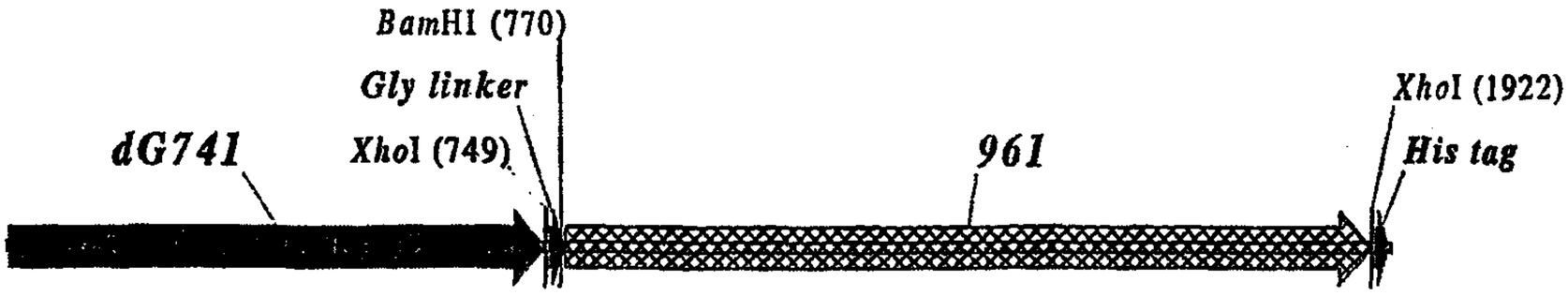


FIGURE 12 — ΔG741-961c

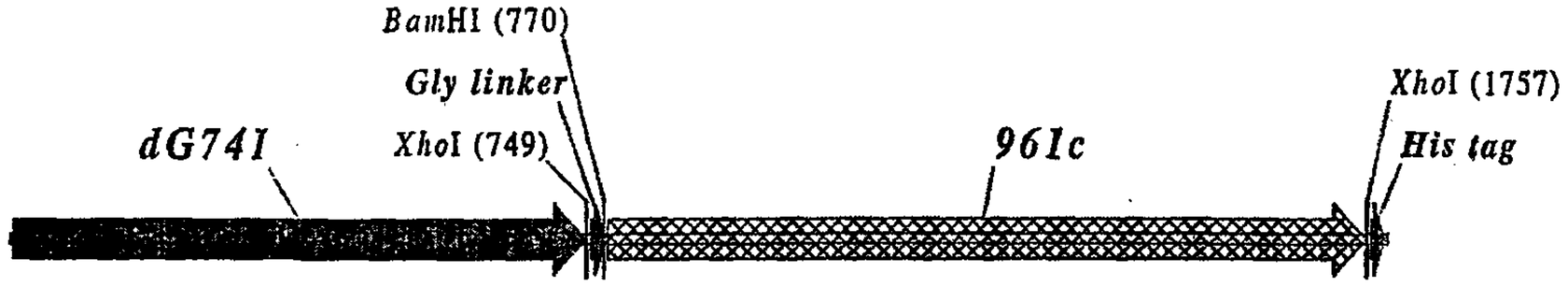


FIGURE 13 — ΔG741-983

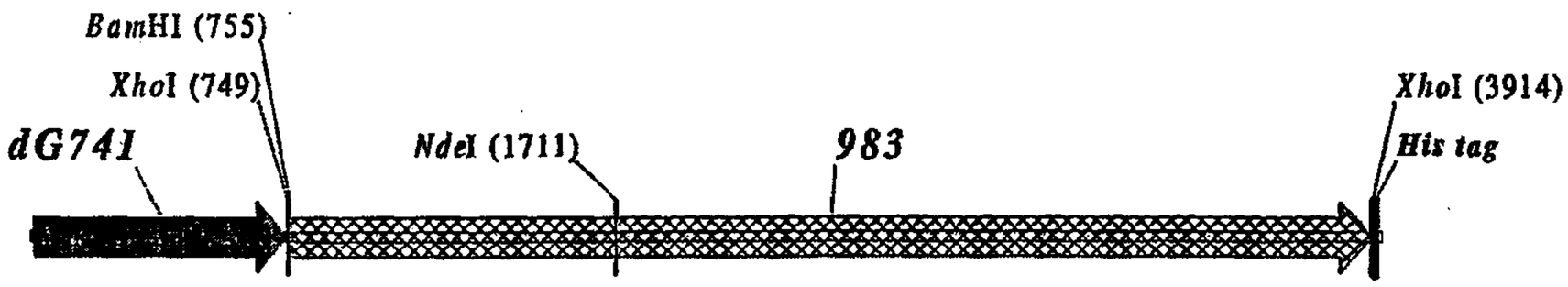


FIGURE 14 — ΔG741-ORF46.1

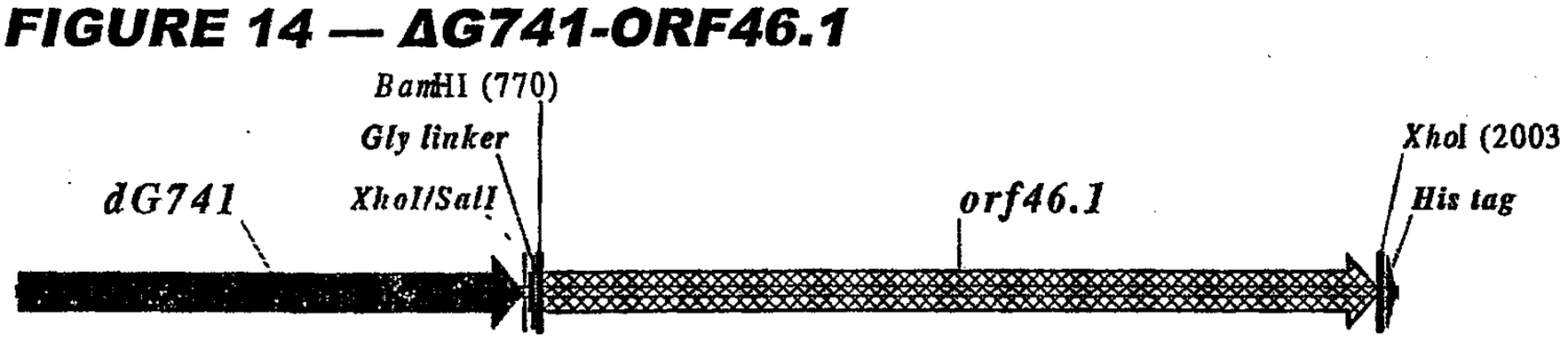


FIGURE 15 — ORF46.1-741

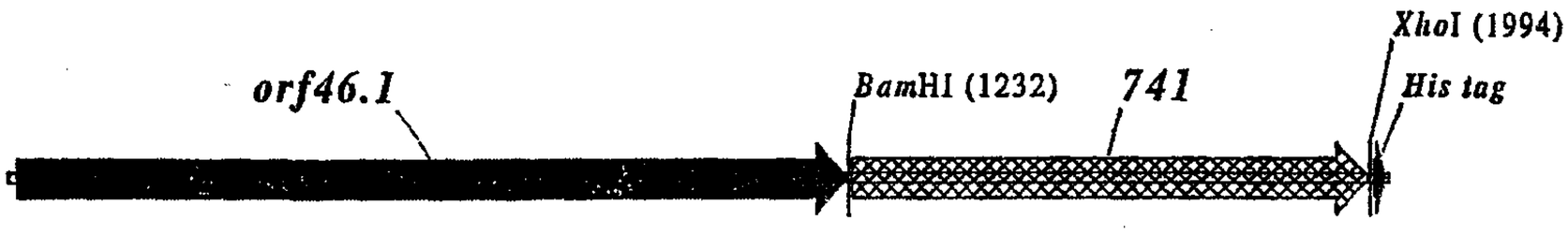


FIGURE 16 — ORF46.1-961

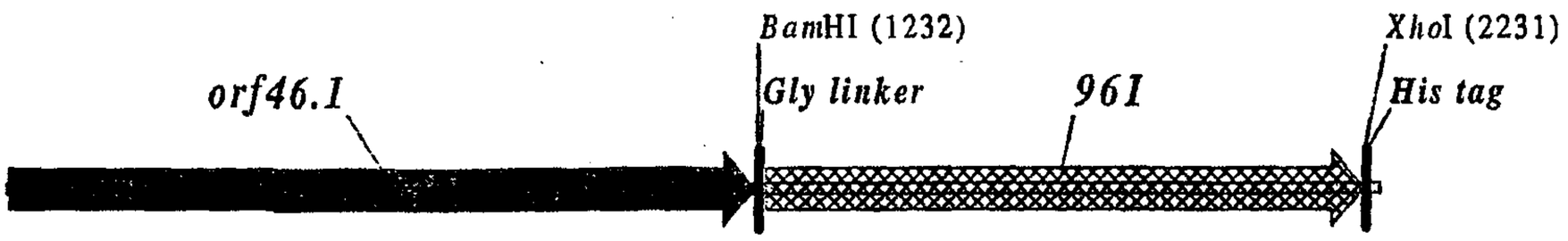


FIGURE 17 — ORF46.1—961c

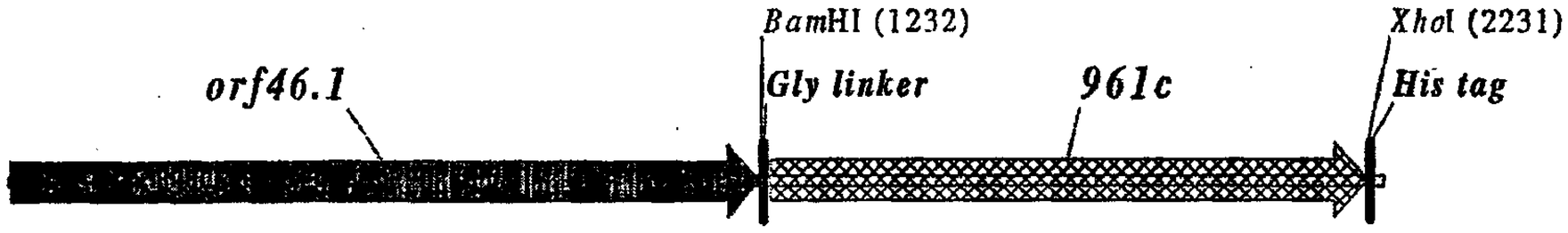


FIGURE 18 — 961-ORF46.1

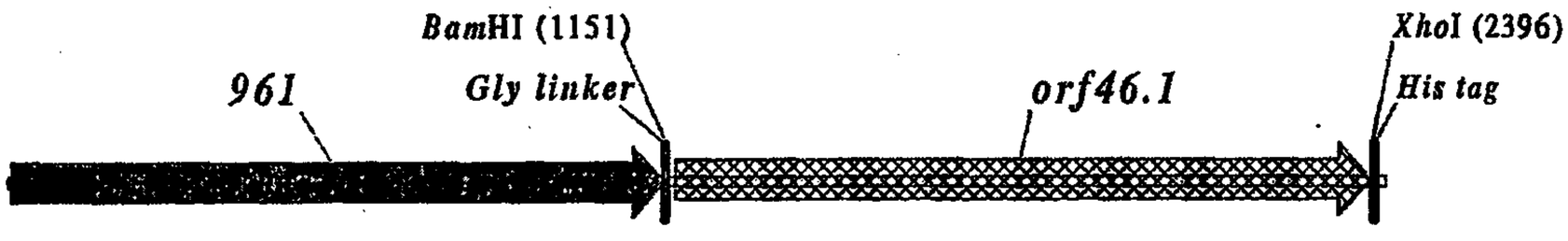


FIGURE 19 — 961-741

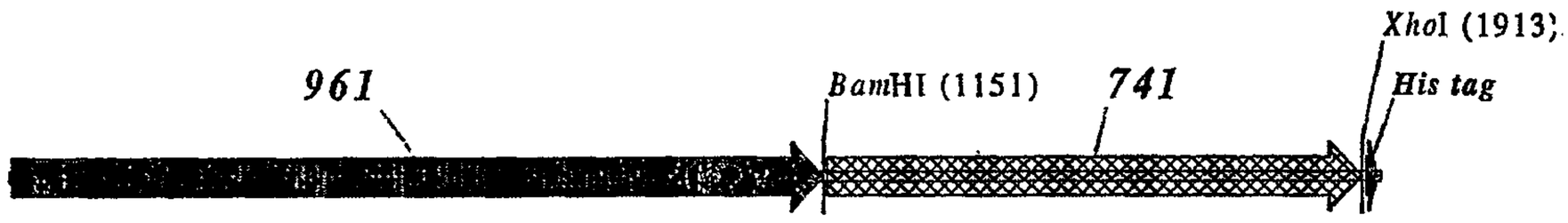


FIGURE 20 — 961-983

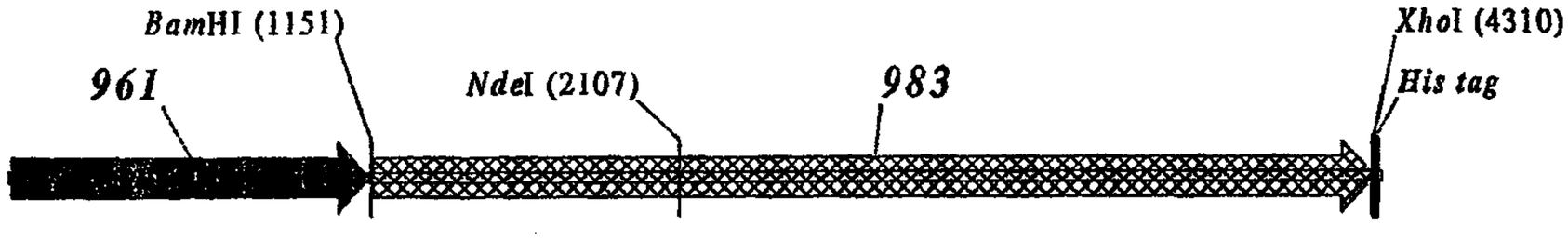


FIGURE 21 — 961c-ORF46.1

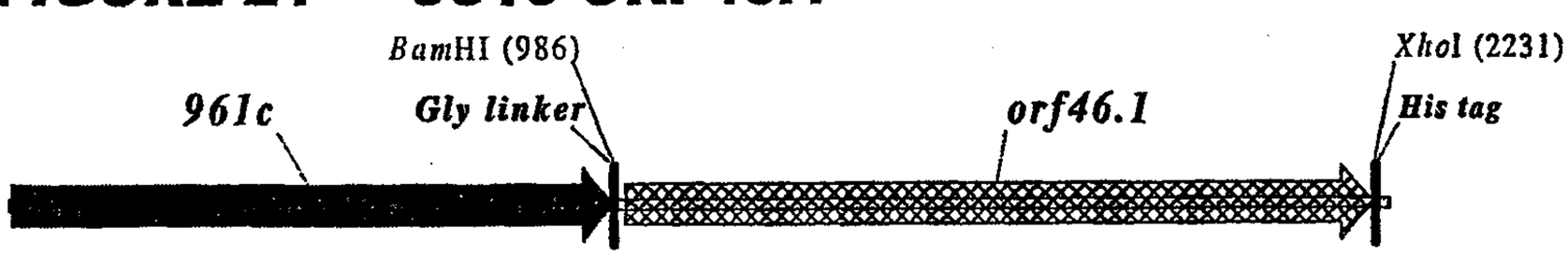


FIGURE 22 — 961c-741

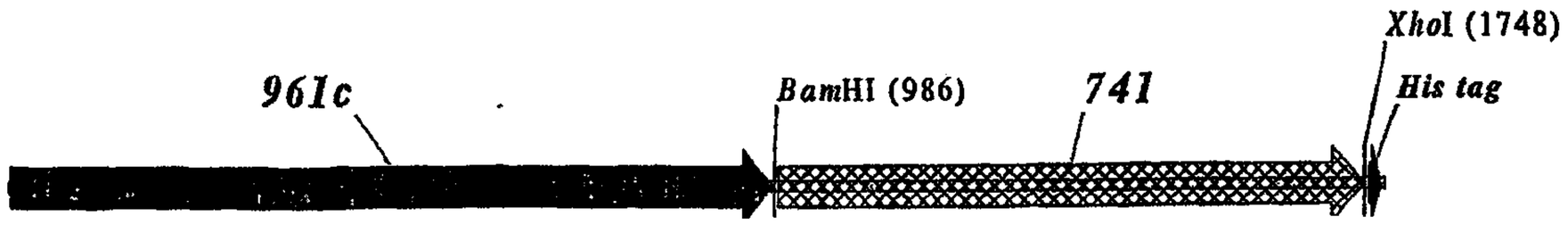


FIGURE 23 — 961c-983

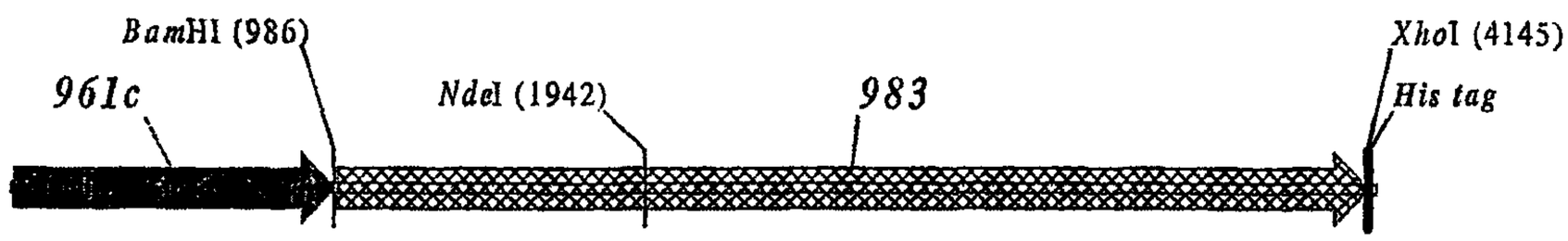


FIGURE 24 — 961cL-ORF46.1

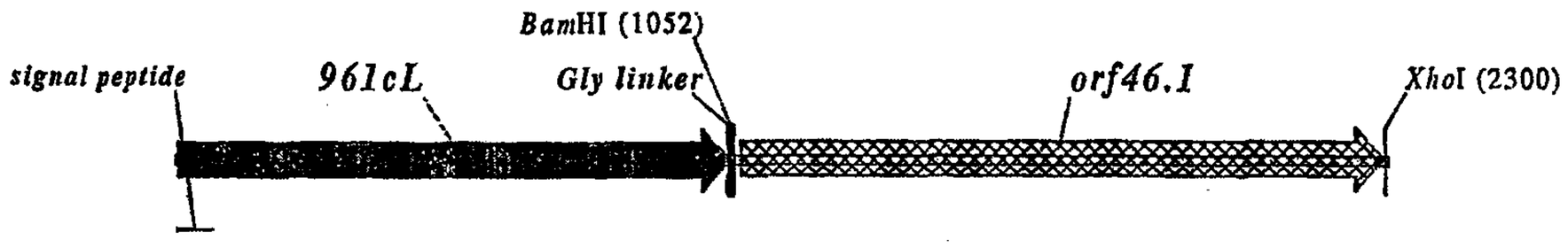


FIGURE 25 — 961cL-741

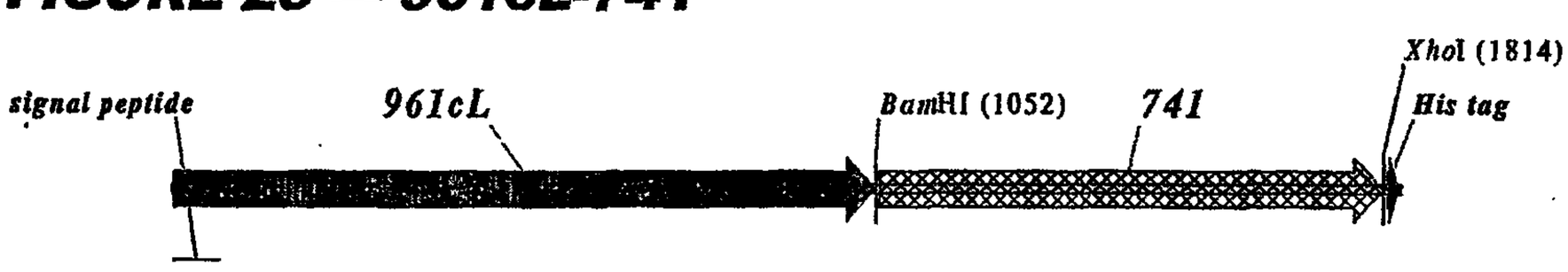
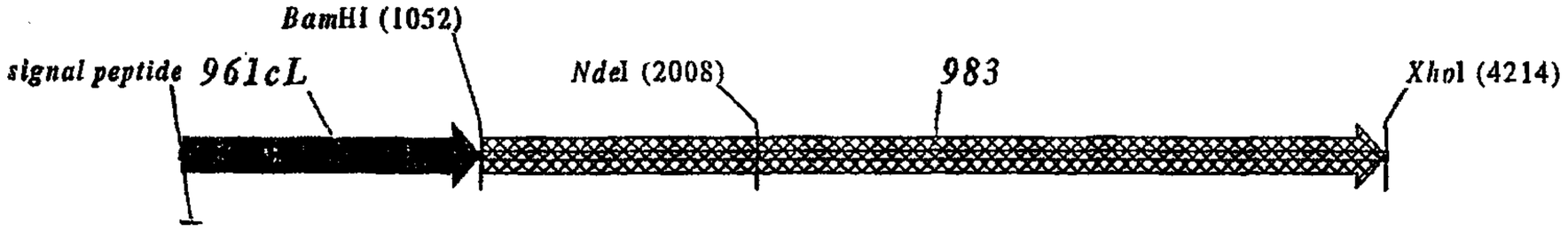
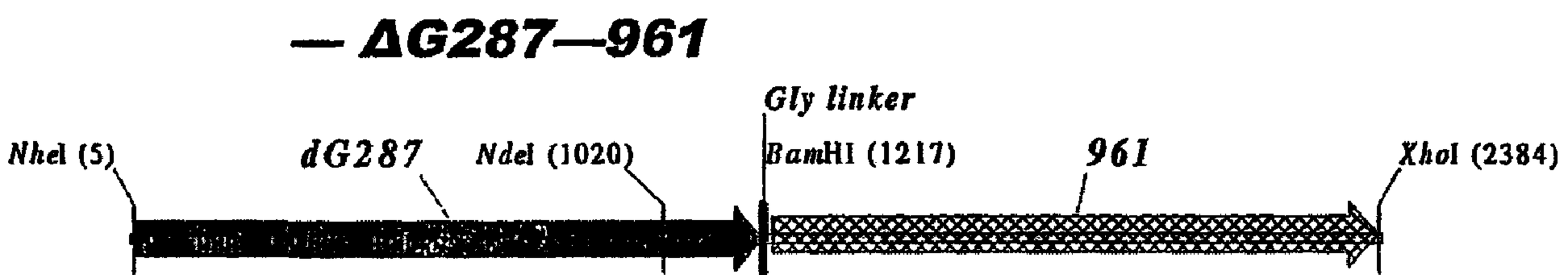


FIGURE 26 — 961cL-983




— ΔG287—961
Gly linker
NheI (5)
dG287
NdeI (1020)
BamHI (1217)
961
XhoI (2384)