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Mosaic-like organization of IgA protease genes in *Neisseria gonorrhoeae* generated by horizontal genetic exchange *in vivo*

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IgA protease is a putative virulence factor that exists in several allelic forms in *Neisseria gonorrhoeae*. However, extracellular secretion of these variant IgA proteases occurs by the same pathway involving three steps of autoproteolytic maturation from a large precursor. Two principal precursor types (H_1 and H_2) can be distinguished with respect to the location of autoproteolytic sites and the sizes of the mature products. By partial DNA sequence analysis, additional variations have been detected which are not unique to one particular gene; rather, otherwise unrelated *iga* genes often share homology, thus revealing a composite organization. In the context of other gonococcal features, this observation implies that recombination has occurred *in vivo* between *iga* genes of different strains, probably via the route of species-specific DNA transformation. This process may be of general significance for the modulation and the natural exchange of virulence properties among pathogenic Neisseriae.

Key words: bacterial virulence/DNA transformation/ extracellular secretion/genetic recombination

Introduction

A variety of pathogenic bacteria colonizing the human mucosa produce proteases that specifically cleave human IgA1 (Plaut *et al.*, 1974, 1975; Kilian *et al.*, 1979; Mortensen and Kilian, 1984; Senda *et al.*, 1985). Indirect evidence suggests that IgA proteases play a role in virulence (for a review see Kornfeld and Plaut, 1981; Kilian and Reinholdt, 1986). They are almost exclusively produced by human pathogenic species whilst non-pathogenic and closely related opportunistic species lack such enzymes (Mulks and Plaut, 1978). A model proposed to explain the biological significance of IgA1 cleavage invokes the binding of Fabα fragments to bacterial surfaces; such binding might mask antigenic epitopes and allow immune evasion by the pathogen (Kilian and Reinholdt, 1986).

All known IgA proteases cleave human IgA1 within the proline-rich hinge region. Enzymes from different species, and even from the same species, may cleave at different positions within this region. In gonococci, for example, two cleavage specificity types are known which correlate with certain physical characteristics of the IgA protease gene (*iga*) (Mulks *et al.*, 1987). A number of *iga* genes have recently been isolated from Gram-negative and Gram-positive species (Koomey *et al.*, 1982; Halter *et al.*, 1984; Fishman *et al.*, 1985; Grundy *et al.*, 1987; Gilbert *et al.*, 1988). A significant degree of homology exists among the genes of *Neisseria gonorrhoeae, N.meningitidis* and, to a minor extent, *Haemophilus influenzae* (Koomey and Falkow, 1984). The *iga* genes of *N.gonorrhoeae*, found as single genomic copies, have thus far been studied in most detail.

Cloned gonococcal *iga* genes have the unique ability to direct selective extracellular secretion of active enzyme both in *Escherichia coli* and *Salmonella* species (Pohlner *et al.*, 1987; Meyer *et al.*, 1987). The mature IgA protease of *N.gonorrhoeae* MS11 has a mol. wt of ~106 kd (Halter *et al.*, 1984), and is processed from a larger precursor of ~169 kd by autoproteolytic cleavage (Pohlner *et al.*, 1987). Four different functional and structural domains can be distinguished in this precursor, (i) an amino terminal signal peptide, (ii) the secreted protease domain, (iii) the α-domain, a hydrophilic α-helical region that is secreted in conjunction with the protease, and (iv) the carboxy terminal β-domain, which is essential for extracellular secretion of the protease (Pohlner *et al.*, 1987). This domain becomes integrated into the outer membrane of gonococci and permits the outer membrane transport of a 121 kd extracellular intermediate (proform C, this is the protease domain plus α-domain). Proform C is released from the membrane bound β-domain by autoproteolysis and develops further into proform B (109 kd) and α-protein. A final autoproteolytic step converts proform B into the mature 106 kd IgA protease (Pohlner *et al.*, 1987).

Our studies presented here demonstrate the existence of two alleles of gonococcal *iga* genes (H_1 and H_2), specifying precursors which give rise to different secretory intermediates and excreted products. We further show differences in size, and serological and enzymatic properties, of IgA proteases prepared from independent *N.gonorrhoeae* strains. Based on a comparative DNA sequence analysis of *iga* genes,

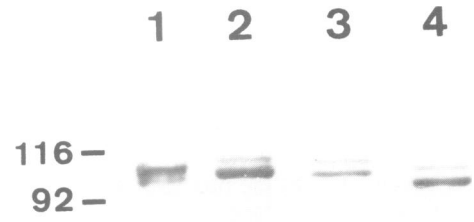

Fig. 1. Gel electrophoresis and immunoblotting of IgA proteases. The enzymes were purified from culture supernatants of strains FA514 (**1**), NG74 (**2**), R16 (**3**) and MS11 (**4**), electrophoresed through a 10% SDS–PAGE gel, immunoblotted and probed with the cross-reactive monoclonal antibody HAL-1 (Pohlner *et al.*, 1987). The upper band in each lane represents proform B, the lower bands represent the mature forms of the enzymes. Size markers are given in kd.

Table I. Mosaic-like organization of *iga* genes

Strain	Cleavage type[a]	*iga* sequence identity[b]					Precursor type[c]	HAL-2[d]
		956–1024	1124–1204	1460–1543	1560–1599	1898–1963		
MS11	2	A	A	A	A	A	H_1	+
R16	2	A	A	B	C	C	H_2	–
FA514	2	(A)	C	C	C	C	H_1	+
NG74	1	C	C	C	C	C	H_2	–

[a]The cleavage specificity of IgA proteases was measured with human IgA1 as a substrate, according to Mulks and Knapp (1985).
[b]Identities between variant sections of *iga* genes as indicated by identical letters (compare with Figure 3A); three single nucleotide changes are seen in the section assigned to (A).
[c]Precursor type with regard to the organization of the helper (i.e. β and α) domain.
[d]The monoclonal antibody HAL-2 is specific for the β_1-domain of H_1 proteases.

we suggest that these properties have been reassorted by genetic exchange between independent strains *in vivo*.

Results

Variables of IgA proteases from different strains

The IgA proteases of four *N.gonorrhoeae* strains, MS11, FA514, R16 and NG74, were partially purified following previously published protocols (Halter *et al.*, 1984; Meyer *et al.*, 1987). Figure 1 shows the purified enzymes in immunoblots labelled with the cross-reactive monoclonal antibody HAL-1 (Pohlner *et al.*, 1987). In each of the four preparations, HAL-1 recognized two bands representing the mature enzyme and the extracellular proform B. The mature IgA proteases have apparent sizes between 106 kd (strain MS11) and 115 kd (strain NG74), and the proforms B are ~3 kd larger as calculated on the basis of DNA sequence data. The final maturation step of the protease, that is the conversion of proform B into mature enzyme (Pohlner *et al.*, 1987), is therefore conserved and independent of the sizes of the respective enzymes. Extracellular proform C was not present in the purified fractions of IgA protease as it converts rapidly during the purification procedure. We demonstrate below that this intermediate differs for two of the strain analysed, R16 and NG74, in comparison with our standard strain MS11.

The size variations among the four purified gonococcal IgA proteases suggested the existence of minor polymorphisms among these enzymes. To test if such polymorphisms have serological implications, we determined the relative inhibition of the enzymes by rabbit antisera raised against the native protease of strain MS11. Although relatively high antibody titres were required to inhibit the MS11 enzyme, inhibition of the heterologous IgA proteases required ~5-fold higher antibody titres (data not shown), hence showing a notable antigenic diversity. We also determined the cleavage specificities of the proteases for human IgA1 (Mulks and Knapp, 1985): One of the proteases (NG74) cleaved at position 237 (Pro–Ser) in the IgA1 antibody and therefore belongs to the type 1 specificity group. The remaining proteases exhibited type 2 specificity, cleaving the Pro–Thr peptide bond in position 235 (see also Table I).

Mosaic-like composition of iga genes

To investigate the genetic basis for the observed polymorphisms among gonococcal IgA proteases, we cloned the *iga* genes of the respective strains and performed physical mapping and partial sequence analyses. All clones were identified in colony blots, using the isolated *iga* gene of strain MS11, and further examined for production of active extracellular IgA protease in *E.coli*. As judged by genomic control hybridizations, the inserts of plasmids pIP2 (FA514), pIP3 (R16) and pIP4 (NG74) proved to have maps identical to their genomic counterparts, as was previously shown for the insert in pIP1 from strain MS11 (Halter *et al.*, 1984).

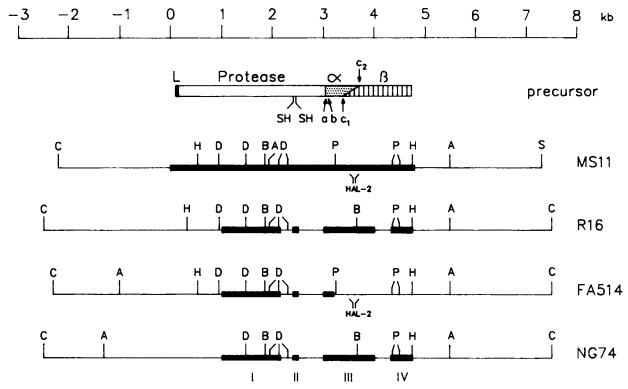

Fig. 2. Physical maps of gonococcal *iga* genes. The precursors of IgA proteases consist of an amino terminal signal sequence (L), the protease domain and the carboxy terminal helper which is split into the α- (dotted) and β-domains (hatched). Reference scale in kb corresponds to the sequence of *iga*-MS11. Locations of two unique cysteine residues (SH) and the autoproteolytic cleavage sites a, b, c_1 and c_2 (for H_1 and H_2 proteases, respectively) are indicated. The physical maps of cloned *iga* genes of strains MS11, R16, FA514 and NG74 are depicted below (A, *AvaI*; B, *BglII*; C, *ClaI*; D, *HpaI*; H, *HindIII*; P, *PstI*; S, *SalI*). Solid bars indicate the regions which have been sequenced and arbitrarily assigned the numbers I–IV. The sequences of regions I and III are presented in Figure 3A and B, respectively; the sequences of regions II and IV are identical among all genes, in agreement with the previously published sequence of strain MS11 (Pohlner *et al.*, 1987).

A comparison of the physical maps of the cloned *iga* genes revealed a number of restriction site polymorphisms (Figure 2). A single *Hin*dIII site is located at the 5′-end of all type 2 genes, followed by an *Hpa*I site further downstream. These restriction sites are lacking in the gene of strain NG74 and is the only pronounced polymorphism that correlates with the cleavage specificity of the respective IgA protease; this 5′-proximal region might therefore specify properties for the differential recognition and/or cleavage of IgA1.

The *Hin*dIII fragment contained in *iga*-R16 is larger than that of *iga*-MS11 as a result of an insertion located between the *Hin*dIII and *Hpa*I restriction sites at the 5′-end of

iga-R16; this is reflected in the increased size of the R16 enzyme (Figure 1). Other polymorphisms are found in regions which determine the amino terminal half of the protease domain and the α-domain. These regions were sequenced in all four *iga* genes (Figure 3).

The polymorphic region spanning part of the protease domain has several clusters of single or multiple nucleotide substitutions. Altogether, 21 (R16), 27 (FA514) and 36 (NG74) amino acid alterations are present in comparison with the amino acid sequence deduced from *iga*-MS11. Strikingly, a single codon insertion is shared between the *iga*-FA514 and *iga*-NG74 genes, in a region which is perfectly homologous between these two genes (1124–1543), though heterologous with the other *iga* genes. Homologies are also evident among the genes of MS11 and R16 (positions 956–1204) and among the genes of R16, FA514 and NG74 (positions 1560–1599 and 1898–1963; Figure 3A).

Another striking example of the patched organization of allelic *iga* genes is seen in the region which specifies the α-domain and the adjacent, amino terminal portion of the β-domain of the IgA protease precursors. This region carries either a characteristic *Bgl*II site or a *Pst*I site in the helper domain and thus allows the distinction of two groups of helper genes, i.e. H_1 genes (MS11 and FA514) and H_2 genes (R16 and NG74) (see Figure 2). Notably, this feature does not correlate with the cleavage type specificity, which is actually different for R16 and NG74 (Table I). Heteroduplex analysis using cloned *iga* genes of strains MS11 and R16 showed a pronounced loop structure, specifying the H_1 and H_2 polymorphism (data not shown). The DNA sequences of this region revealed extensive differences between, but not within, the two groups of *iga* genes (Figure 3B).

Two distinct classes of IgA protease precursors

We have previously demonstrated that the mature IgA protease of *N.gonorrhoeae* MS11 is processed from a 169 kd precursor form which includes the domains for the membrane associated β-protein and the co-secreted α-protein (Pohlner *et al.*, 1987). Here we tested whether the polymorphism detected between H_1 (MS11 and FA514) and H_2 *iga* genes (R16 and NG74) affects the formation of secretory intermediates as well as the final products of autoproteolytic maturation. For this purpose we tried to identify the respective α- and β-proteins of the precursors of both H_1 and H_2 genes. The monoclonal antibody HAL-2 (Pohlner *et al.*, 1987), which is specific for the β-domain of *iga*-MS11, detected the previously described 45 kd β_1-domain in cell lysates of strains MS11 and FA514, but failed to detect a specific protein in the R16 and NG74 lysates (Figure 4B). Instead, a serum that cross-reacts with both groups of β-proteins (anti-$fp80_{R16}$) detected a smaller β-domain (β_2) of 33 kd in strains R16 and NG74 (Figure 4A).

The α-proteins were prepared by concentrating *N. gonorrhoeae* culture supernatants and were subsequently analysed by immunoblotting. Antisera directed against a fusion protein containing the α_2-domain of the R16 IgA protease (anti-$fp80_{R16}$) detected a protein of 24 kd in the preparations from strains R16 and NG74 (Figure 4C). In contrast, an α_1-protein of 15 kd was found in the supernatant of the strain FA514, as observed previously for the strain MS11 (Pohlner *et al.*, 1987). A polyclonal antiserum raised against a fusion of the MS11 protease (anti-$fp42_{MS11}$), detected the MS11 and FA514 α_1-proteins, but reacted only weakly with the larger α_2-protein of R16 and NG74 (data not shown), thus reflecting the differences between the two α-protein types.

As reported previously, fusion proteins derived from the MS11 *iga* gene, containing the autoproteolytic sites, are suitable substrates for purified IgA protease (Pohlner *et al.*, 1987). Similar fusion proteins were obtained by construction of hybrid *iga*-R16 genes (see Materials and methods, and Figure 5B). Cleavage of such fusion proteins with IgA protease yielded α- and β-proteins identical with the naturally observed proteins (Figure 5). Interestingly, the cleavage products obtained from fusion proteins of either R16 or MS11 gene hybrids were identical regardless of whether an enzyme of type 1 (NG74) or type 2 (MS11) cleavage specificity was used (Figure 5A). Consequently, the cleavage type preferences of IgA proteases represent minor differences detectable only with IgA1 as a substrate. The data further show that the generation of the mature products depends on the location of autoproteolytic sites in the precursor rather than on the fine specificity of the enzyme. The respective autoproteolytic sites used (c_1 and c_2) are indicated in the sequence shown in Figure 5B. The actual use of these sites has been confirmed by amino acid sequencing of fusion protein cleavage products (Figure 3B).

Some conserved features of gonococcal iga genes

A few characteristics, already described for the *iga* gene of strain MS11, are conserved among the four genes studied here. These include (i) a short region which contains the only two cysteines of the entire *iga* precursor, located close together at positions 799 and 810 (Figure 2). Previous speculations that this region might be part of the active site of IgA protease (Pohlner *et al.*, 1987) are probably incorrect since site-specific mutagenesis of either or both of the two cysteines did not abolish autoproteolytic maturation or enzymatic activity of the MS11 IgA protease (J.Pohlner, unpublished data). (ii) While the autoproteolytic site c differs for H_1 and H_2 precursors, sites a and b are common to all four precursors studied. The homology includes the flanking regions of sites a and b, with a few minor exceptions (Figure 3B). (iii) Although our sequence analysis was not extended for the entire β-domains of all four genes (see Figure 2), this region appears to be broadly conserved beyond the sequence shown in Figure 3B. (iv) Finally, the putative DNA uptake signal described by Goodman and Scocca (1988), located within the *iga* transcriptional terminator and centred around a *Hin*dIII site, is conserved in the four genes.

Discussion

In this study we compare the structural and functional features of four allelic *iga* genes. They originate from *N.gonorrhoeae* isolates collected between 1971 and 1981 from different locations in Europe and in the USA. The four genes show several DNA sequence polymorphisms which are also reflected by differences in the location of restriction sites. Based on such polymorphisms, Mulks *et al.* (1987) tentatively defined eight different types and subtypes of gonococcal *iga* genes. Consistent with that study, one of the *iga* genes investigated here codes for an enzyme with type 1 specificity for IgA1, while the remaining genes code for enzymes with type 2 specificity or IgA1 (Mulks and Knapp,

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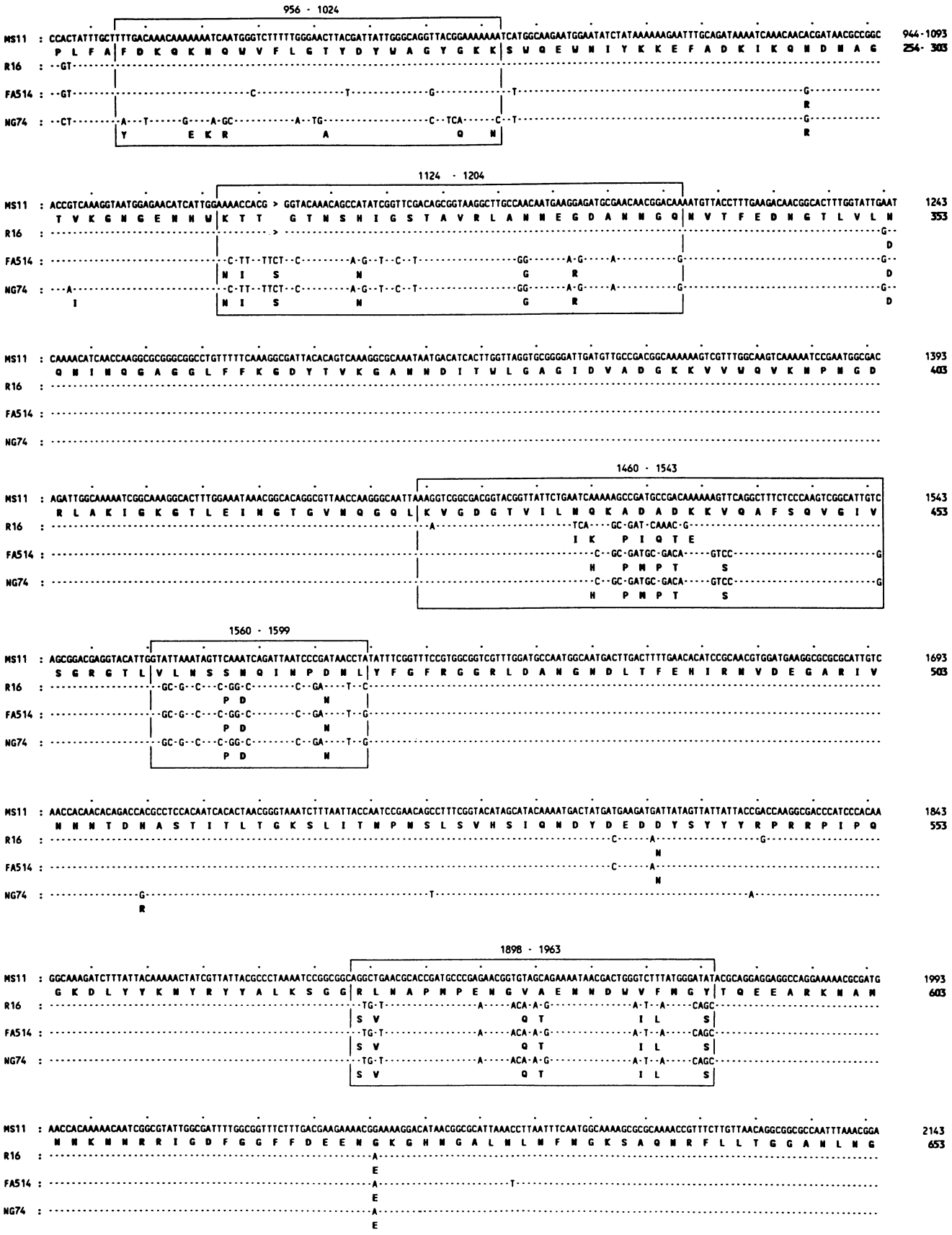
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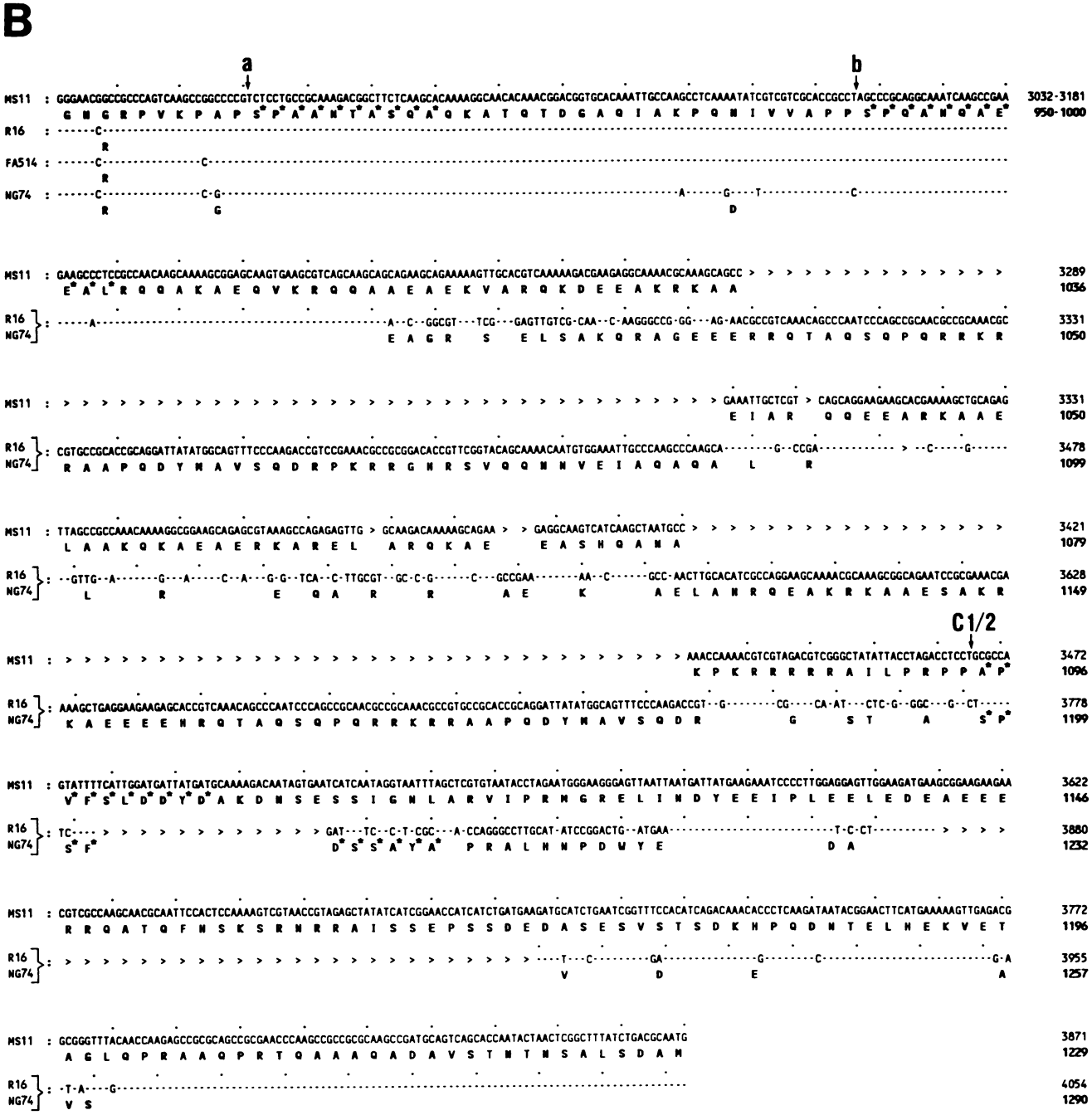

Fig. 3. DNA sequences of variant regions in the *iga* genes from strains MS11, FA514, R16 and NG74. **Panels A** and **B** show the sequences corresponding to regions I and III, respectively (Figure 2). The nucleotide (plain) and amino acid (bold) numbers refer to the *iga* sequence of strain MS11 (Pohlner *et al.*, 1987). The autoproteolytic cleavage site a, b, c_1 and c_2 in the respective precursors were confirmed by amino acid sequence analysis (asterisks) of cleavage products obtained by digestion of fusion proteins with IgA protease (see Figure 5A). Sites c_1 and c_2 differ as regards their sequence and relative location in the precursors (compare with Figure 2). Homologies between the sequences are indicated by dashes; triplet codon deletions are indicated by arrowheads (>). Mosaic-like sections referred to in Table I are boxed.

1985). Other than this specificity difference, which we consider to be less significant since it is only seen with IgA1 as substrate, we can distinguish between two principal precursor types (H_1 and H_2) which give rise to different autoproteolytic products (Figure 5).

The *iga* genes appear to consist of exchangeable building blocks (or determinants) which can be arranged in different combinations (Table I). For example, the unmapped determinant for type 2 specificity can be found in combination with either the H_1 or H_2 determinant of the precursor (e.g. *iga*-MS11 and *iga*-R16). Similar examples of mixed combinations were detected in the protease domain by DNA sequence analysis. The fact that individual building blocks share identity with one group of variant genes, but show striking heterology with another group of *iga* genes, cannot be explained solely by diverging single step

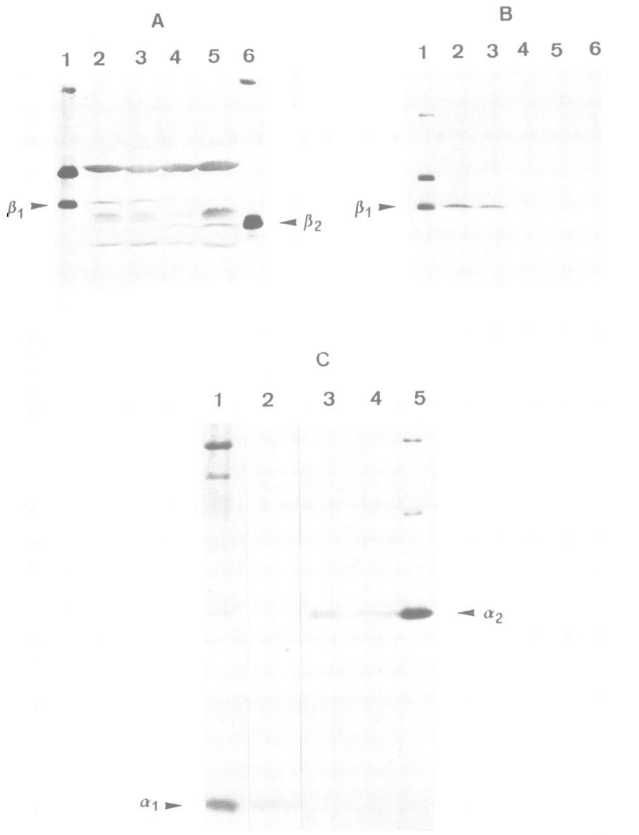

Fig. 4. Identification of α- and β-proteins of H_1 and H_2 proteases by immunoblotting. **Panel A**, identification of β-proteins with rabbit anti-$fp42_{MS11}$ serum. Lanes 1 and 6, β_1 and β_2 proteins obtained by digestion of fusion proteins ($fp180_{MS11}$ and $fp180_{R16}$, respectively) with MS11 IgA protease. Lanes 2 and 3, β_1-proteins in whole cell lysates of the strains MS11 and FA514; lanes 4 and 5, β_2-proteins in lysates of the strains R16 and NG74. (The anti-$fp42_{MS11}$ serum was not saturated against gonococci and hence shows several cross-reacting bands.) **Panel B**, same as panel A, except blot incubated with β_1 specific monoclonal antibody HAL-2 (Pohlner *et al.*, 1987). The epitope for HAL-2 is missing in the β_2-proteins in lanes 4–6. **Panel C**, identification of α_1- and α_2-proteins in culture supernatants of strains FA514, R16 and NG74 (lanes 2–4, respectively) using anti-$fp80_{R16}$ serum. Lanes 1 and 5 are reference lanes showing α-proteins in IgA protease digests of $fp180_{MS11}$ and $fp180_{R16}$ fusions, respectively.

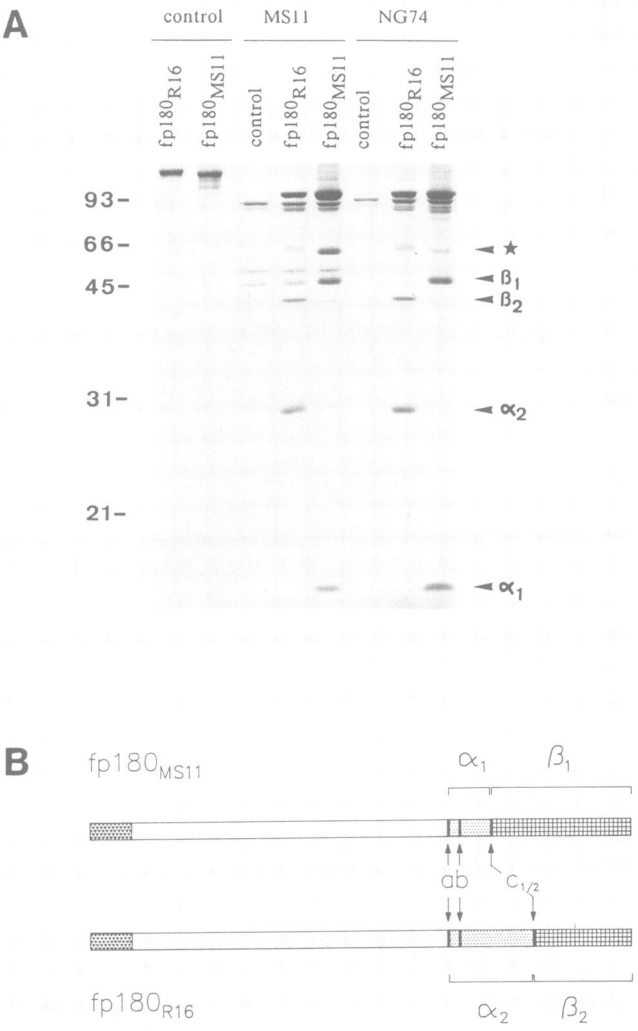

Fig. 5. Fusion proteins cleaved by IgA proteases with different specificities for IgA1. **Panel A**, Coomassie blue stained SDS–PAGE. Control, untreated fusion proteins ($fp180_{R16}$ and $fp180_{MS11}$); MS11 and NG74 controls, IgA protease preparations from strains MS11 (IgA1 specificity type 2) and NG74 (IgA1 specificity type 1). The remaining lanes show digests of the fusion proteins $fp180_{R16}$ and $fp180_{MS11}$ with IgA proteases from MS11 and NG74 respectively. The cleavage products are identical irrespective of the protease used; they differ though, with regard to the fusion proteins used as substrates. Partially digested fragments of the fusion proteins (α + β) are marked by an asterisk. Both the β-proteins and especially the α-proteins mimic increased apparent sizes in SDS gels; all mol. wts given in the text are based on DNA sequence calculations. **Panel B** illustrates the location of the cleavage site a, b, c_1 and c_2 in the fusions $fp180_{MS11}$ and $fp180_{R16}$ and of the fragments generated by cleavage with IgA proteases. The amino terminal ends of the fusion proteins consist of a 12 kd portion of the phage MS2 polymerase (Strebel *et al.*, 1986).

mutations, i.e. genetic drift. Neither can the observed changes be attributed to a convergent evolution in some strains driven by their need for particular amino acid combinations; this possibility can be ruled out because the observed variations include many silent nucleotide exchanges. To explain the mosaic-like composition of variant *iga* genes, we postulate a process of horizontal genetic exchange between the *iga* genes of independent *N. gonorrhoeae* strains.

This assumption is intriguing in the light of several recent observations. *N.gonorrhoeae*, like few other bacterial species, possesses an efficient natural DNA uptake system that allows high frequency transformation of this species (Sparling, 1966; Scocca *et al.*, 1974; Dougherty *et al.*, 1979), with homologous DNA being rapidly incorporated into the chromosome of transformed cells by homologous recombination. In addition, gonococci tend to undergo spontaneous autolysis (Hebeler and Young, 1975), thus providing DNA that might be taken up by healthy cells. Owing to these properties, transformation has been invoked as a mechanism involved in pilus-specific colony variation of gonococci (Norlander *et al.*, 1979). Conclusive evidence for the transformation model was recently provided by demonstrating a decreased frequency of pilus phase transitions in a DNA uptake deficient mutant and by growth of gonococci in the presence of DNase I (Seifert *et al.*, 1988; Gibbs *et al.*, 1989). It is therefore appealing to implicate DNA transformation as the mechanistic principle behind the composite organization of *iga* genes. This hypothesis is further supported by the recent identification of a gonococcal

DNA uptake sequence which is associated with transcriptional terminators of some gonococcal genes (Goodman and Scocca, 1988). Such a DNA uptake signal is also part of the *iga* terminator and is a conserved structure of all *iga* genes investigated here (Figure 2).

Transformation-mediated recombination may play a central role in increasing the genetic flexibility of the gonococcus and modulating its pathogenic potential. As previously demonstrated, this process constitutes the major pathway of pilin variation *in vitro* (Seifert *et al.*, 1988; Gibbs *et al.*, 1989). The mosaic-like organization of the *iga* genes, reported here, suggests that transformation-mediated recombination may also play a significant role *in vivo*. Transformation-mediated processes may also provide a means for the exchange of functional regions between the large *cps* gene complexes of encapsulated Neisseriae (Frosch *et al.*, 1989) as well as for the generation of hybrid gonococcal Protein I genes, which has been shown to occur by transformation *in vitro* (Carbonetti *et al.*, 1988). To affect single copy genes, such as the *iga* or the P.I locus, transformation-mediated recombination would of course require simultaneous infection of the same individual with more than a single strain. In this context it would be interesting to investigate whether transformation-mediated recombination could also promote genetic exchange between heterologous *Neisseria* species.

With regard to the secretory properties of gonococcal IgA proteases, it is the intriguing that the carboxy terminal helper domain can exist in at least two different forms (H_1 and H_2) without affecting the extracellular transport of the protease in *E.coli*. Preliminary observations suggest that the α-domain of the helper is not essential for the extracellular transport of IgA protease (J.Pohlner, unpublished data). Why this highly variant region, which gives rise to the small co-secreted α-protein, is maintained in gonococcal *iga* genes remains to be elucidated.

In contrast, the core of the β-domain seems to be strictly conserved. This region harbours those sequences of the helper which probably form amphipathic β-sheets and may fulfil pore functions necessary for the translocation of proform C through the outer membrane (F.Jähnig and T.Klauser, unpublished; Pohlner *et al.*, 1987). Future studies might therefore concentrate on this conserved amphipathic structure with the aim of explaining the mechanism of the extracellular secretion of IgA protease.

Materials and methods

Bacterial strains and growth medium

N.gonorrhoeae MS11 was originally isolated from an uncomplicated case of gonorrhoea at Mt Sinai Hospital, New York, in 1971/1972 (Swanson, 1973). Strain R16 was also from a case of uncomplicated gonorrhoea and isolated in 1978/1979 at New York Hospital (Salit *et al.*, 1980). Both strains were received from E.C.Gotschlich, New York. Strain FA514, obtained from F.P.Sparling (Chapel Hill), is a derivative of strain 6-73389, one of the initial penicillin-resistant gonococcal isolates in the United States and obtained from the Centers for Disease Control in 1976. *N.gonorrhoeae* NG74, provided by J.C.Piffaretti (Lugano), was isolated in November 1981 in Bern from a case of gonococcal urethritis. *E.coli* strain GC1 (Meyer *et al.*, 1982) was used for transformation with gonococcal DNA and DH1 (recA) (Hanahan, 1983) for the propagation of plasmid DNA. *E.coli* 537, a derivative of C600 containing the plasmid pCI857 (Remaut *et al.*, 1983), was used as the host strain for the production of pEX31-directed fusion proteins. GC agar base for growth of gonococci and meningococci was purchased from BBL and supplemented with IsoVitale X obtained from the same vendor.

Preparation of IgA proteases and α-proteins from culture supernatants

The purification of IgA proteases types 1 and 2, as well as the test system, was described previously (Halter *et al.*, 1984; Meyer *et al.*, 1987). Aliquots of ~100 ng of purified IgA protease were separated on 10% polyacrylamide gels (Laemmli, 1970) and blotted onto nitrocellulose. For preparation of α-protein from culture supernatants, the cells were removed after 12 h cultivation and the proteins of the supernatants were precipitated with 10% TCA. The precipitated proteins were washed with 80% ethanol, dried and diluted in sample solution; equivalents of 3 ml supernatant were separated on 13.75% SDS–PAGE gel (Laemmli, 1970) and immunoblotted.

Preparation of whole cell lysates from gonococci

The gonococcal strains were grown on GC agar plates for 14 h. Cells from one plate were harvested and suspended in 0.5 ml TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) supplemented with 10 mM EDTA. The same volume of sample solution was added, the cells were boiled for 10 min and then sonicated extensively. Samples of 20 μl were separated on 11.25% SDS–PAGE and subjected to immunoblotting.

Immunoblot analyses

Proteins separated on polyacrylamide gels were transferred onto a nitrocellulose sheet at 1 mA/cm^2 for 2 h using a semi-dry blot system (Biotec Fischer). The filters were saturated with 3% BSA in TBS followed by incubation with a monoclonal antibody (1:1000 dilution) or with antiserum (1:100 dilution). The nitrocellulose was washed repeatedly in TBS containing 0.5% Tween-20 and incubated with alkaline phosphatase coupled anti-mouse IgG, anti-rat IgG, or protein A (Sigma), as appropriate. The nitrocellulose was washed with TBS containing 0.5% Tween-20 and incubated with 5-bromo-4-chloro-3-indoxyl-phosphate, a synthetic substrate for alkaline phosphatase.

Preparation of genetically engineered fusion proteins and anti-fusion protein sera

Fusion proteins were produced with the phage λ P_L promoter controlled pEX expression system which gives rise to amino terminal fusions of the phage MS2 polymerase and a desired protein (Strebel *et al.*, 1986). To generate the $fp180_{R16}$ fusion, the *Hin*dIII fragment of *iga*-R16 (Figure 2) was inserted into the vector pEX31B, similar to the method previously described for the fusion protein $fp180_{MS11}$ (Pohlner *et al.*, 1987). The fusion protein $fp80_{R16}$ which includes the α_2-domain of *iga*-R16, was constructed by insertion of the *Bgl*II fragment of the *iga*-R16 gene (Figure 2) into the *Bgl*II site of pEX31B. Transformation of *E.coli* 537 with these recombinant plasmids placed the hybrid genes under temperature-sensitive control of the λ CI_{ts} repressor. The induction of fusion protein synthesis was according to Strebel *et al.* (1986). Fusion proteins were purified by electroelution from Coomassie blue stained SDS–PAGE gels using a Biotrap BT1000 (Schleicher & Schuell; Hunkapiller *et al.*, 1983). The eluted proteins were extensively dialysed against 40 mM Tris–acetate, pH 7.5, to remove SDS and subsequently against buffered saline with 50% glycerol. The eluted $fp80_{R16}$ was used to raise α_2 specific antiserum (anti-$fp80_{R16}$) in rats. Anti-$fp80_{R16}$ serum was collected on the 5th day after three consecutive intraperitoneal injections given at 14-day intervals.

Cleavage of fusion proteins with IgA protease

Fusion proteins eluted from SDS–PAGE gels were digested with purified IgA protease in an enzyme:substrate molar ratio of $<1:50$ in 10 mM potassium phosphate, pH7.5, 150 mM NaCl for 2 h at 37°C. It was essential to perform this reaction in the absence of traces of urea and SDS. The cleavage products were separated on a 13.75% SDS–PAGE gel and stained with Coomassie blue.

Cloning of iga genes and physical analysis of DNA

Chromosomal DNA from gonococcal strains was prepared from colonies grown on solid support (Stern *et al.*, 1986) and digested with *Cla*I enzyme. Fragments of ~9 kb were isolated by sucrose gradient centrifugation and inserted into pBA (Halter *et al.*, 1984), a derivative of pBR322. *E.coli* GC1 transformants were screened by colony hybridization using ^{32}P-labelled *iga*-MS11 fragments as a probe. The chromosomal mapping of gonococcal genes was performed essentially as described (Southern, 1975), with some modifications (Stern *et al.*, 1986). The cloned genes were mapped with enzymes from Boehringer Mannheim, Renner and New England Biolabs. DNA fragments were electrophoresed on either 0.8% agarose (SeaKem-MC) horizontal gels or 12.5% acrylamide gels using TBE buffer (100 mM Tris–borate, pH 8.1, 2.5 mM EDTA).

DNA sequence analysis

*Hin*dIII/*Bgl*II fragments of pIP2 and pIP3 were inserted into the *Hin*dIII/*Bgl*II sites of M13mp9 (Messing and Vieira, 1982) and into the *Hin*dIII/BamHI site of pEMBL-8 and pEMBL-9 (Dente *et al.*, 1983); *Bgl*II fragments (pIP3 and pIP4) were subcloned into the *Bam*HI site of pEMBL-9 in both orientations; the *Bgl*II/*Pst*I fragment of pIP2 was subcloned into the *Bam*HI/*Pst*I sites of M13mp9; the *Cla*I/*Bgl*II fragment of pIP4 was subcloned into the *Eco*RI/*Bam*HI site of pEMBL-8; the *Bgl*II/*Hin*dIII fragments of pIP2 and pIP4 were subcloned into the *Hin*dIII/*Bam*HI site of pEMBL-8 and pEMBL-9. These constructs were sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1980) using a labelled 17mer universal primer (P-L Biochemicals). Five oligonucleotides, JO22 (5′-GGTTAACGCCTGTGCCGT), JO14 (5′-TTGGGTTACAAAAACGGC), JO16 (5′-CGGTATTACCCGGTTGT), JO20 (5′TGCACATCGCCAGGAAGC) and JO24 (5′-TCCAGCGCATCCAAGGGG) were used to sequence the regions encoding the two cysteines (JO14), the processing sites a and b (JO16), the processing sites c_2 of pIP2 and pIP4 (JO20 and JO24) and the vicinity of the *Hpa*I sites (JO22). JO22 corresponds to the antisense (−) strand at nucleotide position 1448, JO14 and JO16 to the sense (+) strand at positions 2402 and 3101. JO20 and JO24 correspond to the sense and antisense strands at positions 3581 and 3874 in *iga*-R16 and *iga*-NG74, respectively. The oligonucleotides were end-labelled and used according to the dideoxynucleotide chain termination method (Sanger *et al.*, 1980). The synthesis and purification of the oligonucleotides was performed as described (Haas and Meyer, 1986).

Amino terminal protein sequence analysis

Proteins were separated by SDS−PAGE and transferred onto PVDF-membranes (Millipore). The proteins blotted onto the membrane were stained with Coomassie blue prior to dissection. Membrane-bound proteins were then sequenced using a pulsed liquid phase protein sequencer from Applied Biosystems (Model 477A), according to protocols provided by the vendor.

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References

Carbonetti,N.H., Simnad,V.I., Seifert,H.S., So,M. and Sparling,P.F. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6841−6845.

Dente,L., Cesarini,G. and Cortese,R. (1983) *Nucleic Acids Res.*, **11**, 1645−1656.

Dougherty,T.J., Asmus,A. and Tomasz,A. (1979) *Biochem. Biophys. Res. Commun.*, **86**, 97−104.

Fishman,Y., Bricker,J., Gilbert,J.V., Plaut,A.G. and Wright,A. (1985) In Schoolnik,G.K., Brooks,G.F., Falkow,S., Frasch,C.E., Knapp,J.S., McCutchan,J.A. and Morse,S.A. (eds), *The Pathogenic Neisseriae. Proceedings of the Fourth International Symposium.* American Society for Microbiology, Washington, DC, pp. 164−168.

Frosch,M., Weisgerber,C. and Meyer,T.F. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 1669−1673.

Gibbs,C.P., Reimann,B.Y., Schultz,E., Kaufmann,A., Haas,R. and Meyer,T.F. (1989) *Nature*, **338**, 651−652.

Gilbert,J., Plaut,A.G., Fishman,J. and Wright,A. (1988) *Infect. Immun.*, **56**, 1961−1966.

Goodman,S.D. and Scocca,J.J. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 6982−6986.

Grundy,F.J., Plaut,A.G. and Wright,A. (1987) *J. Bacteriol.*, **169**, 4442−4450.

Haas,R. and Meyer,T.F. (1986) *Cell*, **44**, 107−115.

Halter,R., Pohlner,J. and Meyer,T.F. (1984) *EMBO J.*, **3**, 1595−1601.

Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557−580.

Hebeler,B.H. and Young,F.E. (1975) *J. Bacteriol.*, **122**, 385−392.

Hunkapiller,M.W., Lujan,E., Ostrander,F. and Hood,L.E. (1983) *Methods Enzymol.*, **91**, 227−236.

Kilian,M. and Reinholdt,J. (1986) In Easmon,C.F.S. and Jeljaszewicz,J. (eds), *Med. Microbiol.*, **5**, 173−206.

Kilian,M., Mestecky,J. and Schrohenloher,R.E. (1979) *Infect. Immun.*, **26**, 143−149.

Koomey,J.M. and Falkow,S. (1984) *Infect. Immun.*, **43**, 101−107.

Koomey,J.M., Gill,R.E. and Falkow,S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7881−7885.

Kornfeld,S.J. and Plaut,A.G. (1981) *Rev, Infect. Dis.*, **3**, 521−534.

Laemmli,U.K. (1970) *Nature*, **227**, 680−685.

Messing,J. and Vieira,J. (1982) *Gene*, **19**, 269−276.

Meyer,T.F., Mlawer,N. and So,M. (1982) *Cell*, **30**, 45−52.

Meyer,T.F., Halter,R. and Pohlner,J. (1987) In McGhee,J.R., Mestecky,J., Ogra,P.L. and Bienenstock,J. (eds), *Recent Advances in Mucosal Immunology, 216B;* Plenum Press, New York, pp. 1271−1281.

Mortensen,S.B. and Kilian,M. (1984) *Infect. Immun.*, **45**, 550−557.

Mulks,M.H. and Plaut,A.G. (1978) *N. Engl. J. Med.*, **299**, 973−976.

Mulks,M.H. and Knapp,J. (1985) In Schoolnik,G.K., Brooks,G.F., Falkow,S., Frasch,C.E., Knapp,J.S., McCutchan,J.A. and Morse,S.A. (eds), *The Pathogenic Neisseriae. Proceedings of the Fourth International Symposium.* American Society for Microbiology, Washington DC, pp. 51−56.

Mulks,M., Simpson,D.A. and Shoberg,R.J. (1987) *Antonie van Leeuwenhoek*, **53**, 471−478.

Norlander,L., Davies,J., Norqvist,A. and Normark,S. (1979) *J. Bacteriol.*, **138**, 762−769.

Plaut,A.G., Genco,R.J. and Tomasi,T.B., Jr (1974) *J. Immunol.*, **45**, 289−291.

Plaut,A.G., Gilbert,J.V., Artenstein,M.S. and Capra,J.D. (1975) Science, **190**, 1103−1105.

Pohlner,J., Halter,R., Beyreuther,K. and Meyer,T.F. (1987) *Nature*, **325**, 458−462.

Remaut,E., Tsao,H. and Fiers,W. (1983) *Gene*, **22**, 103−113.

Sanger,F., Coulson,A.R., Barell,B.G., Smith,A.J.H. and Roe,B.A. (1980) *J. Mol. Biol.*, **143**, 161−178.

Salit,I.E., Blake,M. and Gotschlich,E.C. (1980) *J. Exp. Med.*, **151**, 716−725.

Scocca,J.J., Poland,R.L. and Zoon,K.C. (1974) *J. Bacteriol.*, **118**, 369−373.

Seifert,H.S., Ajioka,R.S., Marchal,C., Sparling,P.F. and So,M. (1988) *Nature*, **336**, 392−395.

Senda,S., Fujiyama,Y., Ushijima,T., Hodohara,K., Bamba,T., Hosoda,S. and Kobayashi,K. (1985) *Microbiol. Immunol.*, **29**, 1019−1028.

Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503−517.

Sparling,P.F. (1966) *J. Bacteriol.*, **92**, 1364−1370.

Stern,A., Brown,M., Nickel.P. and Meyer,T.F. (1986) *Cell*, **47**, 61−71.

Strebel,K., Beck,E., Strohmaier,K. and Schaller,H. (1986) *J. Virol.*, **57**, 983−991.

Swanson,J. (1973) *J. Exp. Med.*, **137**, 571−589.

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