Molecular Microbiology (1987) 1, 5–12

Common mechanism controlling phase and antigenic variation in pathogenic neisseriae

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Summary

The expression of the *Neisseria gonorrhoeae* opacity protein (Op, protein II), a major antigenic determinant of the outer membrane, is subject to frequent phase transitions. At least nine expression loci (*opa*E) are involved in the production of a large number of serologically distinct Op types. Using *opa*-specific oligonucleotides as probes in genomic blots, we detect Op-related gene sequences (*opr*) in *N. meningitidis* as well as in *N. lactamica*. DNA sequence analysis of such *opr* genes derived from *N. meningitidis* reveals distinct regions of homology with gonococcal *opa*E genes. As shown in the immunoblot, the proteins encoded by *opa* and *opr* are serologically related. Like the *opa*E genes, the 5′-coding sequences of the *opr* genes include a repetitive sequence composed of pentameric CTCTT units. The number of these coding repeat (CR) units is variable. This finding, together with the observation that all *opr* genes are constitutively transcribed, regardless of the status of protein production, suggests a translational control mechanism identical to that of the *opa* genes in gonococci. The related structures and control mechanisms of *opa* and *opr* genes imply a general significance of their gene products for the pathogenic character of the investigated *Neisseria* species.

Introduction

The *Neisseria* species form a group of Gram-negative diplococci that colonize the mucosal surfaces of mammals. The genus *Neisseria* includes both pathogenic and harmless commensal species. The pathogenic nature is strongly pronounced in *N. gonorrhoeae* and *N. meningitidis* (Bovre, 1984), but other members, too, may show opportunistic properties causing infections in a variety of anatomical sites (Johnson, 1983). The determinants that are responsible for the infectious character of certain *Neisseria* species are not very well understood.

Received 20 February, 1987. *For correspondence.

Bacterial virulence is a consequence of multiple attributes affecting different stages during the course of an infection. Many efforts have been made to identify specific factors that would help to distinguish between pathogenic species and commensal organisms. Indeed, some predominant characteristics have been implicated in pathogenicity, i.e., the production of an IgA1 protease (Plaut *et al.*, 1975; Pohlner *et al.*, 1987), the ability of bacteria to adhere specifically to mucus-secreting columnar epithelial cells (McGee *et al.*, 1982), the presence of the so-called H.8 antigen in the outer membrane (Cannon *et al.*, 1984; Black and Cannon, 1985), and the expression of iron-regulated proteins (Mietzner *et al.*, 1986).

Another series of investigations focused on variable components on the surface of pathogenic *Neisseria* spp., such as pili, outer membrane proteins and lipopolysaccharides, which are thought to contribute to the virulence of the organisms. Surface variability is considered as a crucial aspect of pathogenic *Neisseriae* which enables them to colonize different microenvironments within the host during the course of a natural infection, and to evade the host's immune response (Lambden *et al.*, 1979; Watt and Ward, 1981; Kristiansen *et al.*, 1984).

In *Neisseria gonorrhoeae*, the pilus protein as well as the opacity protein (Op, also named P.II) show significant inter- and intra-strain variability (Black *et al.*, 1984; Blake and Gotschlich, 1983; Heckels, 1981; Swanson, 1982). In *Neisseria meningitidis*, intra-strain heterogeneity has been demonstrated for outer membrane proteins of class 5 and LPS (Stephens and McGee, 1983; Poolman *et al.*, 1985). Regarding the heat-modifiable mobility during electrophoresis, the sensitivity to proteolytic enzymes, the occurrence of phase variation, and the extensive variability in expression and molecular weight, the class 5 proteins seem to be the equivalents of the Op molecules of the gonococcal outer membrane (Poolman *et al.*, 1985).

From our studies of the *opa* gene system, which is responsible for Op expression in *N. gonorrhoeae*, we have learned that the genome of *N. gonorrhoeae* harbours at least nine gene loci, all of which are able to express variant Op molecules (Stern *et al.*, 1984). All members of this gene family are constitutively transcribed. However, translation to a functional protein can only occur if the translational start codon ATG is in frame. The reading frame is controlled by a structural element, termed the coding repeat (CR), which consists of a variable number of CTCTT repeat units. Such units can be spontaneously added or

lost, manoeuvring the ATG codon into or out of frame (Stern *et al.*, 1986). In addition to its role in gene conversion, CR variation appears to be the basic mechanism involved in phase and antigenic variation of the gonococcal Op expression.

The class 5 proteins in the outer membrane of *N. meningitidis* exhibit similarities to the gonococcal Ops with regard to their heat-modifiable electrophoretic mobility and their extensive antigenic diversity (Poolman *et al.*, 1985). This fact prompted us to extend our molecular analysis of surface variation to other interesting members of the genus *Neisseria*. Our report describes the structure and regulation of Op-related genes (*opr*) that we detected in *N. meningitidis* and also *N. lactamica*, demonstrating the narrow relatedness of the three systems.

Results

Identification of opr *sequences in* N. meningitidis *and* N. lactamica

Using conserved oligonucleotide probes of a gonococcal *opa* gene in a dot blot assay we recently became aware of Op-related (*opr*) sequences in *N. lactamica* and *N. meningitidis*, though not in other *Neisseria* species (unpublished results). One of the probes, AN3, was used to characterize further the *opr* sequences detected in the two *Neisseria* species by Southern blot analysis. The AN3 probe was homologous to the so-called coding repeat (CR) sequence consisting of CTCTT pentamer units. Chromosomal DNA samples prepared from one *N. lactamica* strain and four *N. meningitidis* strains, and from *N. gonorrhoeae* strain MS11 as a reference, were digested with *Cla*I and *Pst*I endonucleases (Fig. 1A and B, respectively) and, after blotting onto nitrocellulose, probed with labelled AN3. The results of the hybridization clearly demonstrate the presence of more than one *opr* gene in genomic DNA of both *N. lactamica* and *N. meningitidis*. In contrast to the complexity of the *opa* gene family in gonococci, only four *opr* loci were found in each of the *N. meningitidis* strains and no more than two were found in *N. lactamica*.

Op-related proteins in N. lactamica *and* N. meningitidis

The detection of *opr* loci in *N. lactamica* and *N. meningitidis* raised some questions: are these genes active in the two non-gonococcal species and, furthermore, do the gene products show significant homology to the gonococcal Op protein. In order to investigate the expression of Op-related proteins, immunoblotting was performed using rabbit antiserum raised against denatured Op protein of the gonococcal strain MS11 V0 (Stern *et al.*, 1984). Four out of five *N. meningitidis* isolates produced one protein each that showed strong cross-reactivity with the anti-Op serum (Fig. 2). The molecular weights of the detected proteins corresponded to those of the class 5 proteins of *N. meningitidis*. In the cell lysates of *N. meningitidis* DMP141 and the *N. lactamica* isolate no Op-related proteins were detectable. Obviously, the number of proteins expressed did not correlate with the number of *opr* loci detected in the Southern blot and therefore not every locus was active in the production of a protein.

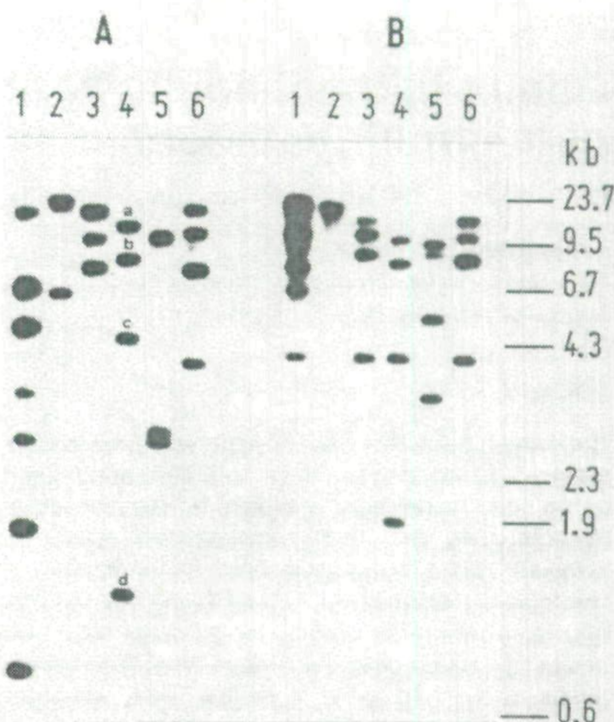

Fig. 1. Genomic blot of chromosomal DNA from different *Neisseria* species. Both panels (A and B) show six lanes with chromosomal DNA from *N. gonorrhoeae* MS11 (O⁺) (lane 1), *N. lactamica* 1855 (lane 2), *N. meningitidis* B1937 (lane 3), *N. meningitidis* C1936 (lane 4), *N. meningitidis* B1940 (lane 5), and *N. meningitidis* AMP140 (lane 6). Genomic DNA was digested with *Cla*I (A) and *Pst*I (B), respectively, and probed with the oligomer AN3 (Fig. 4).

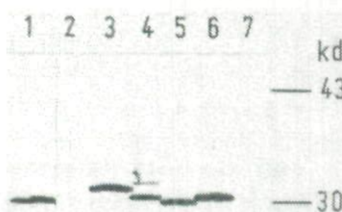

Fig. 2. Immunoblotting of Op-related proteins in cell lysates of various *Neisseria* species. The cell lysates from *N. gonorrhoeae* MS11 (O^+) (lane 1), *N. lactamica* 1855 (lane 2), *N. meningitidis* B1937 (lane 3), *N. meningitidis* C1936 (lane 4), *N. meningitidis* B1940 (lane 5), *N. meningitidis* AMP140 (lane 6), and *N. meningitidis* DMP141 (lane 7) were subjected to immunoblotting using rabbit antiserum raised against dentured Op of MS11 gonococci.

The control region of opr *genes*

The identification of *opr* genes in *N. meningitidis* and *N. lactamica* using the AN3 probe at the same time demonstrated that these loci possessed a so-called coding repeat (CR) which was known to be the crucial element in the control of gonococcal *opa* gene expression (Stern *et al.*, 1986). We therefore characterized the CR region of each *opr* locus, first by chromosomal DNA sequencing of *opr* genes and second, for comparison, by sequencing on the mRNA level. Both experiments were performed on the basis of the primer extension protocol successfully used previously (Stern *et al.*, 1986). The protocol involved an oligonucleotide primer (MB1) that was complementary to a conserved sequence in *opa/opr* genes overlapping the CR sequence and the downstream *opa* leader coding sequence (see Fig. 4). This primer was extended in the presence of dATP and dGTP as the sole triphosphates. Therefore, only the CTCTT pentamer repeat units of the CR sequence would be a sufficient template for DNA polymerase. The primer extension reaction was expected to be immediately terminated beyond the 5′-end of the CR sequence, thus creating discrete bands in a sequencing gel. As a control, to exclude incidental termination, all primer extension assays were repeated with the same triphosphates plus ddTTP. In these latter assays the extended primer should have been exactly one nucleotide longer since an A was expected upstream of all CR sequences (Stern *et al.*, 1986).

For the DNA level, Fig. 3A shows different patterns of extended primers for the various *Neisseria* DNAs investigated. The number of bands, each corresponding to at least one *opr* locus, varies between different species. Moreover, individual *opr* genes contain different numbers of repeat units ranging from 7 up to about 22 CR units. Some of the bands in the sequencing gel appear to be double bands, thus representing more than one *opr* locus. Essentially the same pattern of bands is seen on the mRNA level (Fig. 3B), suggesting that all existing *opr* genes linked with a CR element are constitutively transcribed in the two *Neisseria* species.

Variations in the CR region of opr *genes*

In *N. gonorrhoeae* the variability of Op-expression is controlled by the CR region which affects the translation of *opa* genes by switching the reading frame. To determine if the expression of individual *opr* genes in *N. meningitidis* and *N. lactamica* is subject to the same intriguing control mechanism, we tried to derive spontaneous isogenic variants of some of the isolates by *in vitro* cultivation. This could not be done on the basis of colony morphology

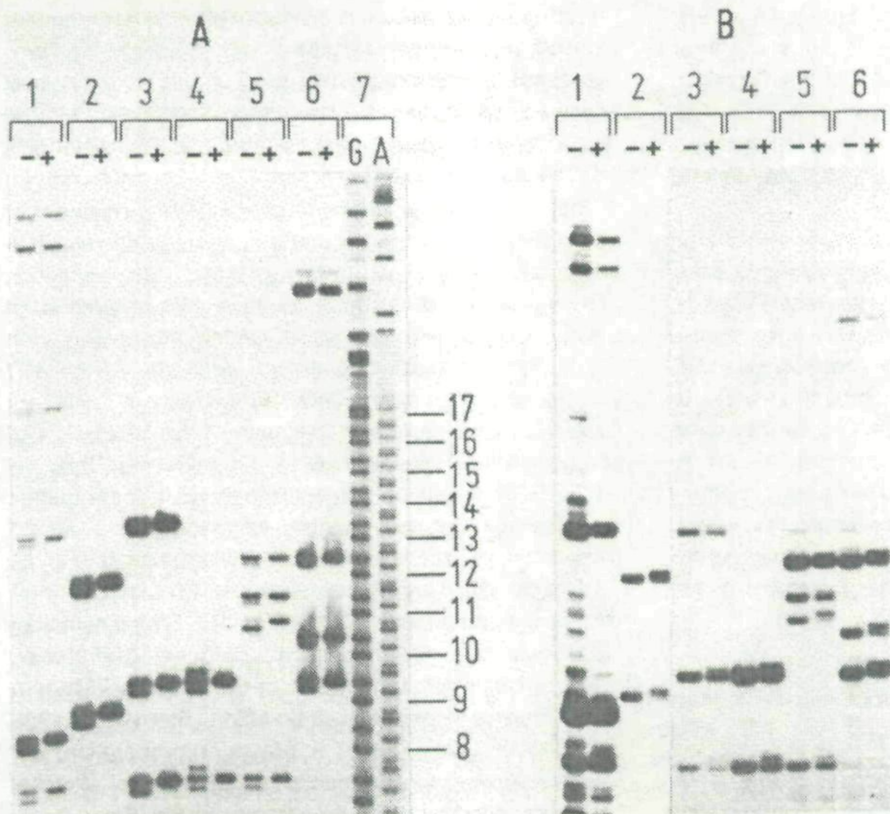

Fig. 3. Primer extension sequencing of chromosomal DNA and total RNA isolated from various *Neisseria* species. Chromosomal DNA (panel A) and RNA (panel B) were isolated from *N. gonorrhoeae* MS11 (O⁺) (lane 1), *N. lactamica* 1855 (lane 2), *N. meningitidis* B1937 (lane 3), *N. meningitidis* C1938 (lane 4), *N. meningitidis* B1940 (lane 5), and *N. meningitidis* AMP140 (lane 6). For sequencing, the 5′-labelled oligonucleotide MB1 was used as a primer. The reactions were performed in the presence of dATP and dGTP (−), or with additional ddTTP (+). As reference (lane 7, panel A), to show the repetitive features of the CR sequence, the G and A reaction of the standard sequencing protocol was performed on plasmid pOPR18 (Stern *et al.*, 1986).

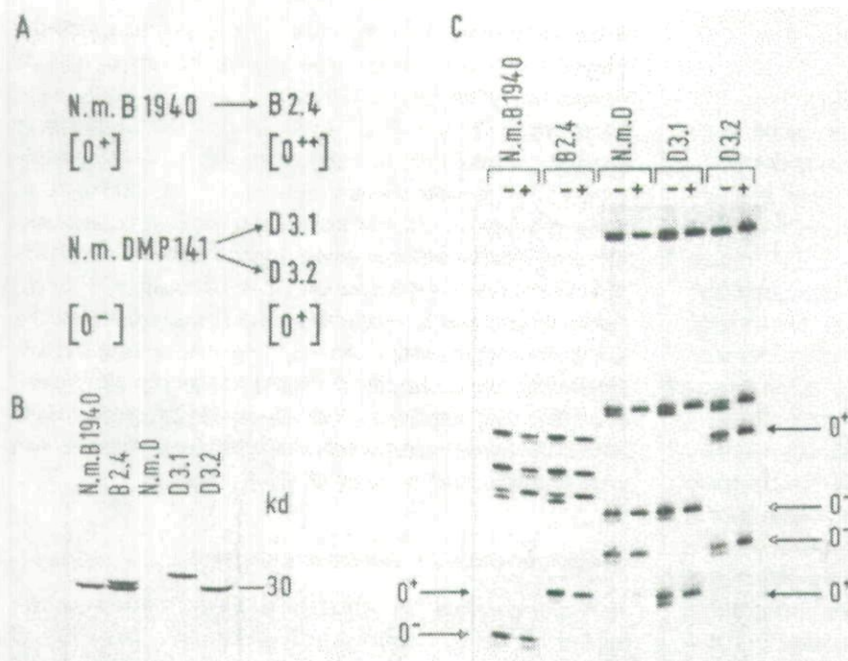

Fig. 4. Analysis of the control mechanism for phase and antigenic variation. (A) Scheme of the derivation of isogenic variants of *N. meningitidis* strains B1940 (O^+) and DMP141 (O^-). (B) Immunoblotting of the variant Op-related proteins in cell lysates according to the protocol in Fig. 2. (C) Primer extension with *opr*-specific mRNA isolated from isogenic meningococci. (−) and (+) reactions of the primer extension experiment are outlined in Fig. 3. The positions of the variable bands, including the putative *opr* expression phenotype of the corresponding transcripts, are indicated at the borders of the gel.

because the variation in Op-related proteins was not reflected by detectable differences (Poolman *et al.*, 1985; unpublished results).

Instead, we screened individual colonies with anti-Op serum and observed occasional changes in the reaction with this serum. Aliquots of variant colonies were taken and lysed for an immunoblot analysis. Figures 4A and B show the derivations and the protein patterns of three spontaneous variants selected from two *N. meningitidis* strains. These are variant B2.4 (O^{++}: 29.5 kDa, 30.5 kDa) derived from the isolate B1940 (O^+: 29.5 kDa), and variants D3.1 (O^+: 32.5 kDa) and D3.2 (O^+: 30 kDa) derived from isolate DMP141 (O^-).

Figure 4C shows the primer extension experiments done with chromosomal DNA of these isogenic meningococcal *opr* variants. The O^+ to O^{++} switch of parental B1940 to variant B2.4 involves a distinct increase in size of one band, corresponding to an insertion of precisely one CR unit. Further, the two independent switches from O^- to O^+ of isolate DMP141 to its isogenic derivatives D3.1 and D3.2 are associated with the loss of a single CR unit in one transcript and an insertion of two CR units in another transcript. This result shows a strict correlation of *opr* gene expression and the variations of the CR unit number, which directly affect the reading frames in the 5′ regions of *opr* genes.

Cloning and DNA sequence analysis of meningococcal opr *genes*

To analyse the relatedness of *opa* and *opr* structural genes we determined the DNA sequences of two *opr* gene copies of *N. meningitidis* C1938. For this purpose we extracted the four *opr*-specific *Cla*I fragments of this strain (Fig. 1A, lane 4) out of a preparative agarose gel. As a reference, a small aliquot of this gel was subjected to Southern analysis using the AN3 probe. Before cloning, we determined the number of CR units of each individual *Cla*I fragment by primer extension. In agreement with the primer extension experiment performed with total genomic DNA, we found for three fragments (a, b, and d) nine CR units each and for fragment c seven repeat units (Fig. 1). The two smaller fragments were inserted into the vector pBA and cloned in *E. coli*.

The physical map of the clones pOPM1 (fragment c) and pOPM3 (fragment d), and the strategy of their DNA sequence analysis using synthetic oligonucleotide primers, are shown in Fig. 5; the nucleotide sequences of the two cloned meningococcal *opr* loci are presented in Fig. 6. Plasmid pOPM1 contains a complete *opr* gene of 780 bp, whereas plasmid pOPM3 harbours a partial *opr* gene that lacks its 3′-end because of an intragenic *Cla*I site used for the digestion of the chromosomal DNA.

The DNA sequence alignment reveals a large degree of homology between the two *opr* genes, including the transcriptional and translational initiation signals (Fig. 6). The major sequence diversities appear to be concentrated on a few hypervariable regions (Fig. 5B). Strong homology also exists with the gonococcal *opa* genes (Stern *et al.*, 1986). A stringent comparison of the amino acid level of six different *opa* genes of strain MS11 (Stern *et al.*, 1986; A. Stern, F. Jähnig and T.F. Meyer, in preparation) with the two meningococcal genes reveals several stretches of continuous homology (see frames in Fig. 6).

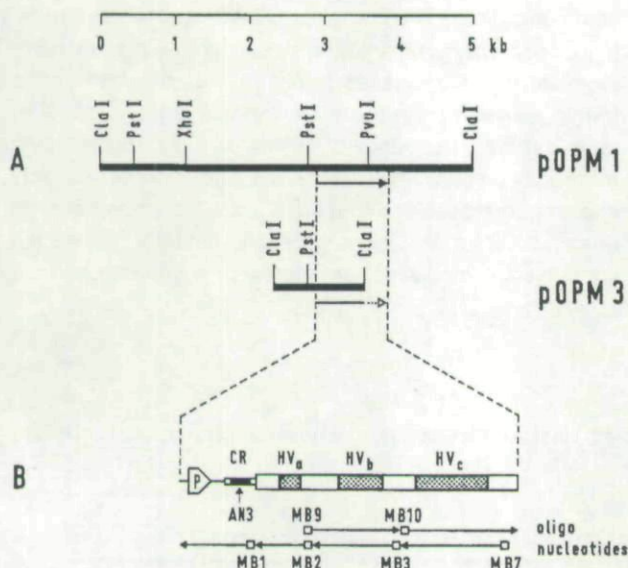

Fig. 5. Physical map and DNA sequencing strategy of the cloned opr_c and opr_d loci of *N. meningitidis* C1938. (A) The top shows the physical maps of pOPM1 (opr_c) and pOPM3 (opr_d). The coding regions of the *opr* genes and the direction of transcription, as deduced from the sequencing data, are marked by an arrow. (B) represents a generalized diagram of the *opr* genes with a CR sequence, hypervariable regions (HV), and the promoter (P). The location of the oligonucleotide sequences used as primers in the DNA sequencing experiments are indicated. The boxes indicate the location of the primers, the arrows designate the direction of the primer extension. AN3 was used as a probe in DNA filter hybridizations. Sequence of the oligonucleotide primers:

AN3: 5'-CTCTTCTCTTCTCTTCTCTTCTCTT-3'
MB1: 5'-TGCGCTGCGGAAGAGAAG-3'
MB2: 5'-ATGCGTACGGATGTTTCT-3'
MB3: 5'-ATAGGGTTTGAATTTATC-3'
MB7: 5'-TTCGTGGGTTTTGAAGCG-3'
MB9: 5'-AACATCCGTACGCATTCC-3'
MB10: 5'-GTCGCCTACGGACACGTC-3'

The reading frame of both cloned *opr* genes is out of frame with respect to the unique initiation codon ATG (Fig. 6). This is consistent with the CR variation control model for an inactive *opr* expression locus (Fig. 7). However, the opr_d locus cloned in plasmid pOPM3 shows only 8 CR units in contrast to the 9 CR units expected from the genomic sequencing experiment of fragment d (Fig. 3A). Clone pOPM1, on the other hand, contains the expected number of 7 CR units. Interestingly, when we extrapolate the pOPM3 clone to the expected 9 CR units, the gene comes into frame (Fig. 6). Because of our cloning strategy we can exclude the possibility of having cloned an *opr* locus other than opr_d. Instead we must account for the loss of one CR unit to a spontaneous CR switch in opr_d. Whether it occurred in a small fraction of the meningococcal population used for the cloning, or after cloning in *E. coli*, is not known. More importantly, we can assume now that opr_d with its original 9 units represents the active *opr* locus of strain C1938, being responsible for the production of the Op-related protein (Fig. 2, lane 4).

Discussion

The suggested significance of gonococcal Op in virulence and the intriguing genetic system involved in the expression of this protein led us to extend our interest on Op-related proteins to other *Neisseria* species. By Southern blotting we revealed two *opr* gene loci in *N. lactamica* and four loci in each of the meningococcal isolates investigated. Although this study shows a much lower complexity of *opr* genes in these two species as compared with the gonococcal *opa* gene family which has at least nine gene loci, nevertheless, the typical regulatory features of gonococcal *opa* genes appear to be conserved in the *opr* genes of *N. lactamica* and *N. meningitidis*: i.e. (a) all *opa* and *opr* genes present in the genome are constitutively transcribed, (b) the 5'-regions of these genes are composed of pentameric repeat units, the so-called CR units that code for the hydrophobic part of a leader peptide, (c) the number of CR units is different in most of the genes of one genome and, finally, (d) the variation of *opa* and *opr* expression is inherently associated with changes in the CR unit number of individual transcripts.

Fig. 7 illustrates the control mechanism of *opa*/*opr* genes that appears to be a common principle in the three different *Neisseria* species investigated. In all three species at least two expression loci are found that can be activated to produce Op and Op-related proteins. The number of CR units present in an individual gene locus determines whether translation into a functional protein can occur. As the majority of the *opa*/*opr* gene sequences code for variable proteins, changes in the repeat number of different gene loci can result in both phase and antigenic variation.

It is conceivable that the number of *opa*/*opr* genes present in a single genome directly influences the frequency of phase transitions. Nonetheless, the *opr* system in *N. lactamica*, harbouring only two gene loci, may be efficient enough to deceive the immune system of the host, provided that these genes can be expressed. A comparable less complex variation system could be found in *Salmonella typhimurium* where the production of only two antigenic types of the flagella is controlled by an invertable element (Simon *et al.*, 1980).

The presence of *opr* sequences in *N. lactamica*, commonly regarded as a commensal, is consistent with a number of reports that *N. lactamica* can occasionally induce infections at different sites in humans (Johnson, 1983). Furthermore, thermal renaturation studies have shown that *N. gonorrhoeae, N. meningitidis* and *N. lactamica* share greater DNA homology with each other than with other *Neisseria* species (Hoke and Vedros, 1982). In addition, the expression of H.8 antigen and the AgR-37K protein in the three species supports the assumption that an evolutionary divergent development separated them from the other members of the genus (Cannon *et al.*, 1984; Black and Cannon, 1985; Mietzner *et al.*, 1986).

The *opa* and *opr* genes share conserved coding sequences. Previous investigations indicate that the meningococcal class 5 proteins, also subject to an extensive variability, represent the equivalent of the opacity proteins in *N. gonorrhoeae* (Frasch and Chapman, 1972; Frasch and Mocca, 1978; Ashton *et al.*, 1983; Poolman *et al.*, 1985). Convincing evidence for the relatedness of the two protein classes was demonstrated by their amino-terminal sequence homology (M. Achtmann, personal communication). The complete DNA sequence of a meningococcal *opr* gene, presented here, shows that the homology of the two classes is not restricted to the amino terminus. Apart from the hypervariable regions, the Op-related protein sequence of *N. meningitidis*, representing a class 5 protein, shows only few additional amino acid exchanges in positions that are conserved among several opacity proteins of *N. gonorrhoeae* strain MS11. The strict conservation of distinct structures of a variable surface

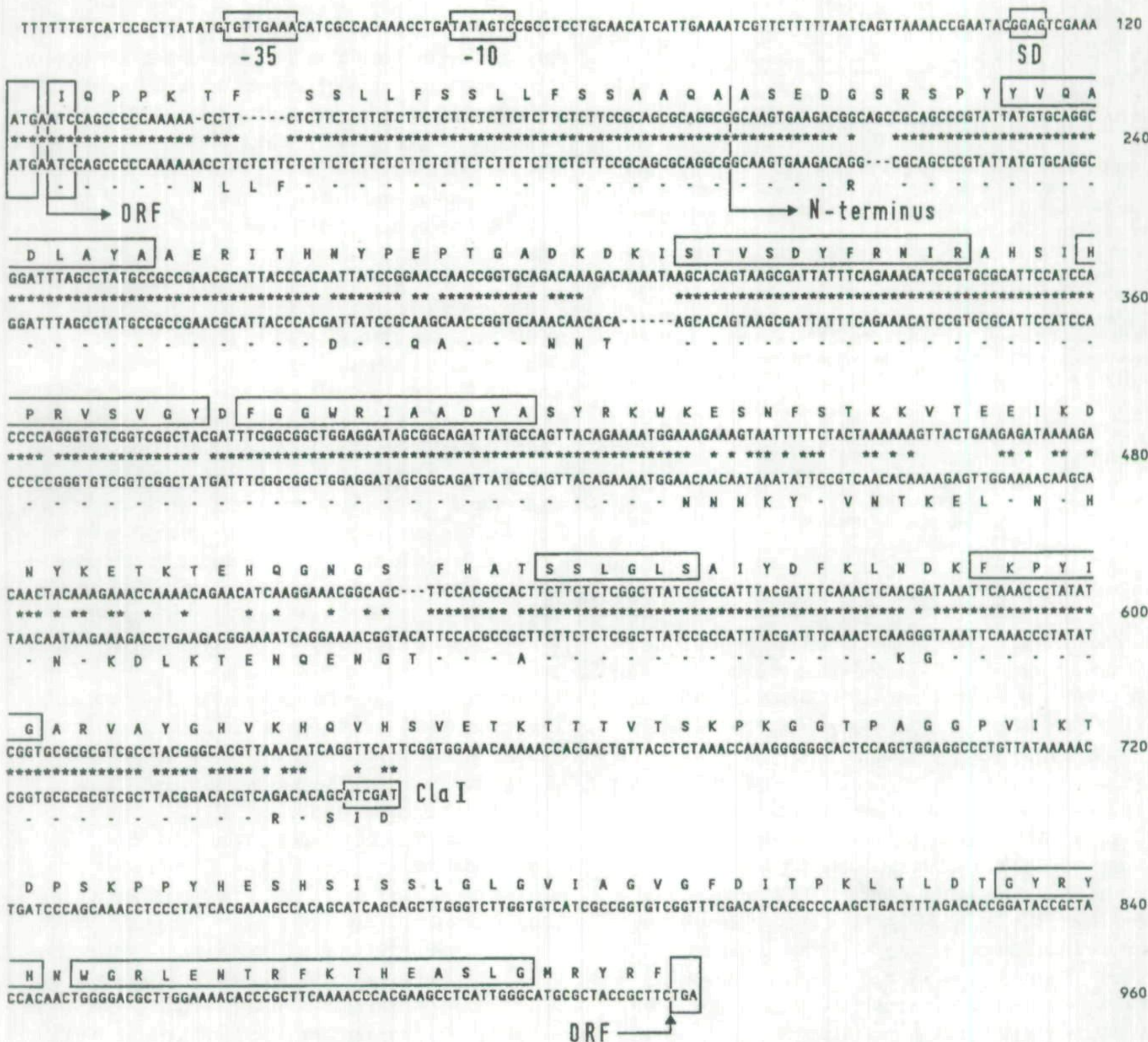

Fig. 6. DNA sequences of the *opr* genes cloned in pOPM1 and pOMB3. The upper DNA and protein sequences refer to pOPM1, the lower ones to pOPM3. Asterisks between the two sequences indicate homology on the DNA level. Upstream of the open reading frame (ORF) and the ATG initiation codon (vertical frames), typical −35 and −10 promoter regions and a Shine-Dalgarno (SD) sequence are marked by horizontal frames. With respect to the ORF the ATG initiation codon is out-of-frame in both gene loci. Homology with conserved *opa* regions of *N. gonorrhoeae* strain MS11 is indicated by horizontal frames at the protein level.

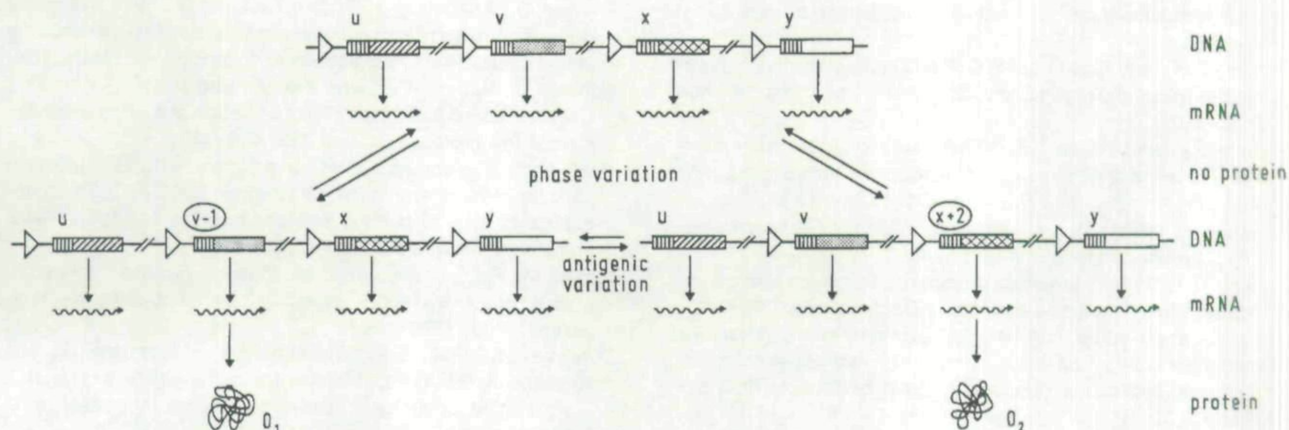

Fig. 7. Model for the control of phase and antigenic variation (for explanations see *Discussion*).

protein among pathogenic *Neisseria* species might provide an essential function associated with the pathogenic character of these bacteria.

Experimental procedures

Bacterial strains

N. meningitidis AMP140, B1937, B1940, C1938, DMP141 of the respective serotypes A to D, and *N. lactamica* 1855 were kindly provided by U. Berger. *N. gonorrhoeae* strain MS11 (Stern *et al.*, 1986) was obtained from E. C. Gotschlich. The *opa* and *opr* expression phenotypes are indicated by $O^{-/+}$. *E. coli* strains GC1 (Meyer *et al.*, 1982) and DH1 (Hanahan, 1983) were used for cloning of meningococcal DNA.

Primer extension and sequencing

5 μg of chromosomal- or 100 ng of gel-purified DNA were digested with *Mbo*I restriction enzyme. The DNA was ethanol-precipitated, and dissolved in 3 μl H_2O and 4 μl of 10 mM NaOH. The DNA was denatured by heating to 100°C and the solution was neutralized with 2 μl 20 mM Tris-HCl pH 7.6 and 1.5 μl 10 mM HCl. 1.5 pmoles of 5′-P^{32}-labelled MB1 primer were coupled to the DNA by incubating at 42°C for 5 min after a second heating. The annealed sample was supplemented with 10 mM Tris-Hcl pH 8.3, 5 mM $MgCl_2$, 200 mM dGTP and 200 mM dATP and split into two aliquots. The second aliquot was supplemented with additional 400 mM ddTTP. 1 U Klenow polymerase was used to extend the primer for 30 min at room temperature. For primer extension with total mRNA cloned 200 U M-MLV reverse transcriptase (BRL) and 0.5 pmol end-labelled oligomer MB1 were used per assay (Stern *et al.*, 1986). The meningococcal clones pOPM1 and pOPM3 were sequenced by a modified supercoil method using end-labelled oligonucleotides as primers (Wallace *et al.*, 1981).

Other techniques

For detection of Op-related proteins in non-gonococcal strains, 100 μg of total cell lysate solved in sample solution was electrophoresed on a 12.5% polyacrylamide gel and blotted onto nitrocellulose sheets. To extract DNA from gels, the agarose pieces were put into dialysis tubings and electro-eluted in 40 mM Tris-acetate pH 8 and 1 mM EDTA. The immunoblotting assay (Blake *et al.*, 1984), the immunological screening of colonies (Stern *et al.*, 1984), DNA filter hybridization (Stern *et al.*, 1984) and oligonucleotide preparations (Stern *et al.*, 1986) have been described.

Acknowledgements

We are grateful for the excellent assistance in these experiments by S. Störzbach, for the preparation of oligonucleotides by B. Tries and J. Pohlner, and for the artwork by K. Lamberty. This work was supported by the Deutsche Forschungsgemeinschaft (grant Me705/2-3).

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