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Horizontal Genetical Exchange in the Evolution of *Neisseria meningitidis* outer Membrane Proteins

Abstract

Antigenic variability contributes significantly to the ability of many pathogens to evade host immune responses. Understanding the genetical mechanisms underlying the evolution of such variation enhances our ability to design and evaluate novel vaccines rationally. Horizontal genetical exchange has played a major role in the evolution of the porin proteins of *Neisseria meningitidis* and the implications of this have to be considered when novel meningococcal vaccines based on these, or other antigens, are being assessed.

Antigenic variation, evolution, and vaccination

A major selective pressure on microorganisms that live in close association with humans is their need to prevent or evade the lethal effects of their host's immune response. There are a number of possible strategies for achieving this including: hiding from the immune response (e.g. intracellular growth); preventing or interfering with the immune response (immune modulation); and evading the immune response (e.g. antigenic variation, or the production of poorly immunogenic surface components). The adoption of these strategies, particularly antigenic variation, has resulted in an 'arms race' between the immune system of the host and the invading or colonising microbes. This has, in turn, lead to the co-evolution of microbe survival strategies and host immune responses (Brunham *et al.* 1993). Many of the complex interactions that have arisen in this way remain poorly understood and it is possible that some of the 'antigenic variation' observed in pathogenic microorganisms is the result of selective pressures other than immune selection (Virji *et al.* 1993).

Since Jenner's experiments into Smallpox vaccination almost 200 years ago, vaccines have become increasingly important for the prevention and control of infectious disease. Priming the immune system by means of vaccination aims to alter the balance between host and parasite in favour of the vaccinated individual; however; mass vaccination can also alter the selective pressures experienced by the target organism. If the vaccine is effective and administered to sufficient members of the host population, the extinction of the parasite may

ensue, the eradication of Smallpox providing a spectacular example (Fenner *et al.* 1988). However, Smallpox remains the sole example of the eradication of a major human pathogen by vaccination, despite a number of international vaccination programs and much research into new vaccines. Of course, it may not always be desirable to eradicate a microbial colonist that rarely causes disease, lest a more aggressive organism should fill the vacated niche. It is therefore desirable to understand the population biology as well as the pathology of disease causing microorganisms.

A further spur to research is the number of disease-causing organisms for which there are no satisfactory vaccines. Antigenic variability is often a major reason for vaccine failure, and also presents difficulties for vaccine design. Bacteria that normally have a commensal relationship with their host, but which can cause disease, present particular problems in vaccine design and assessment. The continual exposure of these bacteria to host immune responses during colonisation has led to the evolution of sophisticated genetical mechanisms for the generation of antigenic variants (Seifert & So, 1988). Populations of such bacteria may contain strains that express diverse surface antigens in almost limitless combinations. This presumably promotes carriage in the host by enabling the bacterium to avoid immune responses induced by its presence. Although progress has been made in defining genetical mechanisms for intra-strain variation for a number of antigens in several microbial species (Robertson & Meyer, 1992), the population genetics of inter-strain variation is less well understood. The increasing demand for novel, defined, vaccines necessitates an understanding of the population genetics of antigenic variation which is essential for rational vaccine design and assessment. It is particularly important in anticipating selective effects of vaccine implementation on the population of the target organism. This article describes the contribution of horizontal genetical exchange to the generation of antigenic diversity of the major outer membrane proteins (OMPs) of *Neisseria meningitidis.*

Vertical and horizontal genetical exchange

In asexual organisms, such as the bacteria which divide by binary fission, cell division normally gives rise to two daughter cells which are clones of their mother cell. In this case genetic information is transferred 'vertically' from single parent to offspring, and the result is a clonal population. The first studies on the population genetics of bacteria identified clonal population structures, characterised by linkage disequilibrium of alleles, and this was considered to be the model for all bacterial populations (Selander & Levin, 1980; Selander *et al.* 1986; Selander *et al.* 1987). However, there is increasing evidence that sexual processes such as transformation, transduction, and conjugation, can play an important part in the evolution of bacterial species (Lorenz & Wackernagel, 1994) and can disrupt such clonal structures (Maynard Smith *et al.* 1993). 'Horizontal genetical exchange' normally involves the movement of relatively

small parts of the chromosome between strains and has been referred to as 'localised sex' (Maynard Smith *et al.* 1991; Maynard Smith, 1995). It requires both a mechanism for DNA transfer and an opportunity for exchange, in other words there must be a sufficient level of association among genetically diverse strains. The balance between horizontal and vertical genetical exchange is different between different species of bacteria and even differs within some species, resulting in different degrees of clonality (Maynard Smith *et al.* 1993; Spratt *et al.* 1995)

The most compelling evidence for horizontal genetical exchange is the observation that bacterial genes are mosaics. A mosaic gene is one in which different segments of the gene have different evolutionary histories; such structures become apparent when genes from many natural isolates are compared (DuBose *et al.* 1988; Coughter & Stewart, 1989; Halter *et al.* 1989; Milkman & Bridges, 1990; Feavers *et al.* 1992a; Spratt *et al.* 1989; Milkman & McKane, 1995). A number of antigen genes from various species have been shown to comprise mosaics, implying involvement of horizontal genetical exchange in their evolution, including: flagella genes in *Salmonella enteritica* (Li *et al.* 1994); *Streptococcus pneumoniae emm*-like genes (Whatmore & Kehoe, 1994); the capsulation genes of *Haemophilus influenzae* (Kroll & Moxon, 1990); IgA protease and pilin genes of *Neisseria gonorrhoeae* (Halter *et al.* 1989; Haas *et al.* 1992); and a number of antigens of *N. meningitidis*, including the porin and *opa* genes (Maiden, 1993; Hobbs *et al.* 1994).

Neisseria meningitidis and horizontal genetical exchange

N. meningitidis is a major cause of childhood disease world wide and is an example of a normally commensal organism that occasionally causes a life-threatening infection. The *Neisseria* are a group of genetically closely related species only two of which, *N. meningitidis* and *N. gonorrhoeae*, commonly cause disease. The meningococcus normally colonises the nasopharynx asymptomatically and carriage rates in a country such as the UK can be high, in some cases exceeding 10% of the population (Cartwright *et al.* 1987). A very small proportion of colonisations, usually fewer than 1 per thousand (Peltola, 1983; Schwartz *et al.* 1989), result in the organism invading the host, crossing the mucus membrane into blood stream. This invasion can develop rapidly into a highly dangerous meningitis and/or septicaemia. If the fulminant stage of the infection is reached prognosis is poor, even with aggressive antibiotic treatment and intensive supportive therapy. Although the serious nature of meningococcal infection has provided much of the impetus for research of this organism, the meningococcus also rewards investigation with the elegance of its mechanisms of antigenic variation and as a model for the interrelationships between epidemiology and population genetics. For example, there are at least four different epidemiologies of meningococcal infection and these are associated with distinct population structures (Maiden & Feavers, 1995).

In common with a number of other bacteria that colonise the upper respiratory tract, *N. meningitidis* has the property of autolysis and is naturally competent for DNA uptake. High carriage rates, autolysis and natural competence combine to provide both the mechanism and opportunity for horizontal genetical exchange mediated by transformation and homologous recombination. Recently, evidence of the major influence of horizontal genetical exchange on the population genetics and epidemiology of the meningococcus has accumulated (Maiden, 1993; Maiden & Feavers, 1995). Nucleotide sequence and other analyses have demonstrated mosaic DNA structures in numerous meningococcal genes including those encoding: 'housekeeping' genes (Zhou & Spratt, 1992); penicillin binding proteins (Spratt *et al.* 1989; Spratt *et al.* 1992); IgA protease (Morelli *et al.* 1994); Opa proteins (Hobbs *et al.* 1994); sulphonamide resistance (Radstrom *et al.* 1992); and outer membrane proteins (OMPs) (Feavers *et al.* 1992a). In addition to evidence inferred by comparison of nucleotide sequence data, there have been a number of *in vitro* demonstrations of horizontal genetical exchange: (i) exchange of putative virulence determinants on co-cultivation of distinct *N. meningitidis* strains (Frosch & Meyer, 1992); (ii) the transfer of *penA* genes, important in penicillin resistance, from *Neisseria flavescens* to *N. meningitidis* (Bowler *et al.* 1994); and (iii) the use of transformation to construct multivalent meningococcal vaccine strains (van der Ley *et al.* 1993).

The outer membrane proteins of the *Neisseria*

In common with other Gram negative organisms the *Neisseria* express porins, pore proteins, in their cell envelope (Nikaido, 1992). These are the most abundant OMPs and constitute a major part of the outer membrane. Most species of *Neisseria* express one porin, the meningococcus being unusual in expressing two simultaneously (Suker *et al.* 1993). On isolation most meningococcal strains express: (i) a class 1 outer membrane protein (OMP), encoded by the gene *porA*; and (ii) either a class 2 or a class 3 OMP, encoded by separate alleles of the *porB* locus (Hitchcock, 1989; Tsai *et al.* 1981). There are many antigenic variants of each of these porin classes (Mocca & Frasch, 1982; Maiden *et al.* 1991; Wolff & Stern, 1991; Feavers *et al.* 1992b; Mee *et al.* 1993).

The antigenically variable porins of both of the pathogenic *Neisseria*, the meningococcus and the gonococcus, have formed the basis of serological typing of isolates for a number of years (Frasch *et al.* 1985). They have also been proposed as vaccine components, particularly against the meningococcus (Saukkonen *et al.* 1989; Bjune *et al.* 1991; Frasch *et al.* 1991; Zollinger & Moran, 1991; van der Ley & Poolman, 1992). These considerations have stimulated work on the antigenic variation of the *Neisseria* porins. The availability of rapid nucleotide sequencing techniques, based on the polymerase chain reaction (PCR), has enabled the determination of the sequences of genes encoding many antigenic variants of these proteins (Barlow *et al.* 1989; McGuinness *et al.* 1990; Maiden *et al.* 1991; McGuinness *et al.* 1991; Feavers *et al.*

1992b; McGuinness *et al.* 1993; Zapata *et al.* 1992; Suker *et al.* 1994). Comparisons of the sequences obtained have demonstrated that all the *Neisseria* porins are related, forming a family (Ward *et al.* 1992; Suker *et al.* 1993). These relationships are illustrated by the phenogram in Fig. 1.

Evidence for horizontal genetical exchange between species can be inferred from these interrelationships, particularly when the meningococcal class 2 and 3 OMPs (Nme P2 and Nme P3 in Fig. 1) and the equivalent porins from *N. gonorrhoeae*, PIA and PIB (Ngo PIA and Ngo PIB in Fig. 1) are considered. The three meningococcal porins are located on different branches and, with the exception of the class 1 OMP which is distantly related to all of the other porins, they are more closely related to porins from other species than they are to each other. This implies that during evolution different species of the *Neisseria* have exchanged porins. The same is true for the gonococcal PIA and PIB porins, each of which is more closely related to porins in other species than they are to each other.

At the nucleotide sequence level, the meningococcal class 2 and 3 OMPs share around 70% nucleotide sequence identity, whereas the class 3 OMP gene is about 80% identical to the PIA protein of the gonococcus. On the other hand,

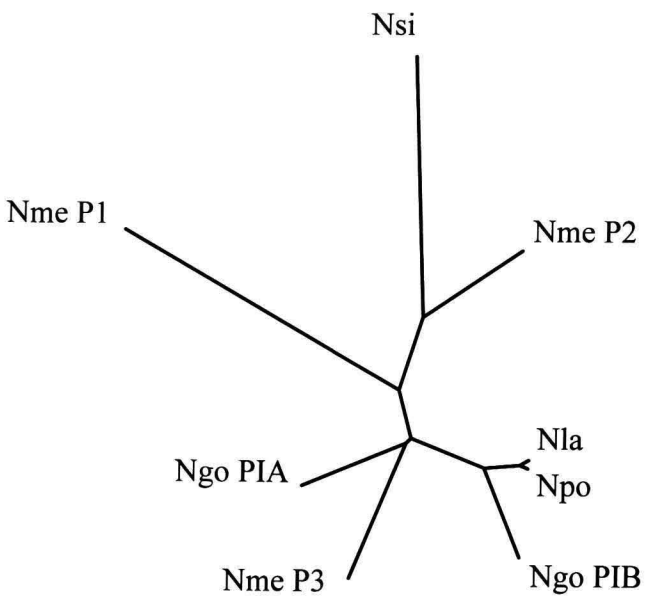

Fig. 1. Relationships among the *Neisseria* Porins. The (unrooted) tree was constructed from aligned amino acid sequences with the programs DRAWTREE and NEIGHBOR using a distance matrix constructed by the program PROTDIST. All these programs were from the PHYLIP (Phylogeny Inference) package written by J. Felsenstein, Department of Genetics, University of Washington, Seattle, WA, USA, down loaded by anonymous FTP from evolution.genetics.washington.edu (directory pub/phylip). The *Neisseria* porins included are: *N. meningitidis* class 1 OMP, NmeP1; *N. meningitidis* class 2 OMP, NmeP2; *N. meningitidis* class 3 OMP, NmeP3; *N. gonorrhoeae* PIA protein, NgoPIA; *N. gonorrhoeae* PIB protein, NgoPIB; *N. sicca* porin, Nsi; *N. lactamica* porin, Nla; *N. polysaccharae* porin, Npo.

the PIB protein from *N. gonorrhoeae* is nearly 90% identical to the porins from the commensal organisms *N. lactamica* and *N. polysaccharae*, although it shares less than 80% identity with the PIA protein. In all cases the precise figures for per cent sequence identity vary slightly, depending on the antigenic variants used in the comparison. It is also noteworthy that the meningococcal class 1 OMP (Nme P1) is the most distantly related *Neisseria* porin described to date, and only shares around 50% nucleotide sequence identity with each of the other porins (Maiden, 1993).

These patterns of sequence identity have important implications for horizontal genetical exchange between and within *Neisseria* species. Efficient homologous recombination is unlikely to occur in sequences that are less than 77% identical. Thus, whilst it is unlikely for a class 2 OMP gene from the meningococcus to recombine with a class 3 OMP gene to form a mosaic, it is much more likely for hybrid class 3 - PIA genes to occur or for the gonococcal PIB gene to recombine with the porin from *N. lactamica*, assuming that there is adequate mixing of the populations. Recombination may occur on either side of the porin genes, if regions of sufficient homology are present, and this is presumably the mechanism for the gene replacement from a class 2 OMP gene to class 3 OMP gene in the meningococcus or from a PIA to PIB gene in the gonococcus. One example of the transfer of a gonococcal PIB porin to a meningococcal strain has been reported (J. Vazquez and B. Spratt, personal communication), and such events may explain the occasional reports of isolates that appear to belong to both species.

Patterns of recombination in the PorA proteins

The class 1 OMP, or PorA, protein of the meningococcus has attracted more study than the other *Neisseria* porins to date, largely because of interest in its use as a major component of novel meningitis vaccines. Initial work on the protein was related to its use as the meningococcal serosubtyping antigen and studies using the monoclonal serosubtyping antibodies targeted against this protein suggested that a limited number of antigenic variants of the PorA proteins existed (Abdillahi & Poolman, 1987; Abdillahi & Poolman, 1988; Poolman & Abdillahi, 1988). This has subsequently proved not to be the case: nucleotide sequence analysis has shown that PorA is antigenically more variable than suggested by the data obtained with monoclonal serosubtyping reagents. The structure of the PorA protein and the genetic basis of variation of the *porA* gene also make it an excellent model for the study of the evolution of antigenic variation by horizontal genetical exchange.

Molecular analyses and sequence comparisons have identified the structural basis for the antigenic variation of the PorA protein (Maiden *et al.* 1991; van der Ley *et al.* 1991). A model for the structure of the porin is shown in Fig. 2A. In common with other porins it is assumed to have a ß-barrel structure, with the C- and N-termini of the polypeptide located on the periplasmic side of the outer

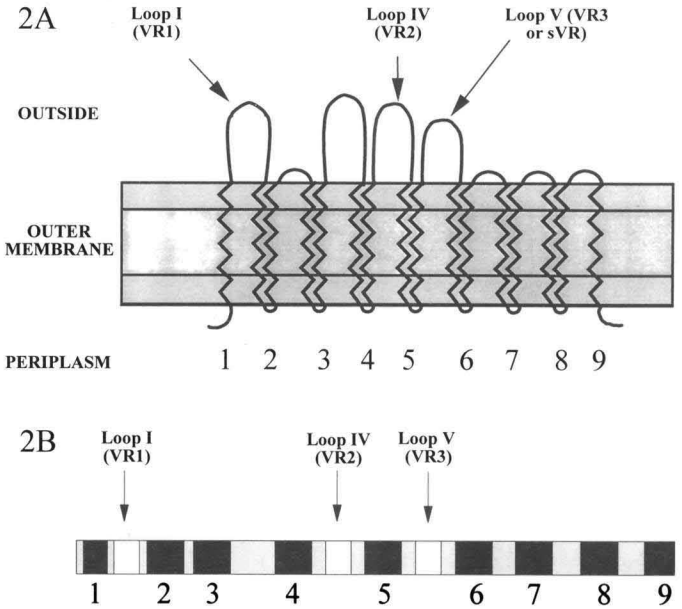

Fig. 2. Molecular basis of the antigenic variation of the class 1 OMP. A: Structural model of the PorA protein adapted from Maiden *et al.* (1991) and van der Ley *et al.* (1991). Antigenic variability resides in the surface exposed loops particularly, in PorA, loops I, IV and, to a lesser extent, loop V. Conserved regions of the protein are indicated by arabic numerals. B: Sequence features of the *porA* gene reflect the protein structure. The regions encoding the structural regions are conserved in different strains (CRs 1–9; dark shading and indicated by arabic numerals) while the regions encoding the surface loops, particularly loops I, IV, and V are variable in different strains (VRs 1, 2 and 3, light shading).

membrane (Nikaido, 1992). The ß-barrel structure comprises eight anti-parallel ß-strands, seven of which are formed by contiguous peptide sequences folding back on themselves by means of a turn located on the periplasmic side of the membrane. The eighth ß-strand is formed from the C- and N-terminal segments of the protein which form an anti-parallel structure with each other. This has the effect, in three dimensions, of forming the barrel which enables the protein to fulfill its function as an aqueous pore in the outer membrane. Between these ß-strands, on the outside of the cell surface, are eight loops of variable length that project away for the cell surface. It is in these loops that antigenic variability of these proteins reside. In the PorA protein most variation occurs in loops I (VR1) and IV (VR2) with minor variation in loop V (VR3 or sVR). In other *Neisseria* porins different surface loops are significant in antigenic variation (Ward *et al.* 1992; Feavers *et al.* 1992b; Suker *et al.* 1993), with the exception of loop III, which by analogy with the crystal structure of the *E. coli* porins, is probably folded into the pore and not surface exposed (Cowan *et al.* 1992).

As a result of the constraints imposed by the protein's structure, there are nine conserved regions (CRs 1–9) in all of the porin genes (illustrated by dark shading and arabic numerals in Fig. 2B) where little variation between different porin

classes or the porin genes of different species. The nucleotide sequence changes observed in these regions are normally synonymous or biochemically conservative. In the meningococcal *porA* genes there are three variable regions (illustrated by the open boxes in Fig. 2B) encoding loops I, IV and V. There are a range of very diverse sequences which encode distinct epitopes for each of these locations, particularly VR1 and VR2. Some sequence diversity also occurs in regions of the gene encoding other loops, these are illustrated in Fig. 2B with light shading. The structure of a conserved gene with a number of variable segments is ideal for the detection of mosaics as it is relatively simple to detect the diverse variable regions and therefore to investigate the reassortment of the variable regions without needing to sequence the entire gene. In addition, although there are relatively few mutations in the conserved regions, it is possible to identify fingerprints of mutations within the CRs. These mutations enable the evolutionary lineage of the entire sequence to be determined and the location of recombination events outside the VRs to be identified.

The epitopes present in loops I and IV of different meningococcal isolates are highly diverse. The relationships of some of the amino acid sequences described to date are illustrated in Fig. 3. The variation of epitope-containing sequences is not continuous and they can be divided into families. The families are named using an extension of the serosubtype designations originally adopted for typing with monoclonal antibodies. Each of the epitope-containing sequences is

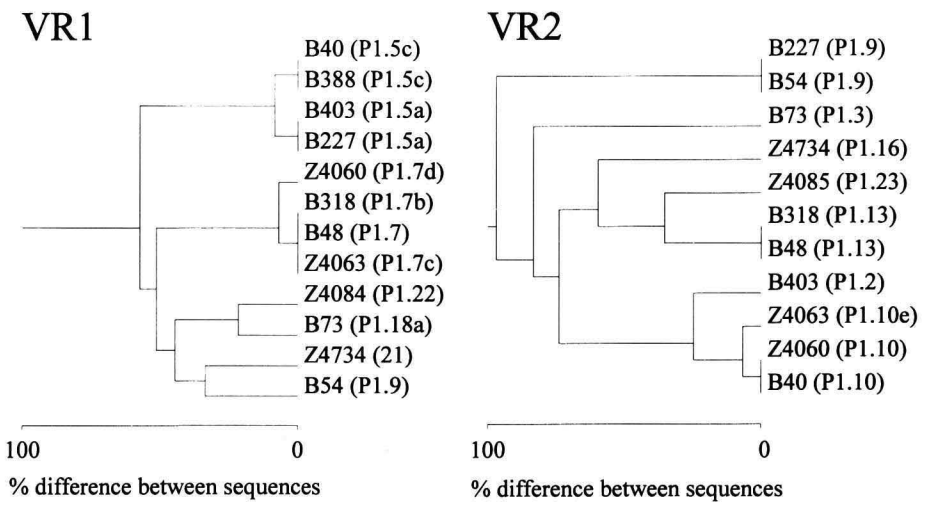

Fig. 3. Variable region families. There are a number distinct amino acid sequence families in the loop I and loop IV regions, originally defined by their reaction with monoclonal antibodies. Within each sequence family there are minor variants. The peptide sequence variants of VR1 and VR1 found in the serogroup A are shown on the dendrograms, with the variant name (P1.5 etc.) and the strain name given for each branch. The dendrograms are based on the similarity of the aligned peptide sequences calculated by the program DISTANCES from the GCG software package (Devereux *et al.* 1984). Note that the two dendrograms are not congruent, indicating that there has been reassortment of the DNA encoding loops I and IV of the PorA protein during the evolution of these genes.

assigned a number which is placed after the notation 'P1.' to indicate a class 1 OMP (P1.1, P1.2 etc.) Unfortunately, because the original designation was adopted before the molecular basis of the antigenic variation was established the numbers are arbitary and do not indicate the location of the epitope-containing sequence in loop I (VR1) or loop IV (VR2). This is addressed by placing the epitopes in the order VR1 VR2 separated by a comma, e.g. P1.7,16 (P1.7 is a VR1 epitope whereas P1.16 is a VR2 epitope). The original monoclonal antibodies did not identify minor variants within these families, which are now distinguished with lower case letters e.g. P1.7, P1.7a, P1.7b etc. So far a total of 24 distinct families have been described, 9 in VR1 and 15 in VR2 and many of these families have several minor variants (Maiden and Feavers, unpublished observations).

Many complete *porA* genes have now been sequenced and there is nucleotide sequence data for the VRs of many more (Barlow *et al.* 1989; McGuinness *et al.* 1990; Maiden *et al.* 1991; McGuinness *et al.* 1991; McGuinness *et al.* 1993; Rosenqvist *et al.* 1993; Suker *et al.* 1994). There are a number of general conclusions from this data set which will be discussed briefly before a more detailed discussion of the evolution of *porA* genes in different epidemiological situations. First, there is a global gene pool of *porA* genes; second this gene pool contains numerous mosaics as determined by reassortment of VRs; third, the rates of change and mechanisms of change of *porA* genes differ in meningococci associated with distinct epidemiologies of meningococcal disease.

Horizontal genetical exchange of *porA* genes in different epidemiological situations

Four epidemiologies of meningococcal disease have been described: epidemic/pandemic; localised epidemic; hyperendemic; and endemic (Schwartz *et al.* 1989). Comparison of data on the electrotypes (ETs) of meningococcal isolates, obtained by multilocus enzyme electrophoresis, with epidemiological data has shown that genetically distinct meningococci are associated with different meningococcal populations (Maiden & Feavers, 1995). As well as being genetically different, these groups of meningococci also exhibit diverse population structures, as defined by Maynard Smith *et al.* (1993). The epidemic/pandemic meningococci (largely serogroup A) are clonal, the localised epidemic and hyperendemic meningococci are 'epidemic' clonal (the result of the rapid spread of a recently arising clone), and the endemic meningococci are non-clonal or panmictic. The population structures of the *Neisseria* and the inter-relationships of population genetics and epidemiology are discussed in more detail in Spratt *et al.* (1995) and Maiden & Feavers (1995).

As the PorA protein is likely to be under immunological selection, it is a sensitive measure of the opportunity for horizontal genetical exchange to occur among different groups of meningococci, as novel products of recombination are likely to be positively selected and recovered during strain isolation. If the

disruption of clonality is due to widespread recombination, as envisaged by Maynard Smith *et al.*, there would be relatively little recombination in the *porA* genes of the clonal organisms, whereas extensive recombination would be found in the panmictic meningococci. A different pattern of recombination might be expected in the 'epidemic clonal' meningococci. The data is at present incomplete for some of these groups and is almost certainly biased by epidemiological sampling (Maiden & Feavers, 1995). However, the available sequence data from *porA* genes confirms these predictions. The most complete data are available for serogroup A meningococci and these will be discussed first.

Pandemic meningococci

Meningococci belonging to serogroup A are responsible for the most epidemiologically serious form of meningococcal disease: large scale epidemics and pandemics, spreading across several continents causing a high incidence of disease in third world countries (Achtman, 1990; Moore, 1992). Extensive genetical analyses have demonstrated that each of the pandemics that have occurred this century have been caused by one of the genetically related subgroups of serogroup A (Olyhoek *et al.* 1987; Wang *et al.* 1992; Achtman, 1994). These subgroups represent clones which are genetically homogenous. In a survey of the *porA* genes in serogroup A subgroups, 55 strains chosen to be genetically, geographically and temporally representative were examined either by nucleotide sequence analysis or 'T-track' analysis (comparing multiple samples by running only one of the nucleotide sequence termination reactions) of the entire gene (Suker *et al.* 1994).

These analyses gave detailed information of the evolution of the genes which allowed the following conclusions to be drawn: (i) there was a limited number of *porA* gene types (4) in the serogroup A meningococci; (ii) these were stable over the sample period (up to 50 years) and during intercontinental spread; (iii) the gene types had originally arisen by recombination, and ultimately derived from a global pool of *porA* genes shared by all meningococci; (iv) with very rare exceptions all members of the same subgroup had the same *porA* gene, probably reflecting the acquisition of a novel *porA* gene type on subgroup divergence; (v) therefore, the *porA* gene types present in serogroup A meningococci were probably older than the subgroups in which they occurred; (vi) the few recombination events that had occurred in these meningococci over the time period sampled had not spread; (vii) two of the gene types had been spread around the world by successive waves of pandemic disease. The association of *porA* gene types with subgroup are illustrated in Fig. 4.

Thus the *porA* genes of serogroup A behave as predicted by the clonal population structure of these meningococci and show little evidence of recent recombination events. There were a number of possible reasons for the limited amount recombination observed in these bacteria. One is that during the rapid epidemic spread of these organisms they simply do not meet other meningococci, and the opportunity of exchange is thereby reduced. It is also possible that serogroup A

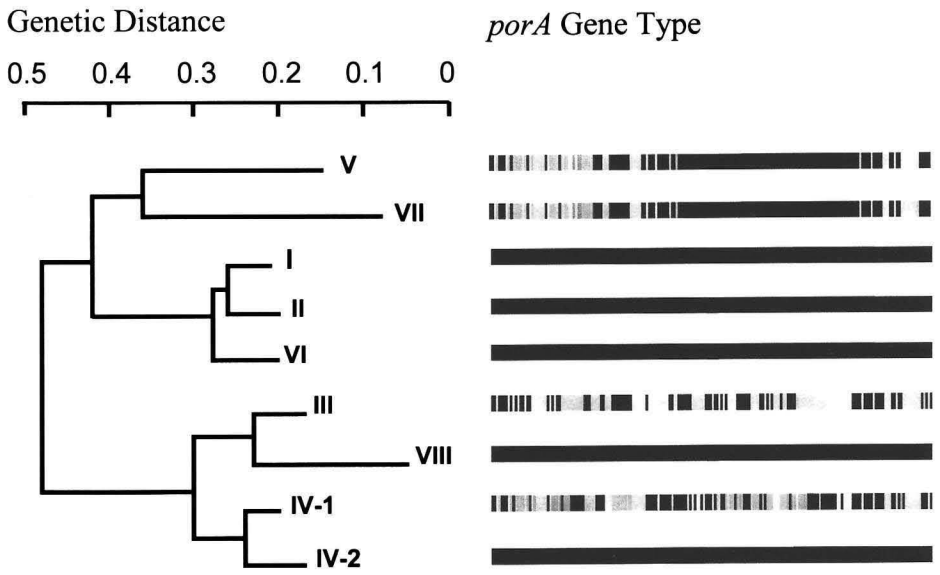

Fig. 4. Distribution of *porA* gene types in the subgroups (clones) of serogroup A meningococci. There are only four main gene types found in the serogroup A meningococci and these are stable within clonal subgroups. The genetic relationships of each of the subgroups is shown, together with a graphical representation of the sequence relationships between the each of the *porA* genes. Similar patterns of shading indicate where two genes are similar, diverse patterns show where the genes differ.

meningococci are less competent for DNA uptake than other meningococci. It should also be noted that antibodies against the serogroup A capsule, in contrast to antibodies against serogroup C and B capsules prevent both disease and carriage and this may have a substantial effect on the selective pressures experienced by the subcapsular antigens, including the porins. The stability of the serogroup A subgroups and their fate between pandemics are yet to be fully explained.

Localized epidemic and hyperendemic meningococci

Meningococci associated with localized epidemic and hyperendemic disease are thought to exhibit the 'epidemic' clonal population structure. There is rather less comprehensive evidence for the evolution of the *porA* gene in these meningococci but two distinct complexes of related meningococci have been studied in sufficient detail for some conclusions to be drawn: the ET-37 complex, which has been responsible for numerous localised epidemics throughout the world over the last 50 years or so (Wang *et al.* 1993); and the ET-5 complex which spread causing hyper-endemic disease in a number of countries in the late 1970s and 1980s (Caugant *et al.* 1986; Caugant *et al.* 1987).

Meningococci belonging to the ET-37 complex generally only express one type of *porA* gene which encodes the P1.5,2 epitopes or close relatives which

have probably arisen by accumulation of mutations in the gene. A few gene replacements were observed (Wang *et al.* 1993). The ET-5 meningococci are antigenically much less stable and there are a number of class 1 OMPs which have been associated with this complex, most of which appear to been imported by gene replacement events, although there is one mosaic reported. Interestingly one gene replacement event found in a ET-5 strain isolated in Worcester, England, was the import of a gene identical at every base position to the predominant gene type found in several serogroup A subgroups, perhaps providing evidence of the exchange of genes between serogroup A and ET-5 meningococci.

Endemic strains

Most disease in western European countries is endemic, sporadic, and difficult to predict. There are two sources of information on *porA* genes for meningococci causing this type of disease: the nucleotide sequence of serological reference strains (Feavers *et al.* 1992a); and a study of VRs 1 and 2 in 250 strains, chosen to be representative of isolates from England and Wales during the period 1989-1991 (Maiden, Fox, and Feavers, unpublished observations). Unlike the genetically related collections of Ets discussed above, the serological reference strains were chosen because of their antigenic diversity, rather than their genetical relatedness. Comparative analysis of a set of 15 strains revealed mosaic gene structures which had been generated by at least five separate recombinational events. In addition, analysis of the chromosome structure of the isolates by pulsed field gel electrophoresis fingerprinting, demonstrated that in some cases similar or identical *porA* genes occurred in genetically distinct meningococci and, in other cases, genetically similar strains had diverse *porA* genes.

More extensive data on the reassortment of VRs comes from the study of 250 isolates by a combination of DNA-dot blot analysis and nucleotide sequence analysis. In this study the epitopes present in both VR1 and VR2 were identified for 240 of the 250 isolates examined: the results are summarised in Fig. 5. There were representatives of seven VR1 families and 13 VR2 families in this strain collection. In one strain the region of the *porA* gene encoding VR2 had been deleted. Some VR families, e.g. the P1.13 VR2 family, were associated with a number of VR1 family sequences. Others, for example the P1.4 and P1.10 VR2 families, occur with a limited range of VR1 sequence families.

These data show that whilst recombination can lead to reassortment of VR1 and VR2 within *porA* genes, strain collections of endemic meningococci do not comprise a completely random assortment of VR1 and VR2 associations. There are three possible explanations for this: (i) sampling; (ii) presence of predominant clones in the meningococcal population; and (iii) structural constraints. As most meningococci derive from case isolates the strain collections may be biased by sampling and some epitope combinations, e.g. P1.5,10, may be prevalent in strains with a higher pathogenic potential. On the other hand, at any one time it is possible that certain clones which happen to have a particular epitope

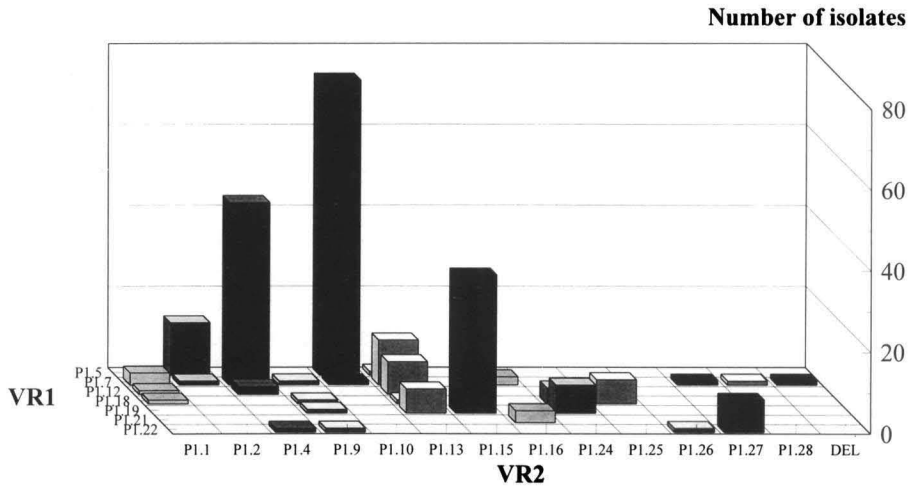

Fig. 5. Non-random association of variable region families in meningococci. The association of VR1 with VR2 epitope families in a set of 250 meningococci is illustrated. Note that while some VR2 epitope families, e.g. P1.13, occur with a number of VR1 epitope families others, e.g. P1.10 are much more limited in the range of VR1 with which they are associated.

combination predominate in the population. Finally, all meningococcal porins have to be able to fold correctly in the outer membrane and to perform their biochemical function. While the structural versatility of the ß-barrel is indicated by the diverse sequences that occur in VR1 and VR2, it is possible that certain epitope combinations lead to unstable or dysfunctional porin. These explanations are not mutually exclusive and it is possible that a combination of more than one of these explanations accounts for the results observed.

Conclusions

Nucleotide sequence comparisons provide extensive evidence of horizontal genetical exchange in the genes encoding the porin proteins of *N. meningitidis.* The differences in the clonal and epidemiological behaviour of different meningococci enable the effect of horizontal genetical exchange in different timeframes to be observed in one species. In serogroup A, where clones persist over decades and during global spread, evidence for 'ancient' exchange events, with a subsequent accumulation of point mutation can be seen. This is similar to the types of mosaics observed in clonal species such as *Escherichia coli* (Hartl *et al.* 1986; DuBose *et al.* 1988; Milkman & Bridges, 1990; Milkman & McKane, 1995). The antigenic diversity of the serogroup A subgroups, or clones, is limited and reassortment of the lineages suggests that the mosaics are in this case older than the clonal subgroups in which they occur.

In other meningococci there is evidence for much more recent exchange, resulting in either the reassortment of PorA epitopes within the *porA* gene or

gene replacement of the complete *porA* gene. Gene replacement has enabled the ET-5 meningococci, for example, to express a number of antigenically different porin genes during its spread around the world. It is interesting to note, however, that there is little evidence for exchange playing a major role in antigenic variation of ET-37 meningococci, although isolates from around the world over a protracted time-scale have been collected. When isolates representing endemic meningococci are examined, there appears to be much recombination of relatively small fragments of the genome resulting not in gene replacement, but reassortment of PorA epitope families within *porA* genes. Thus horizontal genetical exchange has played a major role in the evolution of novel antigenic variants of the porin proteins but this role, and the balance of exchange events to other mutational events, varies in genetically and epidemiologically distinct meningococci.

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