Recombinational reassortment among *opa* genes from ET-37 complex *Neisseria meningitidis* isolates of diverse geographical origins

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Opacity (Opa) proteins are a family of antigenically variable outer-membrane proteins of *Neisseria meningitidis*. ET-37 complex meningococci, defined by multilocus enzyme electrophoresis, have been isolated on different continents. Twenty-six different Opa proteins have been observed within strains of the ET-37 complex isolated between the 1960s and the 1980s, although individual strains have only four *opa* genes per chromosome. In this work the *opa* genes of four closely related ET-37 complex *N. meningitidis* strains recently isolated from Mali, West Africa were characterized and compared with the *opa* genes of strain FAM18, an ET-37 complex isolate from the USA. DNA sequence analysis and Southern blot experiments indicated that recombinational reassortment, including gene duplication and import by horizontal genetic exchange, has occurred in the *opa* genes within the ET-37 complex, resulting in two partially different Opa repertoires being present in FAM18 and the Mali isolates. Using synthetic peptides derived from the hypervariable (HV) regions of *opa* genes, the epitopes for nine mAbs were mapped. These bacteria, isolated on different continents, contain both shared and unique *opa* HV regions encoding epitopes recognized by mAbs and show evidence of recombinational reassortment of the HV regions.

Keywords: *Neisseria meningitidis*, gene conversion, opacity protein, hypervariable regions, global gene pool

INTRODUCTION

Neisseria meningitidis is a common cause of meningitis and septicaemia throughout the world (Peltola, 1983; Achtman, 1995). Meningococci have been classified into serogroups based on the immunological reactivity of the polysaccharide capsule and most isolates from invasive meningococcal disease belong to serogroups A, B or C (Peltola, 1983). Serogroup A meningococci are responsible for epidemics, particularly in sub-Saharan Africa (Olyhoek *et al.*, 1987) and China (Wang *et al.*, 1992),

whereas endemic disease and limited outbreaks in developed countries are mainly associated with serogroups B and C (Achtman, 1995).

Multilocus enzyme electrophoresis (MLEE) of serogroup B and C meningococci has been used to identify three epidemic lineages consisting of related electrophoretic types (ETs) called the ET-5 complex, the ET-37 complex and the A4 cluster (Achtman, 1995). Endemic disease and outbreaks caused by serogroup C meningococci of the ET-37 complex have been reported during the 1990s from North America (Ashton *et al.*, 1991; Pinner *et al.*, 1991), Europe (Kriz *et al.*, 1994), Brazil (Sacchi *et al.*, 1992) and the Sahel region of West Africa, including Mali (Broome *et al.*, 1983; Wang *et al.*, 1993). Although serogroup B and C bacteria are heterogeneous with regard to the cell surface antigens used for serotyping and serosubtyping, serogroup C, ET-37

Abbreviations: ET, electrophoretic type; HV, hypervariable; MLEE, multilocus enzyme electrophoresis.

The GenBank/EMBL/DDJB accession numbers for the *N. meningitidis* Z4197 *opa* sequences reported in this paper are U37255–U37257 and U77881. The accession numbers for the FAM18 *opa* sequences are X63108–X63111.

complex bacteria are fairly uniform, even with respect to expression of variable antigens such as porins and pilin (Wang *et al.*, 1993). Endemic disease isolates from Mali, collected from 1989 to 1991, form a homogeneous group that is related but not identical to other bacteria of the ET-37 complex (Wang *et al.*, 1993).

Characterization of Opa proteins has been useful in detecting microevolution among closely related meningococcal strains (Hobbs *et al.*, 1994; Morelli *et al.*, 1997). Opa proteins are a family of variable outer-membrane proteins in *N. meningitidis* (Stern & Meyer, 1987; Woods & Cannon, 1990; Aho *et al.*, 1991; Hobbs *et al.*, 1994; Morelli *et al.*, 1997) and *Neisseria gonorrhoeae* (Stern *et al.*, 1986; Stern & Meyer, 1987; Connell *et al.*, 1988, 1990; Murphy *et al.*, 1989; Bhat *et al.*, 1991). Opa expression can influence a variety of neisserial interactions with host cells *in vitro* (Rest *et al.*, 1985; Makino *et al.*, 1991; Weel *et al.*, 1991; Belland *et al.*, 1992; Kupsch *et al.*, 1993; Virji *et al.*, 1993; Blake *et al.*, 1995; De Vries *et al.*, 1996), and Opa variation occurs during infection with *N. meningitidis* (Woods & Cannon, 1990; Achtman *et al.*, 1991) and *N. gonorrhoeae* (James & Swanson, 1978; Swanson *et al.*, 1988; Jerse *et al.*, 1994). Phase variation (reversible on-off switching) of Opa proteins results from alterations in reading frame caused by changes in the number of pentanucleotide repeats (CTCTT) in the signal-peptide-encoding region of *opa* genes (Stern *et al.*, 1986; Stern & Meyer, 1987; Kawula *et al.*, 1988; Belland *et al.*, 1989, 1997; Murphy *et al.*, 1989). Antigenic variation among the Opa proteins of an individual strain is a consequence of alternate expression from multiple *opa* loci in the bacterial chromosome, encoding proteins with different antigenic characteristics. Meningococci possess either three or four *opa* genes, with differences between them occurring mainly in two hypervariable (HV) regions within a conserved framework (Stern & Meyer, 1987; Aho *et al.*, 1991; Hobbs *et al.*, 1994; Morelli *et al.*, 1997). Several epitopes for mAbs recognizing surface-exposed determinants and distinguishing between individual Opa proteins have been mapped to HV regions (Connell *et al.*, 1988; Robinson *et al.*, 1988; Aho *et al.*, 1991; Hobbs *et al.*, 1994). At least within serogroup A bacteria, these *opa* genes are located at widely separated chromosomal loci called *opaA*, *opaB*, *opaD* and *opaJ* (Dempsey *et al.*, 1995; Morelli *et al.*, 1997).

Twenty-six Opa proteins differing in electrophoretic mobility and/or reactivity with mAbs have been observed within meningococci of the ET-37 complex (Wang *et al.*, 1993), although all ET-37 strains examined also have four *opa* genes in the chromosome (Aho *et al.*, 1991 and our unpublished observations). Furthermore, only three distinct Opa proteins were expressed by the recent isolates from Mali (Wang *et al.*, 1993). Western blot analysis of outer membranes revealed some antigenic similarities of the Opa proteins from Mali bacteria with those of FAM18, an ET-37 complex strain isolated in North Carolina, USA in 1983 (Wang *et al.*, 1993). Recombinational reassortment of HV regions, gene duplication and import by horizontal genetic exchange

can occur among meningococcal *opa* genes, leading to antigenic variation and differences in the Opa repertoires of clonally related isolates of serogroup A, sub-group IV-1 (Hobbs *et al.*, 1994; Morelli *et al.*, 1997). Horizontal genetic exchange can also result in essentially identical alleles being present in clonally unrelated strains (Maiden *et al.*, 1996). It seemed likely that similar processes of recombination had been responsible for generating the observed variation among Opa proteins expressed by ET-37 complex strains. This study was designed to test that possibility by comparing the sequences of *opa* genes of serogroup C meningococci of the ET-37 complex from Mali with those from strain FAM18. Because former analyses had used epitopes recognized by mAbs to compare Opa proteins, we also delineated the continuous B-cell epitopes in the HV regions of these strains.

METHODS

Bacterial strains. *N. meningitidis* serogroup C isolates from the United States (FAM18) (Aho *et al.*, 1991) and Mali (Z4183, Z4184, Z4193 and Z4197) (Wang *et al.*, 1993) were previously identified as belonging to the ET-37 complex by MLEE (Wang *et al.*, 1993). Meningococci were grown on GCB agar (Difco), with the supplements of Kellogg *et al.* (1963), at 37 °C in a 5% CO₂ atmosphere. *E. coli* DH5αF' was grown on LB agar with or without ampicillin (50 µg ml⁻¹).

Genetic nomenclature. A listing of former and current designations is presented in Table 1. *opa* alleles were designated by their chromosomal locus and a unique number assigned to each allele with a unique sequence (Morelli *et al.*, 1997). Opa proteins were assigned the same number as the encoding allele, except that the mature proteins encoded by *opaA5200* and *opaD5201* are identical and are both referred to as Opa5200 (Table 1).

mAbs. These are listed in Table 2, including the sources of those that had been formerly described. New hybridomas were isolated after immunization of BALB/c mice with heat-inactivated serogroup C bacteria from Mali as described by Achtman *et al.* (1992). After two rounds of cloning by limiting dilution, antibodies were purified from hybridoma supernatants by protein G affinity chromatography.

Electrophoresis and hybridization techniques. Meningococcal outer membranes were prepared as previously described (Cannon *et al.*, 1984). Proteins were solubilized at 37 °C in SDS sample buffer and separated by SDS-PAGE in resolving gels containing 11% (w/v) acrylamide and Laemmli buffer. Western blots were probed with mAbs using a peroxidase-conjugated goat anti-mouse IgG secondary antibody (Sigma) and the Enhanced Chemiluminescent (ECL) detection system (Amersham).

Meningococcal DNA was prepared in agarose blocks, digested with *Bgl*II, resolved by contour-clamped homogeneous electric field (CHEF) electrophoresis and transferred and cross-linked to BAS membrane (Schleicher & Schuell) as previously described (Aho *et al.*, 1991; Dempsey *et al.*, 1991). Southern and plaque blots were performed using hybridization with radiolabelled oligonucleotide probes as previously described (Hobbs *et al.*, 1994).

Cloning and sequencing techniques. The *opa* genes of strain Z4197 were cloned into M13mp18 from DNA fragments amplified by PCR using primers to conserved sequences

Fig. 1. Map of Opa proteins and *opa* genes. Top line, Opa proteins, including the signal peptide (-19 to -1) and the HV1 and HV2 regions from which peptides were synthesized. Numbers correspond to amino acids. Bottom line, *opa* genes and locations of oligonucleotides used. Numbers correspond to nucleotides.

flanking meningococcal *opa* genes as previously described (Hobbs *et al.*, 1994). Plaque blots were screened with oligonucleotide probes derived from the HV1 and HV2 regions of the *opa* genes of strain FAM18 (Aho *et al.*, 1991) to identify clones containing individual *opa* genes from Z4197. Single-stranded phage DNA was sequenced by dideoxy chain-termination with the Sequenase system (USB) using the M13 forward sequencing primer and *opa*-specific internal primers (MH1-MH4) as previously described (Hobbs *et al.*, 1994). Two to four independently isolated clones were sequenced for each *opa* locus.

We used HV-specific oligonucleotides (Fig. 1) from FAM18 *opa* sequences that were present in the *opa* genes of Z4197 [540 HV1, 5' GTTACTGAAGATATAGCAGAC 3'; 540 HV2, 5' GACGGCAACCTCTCCACAGGGAGG 3'; 900 HV2, 5' CAGGGGTGGCGTTACAGAGCC 3'; 1700 HV1, 5' CAAAAGAGTTGGAAAACAAGC 3'; 1700 HV2, Aho *et al.* (1991); 1800 HV1, 5' AGAAACCCAGGACAATAGGG 3'] and an oligonucleotide derived from the HV1 sequence in *opa*5100 from Z4197 (5100 HV1, 5' GCAGAAAATAGAG-ATAATGC 3') as radiolabelled probes in Southern blot analysis of Z4197 DNA to confirm that the PCR-generated clones represented sequences present in the chromosome. We did obtain some clones with HV1/HV2 combinations not found in the chromosome (data not shown); these may have been artefacts generated by PCR and were not analysed further. DNA sequences were analysed using GCG software (Genetics Computer Group, Madison, WI, USA, version 8).

Direct sequencing of individual *opa* genes. *Bgl*II fragments containing *opa* genes were excised from pulsed-field gels and the agarose slices were digested with β -agarase according to the manufacturer's instructions (New England Biolabs). PCR amplification of the *opa* genes (30 cycles of 1 min at 95 °C, 1 min at 57 °C and 1 min at 72 °C, followed by 10 min at 72 °C) was performed with the primers O3510 (5' TACGCT-GCAGAAAATGAATCCAGCCCC 3') and O87 (5' GCG-CACGCCCAATGAGACTTCGTGGG 3') (Fig. 1). The PCR products were adjusted to 12.5% (w/v) PEG 8000 (Sigma), 1.6 M NaCl and precipitated at 37 °C for 10 min. The pellet was harvested by centrifugation in a microfuge for 10 min, washed with 80% (v/v) ethanol, centrifuged again and dried. The pellet was dissolved in water and sequenced from both

strands by automated cycle sequencing (ABI 377) using the oligonucleotide primers O3510, O83 (5' GCGCGTCGC-CTACGGAC 3'), O80 (5' AAGCAACCGGTGCAAACA-ACACAAGC 3'), O82 (5' TGTCCTAGGCGACGCGC 3'), O85 (5' GGCATAATCTGCCGCTATCC 3') and O87. Individual strands were sequenced from independent PCR products.

***opa* loci.** The chromosomal loci of the individual alleles were assigned as described by Morelli *et al.* (1997). Briefly, for each *opa* locus, PCR amplification was performed using a conserved oligonucleotide primer from the N-terminal *opa* region plus a primer from a unique downstream flanking sequence. These PCR products were sequenced as above.

Epitope mapping. Multiple synthetic N-terminally acetylated peptides were synthesized on pins as previously described (Geysen *et al.*, 1987), using an Epitope Scanning Kit (Cambridge Research Biochemicals) with a dilution aid (Epiguide, Labsystems) and the modifications of McGuinness *et al.* (1990). The pins were screened by ELISA for reactivity after incubation for 90 min with mAbs diluted to 1–10 μ g ml⁻¹. Reactivity was detected using alkaline-phosphatase-conjugated, affinity-purified rabbit immunoglobulins to mouse immunoglobulins (Dakopatts) as previously described (Achtman *et al.*, 1992).

RESULTS

N. meningitidis strain FAM18 can express four electrophoretically distinguishable Opa proteins, OpaA through OpaD (Woods & Cannon, 1990). The sequences of the four *opa* genes from strain FAM18 contain four distinct versions of HV1, three distinct versions of HV2 and two minor variants of one of the HV2 versions which differ by three nucleotides (Aho *et al.*, 1991; see below). Wang *et al.* (1993) demonstrated that serogroup C meningococci of the ET-37 complex isolated between December 1989 and March 1990 in Mali, West Africa expressed three Opa proteins with similar electrophoretic mobilities to those of the FAM18 Opa proteins and that some mAbs recognized epitopes

Fig. 2. Immunoreactivity of Opa proteins expressed by ET-37 complex *N. meningitidis* isolates. Outer-membrane proteins were solubilized at 37 °C, separated by SDS-PAGE and stained with Coomassie brilliant blue. The results from Western blots with the same preparations are summarized by symbols indicating reactivity of individual Opa proteins with mAbs H.21 (circles), H.22 (squares) and 7-24-D9 (triangles). RMP, reduction modifiable protein, also known as Class 4 protein.

Table 1. Designations of *opa* genes, clones and Opa proteins

Strain	<i>opa</i> gene	Clone	Opa protein		Reference
			Former name	Current name	
FAM18	<i>opaA1800</i>	pFLOB1800	OpaA	Opa1800	Aho <i>et al.</i> (1991)
	<i>opaB900</i>	pFLOB900	OpaC	Opa900	
	<i>opaD1700</i>	pFLOB1700	OpaB	Opa1700	
	<i>opaJ540</i>	pFLOB540	OpaD	Opa540	
Z4197	<i>opaA5200</i>	pFLOB5200	P5.III- α	Opa5200	Wang <i>et al.</i> (1993)
	<i>opaB5100</i>	pFLOB5100	P5.IIa- α	Opa5100	
	<i>opaD5201</i>	—*	P5.III- α	Opa5200	
	<i>opaJ5000</i>	pFLOB5000	P5.IIb- α	Opa5000	

* The *opaD5201* allele was not cloned.

on the Opa proteins from both sets of strains. These results were confirmed using SDS-PAGE and Western blot analysis with outer membranes from variants of strain FAM18 expressing each of the four Opa proteins and from four independent, representative ET-37 isolates from Mali (Fig. 2). We tested reactivity with a total of nine mAbs that react with one or more Opa proteins in these strains, including mAbs secreted by four newly isolated Opa-specific hybridomas. These mAbs can be assigned to five classes based on the Opa proteins with which they react in Western blots and the epitopes they recognize (see below). Together with the electrophoretic data, the results confirmed that the Mali strains expressed a total of three Opa proteins that exhibited similar electrophoretic mobility and mAb binding to three of the four Opa proteins from FAM18 (P5.III- α and OpaB; P5.IIb- α and OpaD; P5.IIa- α and OpaA). A

protein resembling the FAM18 OpaC protein was not observed in outer-membrane preparations from ET-37 meningococci from Mali.

As a consequence of the variability of *opa* gene sequences between different meningococcal strains, *opa* gene and Opa protein nomenclature has become confusing and partially repetitive. We will henceforth refer to *opa* alleles by unique numbers which in this case also reflect the clones which were sequenced (Table 1). The new protein designations also include the corresponding allele numbers rather than letters as in the past. Table 1 presents a summary of the designations of the *opa* alleles, Opa proteins and clones used in this study.

Reactivity of Opa proteins from different isolates with a given mAb indicates the presence of an epitope with a

Fig. 3. Sequence variation of *opa* alleles and Opa proteins. (a) Consensus nucleotide and amino acid sequences in the region encoding the signal peptide. (b) Sequence polymorphisms in the region encoding the mature Opa proteins. Only polymorphic sites are shown, with the nucleotide position indicated by vertical numbers. The HV1 and HV2 hypervariable regions are boxed. (c) Amino acid polymorphisms in the HV1 and HV2 regions. Epitopes recognized by mAbs are boxed. Points indicate identity to the consensus except in (c) where all amino acids are shown; dashes indicate deletions.

high degree of homology, usually located within an HV region. Thus, shared reactivity with a single mAb suggests, but does not prove, that different Opa proteins contain the same HV region in which the mAb epitope is encoded. Likewise, reactivity with both HV1- and HV2-specific mAbs suggests the presence of the same HV1 and HV2 regions in different Opa proteins. However, even Opa proteins that are indistinguishable electrophoretically and in reactivity with mAbs might differ to some extent in sequence, especially considering the different sources of the bacteria and the variability of Opa proteins identified within the ET-37 complex meningococci from different countries. In addition, the analysis of Opa proteins by mAbs is limited to the products of the genes expressed at a given time. Because Opa expression is subject to phase variation, the absence of a particular Opa protein at the time of analysis does not necessarily indicate its absence from the repertoire of an isolate.

Sequences of *opa* genes

Since Opa proteins expressed by the Mali isolates reacted with mAbs recognizing HV-encoded epitopes present in the *opa* genes of strain FAM18, we used oligonucleotides derived from FAM18 HV sequences to probe Southern blots of Z4197 DNA digested with *Bgl*II. An oligonucleotide probe specific for the 5' repeat region of *opa* genes (CTCTT_n) identified four *opa* loci in the chromosome of the West African strain, present on *Bgl*II restriction fragments of similar size to those from FAM18 (data not shown). Two *Bgl*II fragments reacted with the HV1 probe derived from *opa*1700, raising the possibility that a duplication of one gene had occurred. Such a duplication could account for only three different Opa proteins having been detected by Western blots of isolates from Mali. The other hybridization patterns were fully consistent with the sequence results presented below (data not shown).

Fig. 4. Schematic representation of *opa* genes in ET-37 complex meningococcal isolates. The order of the alleles from top to bottom parallels the decreasing sizes of the *Bgl*III fragments. Four independent isolates from Mali (Z4182, Z4184, Z4193 and Z4197) were identical by Southern blot analysis with HV-specific oligonucleotide probes (data not shown). Paired boxes indicate sequences present in the HV1 and HV2 regions of each *opa* gene. Each pattern represents a different version of an HV sequence. #, three different base pairs in the HV2 sequence of *opaA1800* versus *opaJ540*, *opaJ5000* or *opaB5100*.

Table 2. Reactivities of mAbs with Opa proteins

Antibody*	Opa protein	HV region	Minimal epitope
H.21 ^a , AG612 ^b , AG606 ^b	Opa1700, Opa5200	HV2	DTK
H.22 ^a , AG108 ^b , AF313 ^b	Opa1800, Opa540, Opa5100, Opa5000	HV2	TATSP
15-1-P5.5 ^c	Opa1800, Opa540, Opa5100, Opa5000	HV2	IIQ
7-24-D9 ^c	Opa540, Opa5000	HV1	YKETK
9-1-P5.4 ^c	Opa5100	HV1	NYVKI

* References or source: a, Aho *et al.* (1991); b, this study; c, Wang *et al.* (1993).

To further characterize the Opa repertoire of ET-37 complex meningococci from Mali, the *opa* genes of strain Z4197 were cloned into M13mp18. Clones containing *opa* genes corresponding to the different HV combinations present on the CTCTT-binding *Bgl*III fragments were identified in plaque blots with the FAM18 HV oligonucleotide probes, and inserts were

sequenced from single-stranded phage DNA. Only three different *opa* sequences were obtained. Southern blot analysis with HV-specific probes, including the novel HV1 region from *opa5100* (see below), indicated the same distribution of HV sequences in three other Mali isolates belonging to the ET-37 complex as in strain Z4197 (data not shown). To ensure that these sequences corresponded to all four *opa* loci, the *Bgl*III fragments containing *opa* alleles were excised from pulsed-field agarose gels, and the *opa* genes amplified by PCR and sequenced directly. Finally, to determine the chromosomal loci of the individual alleles (Dempsey *et al.*, 1995, Morelli *et al.*, 1997), the four *opa* alleles from both FAM18 and from Z4197 were sequenced from PCR products generated using primers from unique regions flanking each *opa* locus in serogroup A bacteria. PCR products were obtained for all four loci and the sequences were the same as those obtained from excised *Bgl*III fragments, showing that the *opa* alleles in ET-37 complex bacteria are located at the same chromosomal locations as those in serogroup A. The complete designation of each *opa* allele, including the chromosomal location, is shown in Table 1 and a summary of the sequence variability between these different *opa* alleles is presented in Fig. 3. The complete coding sequences of the Z4197 *opa* genes have been deposited in the EMBL/GenBank/DBJ nucleotide sequence data libraries under accession numbers U37255–U37257 and U77881. The sequences obtained for the *opa* alleles from FAM18 were longer than those originally available and also corrected some minor sequence errors. They have been deposited under the original accession numbers X63108–X63111.

In agreement with the hybridization data, two of the *opa* alleles from strain Z4197, *opaA5200* and *opaD5201*, were identical (Fig. 3b), except for the region encoding the signal peptide where the number of CTCTT repeats differed and the poly(A) stretch directly preceding the CTCTT repeats was one nucleotide shorter (five As) in *opaA5200* (Fig. 3a). The shorter poly(A) stretch was also present in *opaD1700*, *opaB5100* and *opaA1800* and results in in-frame translation when the number of CTCTT repeats is 2, 5, 8, etc. as opposed to the pattern of 3, 6, 9, etc. associated with in-frame translation in *opaB900*, *opaJ5000*, *opaJ540*, *opaD5201* and most other *opa* alleles that have been sequenced (Morelli *et al.*, 1997). Due to the CTCTT repeats, the frameshift introduced by the shorter poly(A) stretch only changes the signal peptide sequence of *opa* proteins by three amino acids and does not affect the sequence of the mature proteins (Fig. 3a). Within strain Z4197, only *opaB5100* was in-frame and only Opa5100 was expressed (Fig. 1).

Translocation and import of *opa* alleles

Part of the HV1 region (nucleotides 237–255) and the sequences (316–654) from the end of HV1 to near the 3' end of the gene were identical between *opaB5100* and

Fig. 5. mAb reactivity with peptides derived from the HV regions of Opa proteins. The peptides indicated along the x axes were synthesized on pins and tested by ELISA with the mAbs indicated in each panel. Peptide sequences were derived from (a) Opa5200 HV2, (b) Opa5000 HV2, (c) Opa5000 HV1 or (d) Opa5100 HV1.

opaJ5000 from strain Z4197 (Fig. 3a, Fig. 4). These identities probably reflect partial gene conversion since these alleles differed extensively at their 5' ends and throughout most of the HV1 region (Fig. 3a). Because *opaJ5000* (strain Z4197) is identical to *opaJ540* (FAM18), except for the number of CTCTT repeats (Fig. 3), it seems likely that the sequence at the *opaJ* locus is ancestral and that the stretches from 237 to 255 and 316 to 654 had translocated from the *opaJ* locus to the *opaB* locus. Similarly, *opaD5201* (Z4197) is identical to *opaD1700* (FAM18) in the region promoter distal to nucleotide 186 (Fig. 3b), suggesting that the *opaD* locus is ancestral and that the almost identical *opaA5200* allele arose by translocation from the *opaD* locus. Similar translocation events may account for the distinct polymorphisms between nucleotides 13 and 174 characteristic of *opaB900* and *opaD1700* of FAM18.

A large part of HV1 (259–351) differs between *opaB900* (FAM18) and *opaB5100* (Z4197) as well as from the other *opa* alleles in these two strains and cannot have arisen by translocation. These differences between

opaB900 and *opaB5100* are also too extensive to have arisen by accumulation of mutations and at least one of these regions must have been imported by horizontal genetic exchange, presumably via transformation with DNA from unrelated neisseriae (Maiden *et al.*, 1996). The remainder of the differences between these two alleles can be attributed to translocation in an ancestor of Z4197 (see above). An independent translocation event in an ancestor of strain FAM18 may have resulted in the similar HV2 regions of *opaJ540* and *opaA1800* (Fig. 3b, Fig. 4). These alleles differ by only four nucleotides between 444 and 598. Three of these polymorphisms are in the HV2 region and one results in an amino acid change (Fig. 3b, c).

As a result of the putative translocation and import events, FAM18 and Z4197 differ extensively at the *opaA* and *opaB* loci. Although the HV1 and HV2 regions are identical at the *opaD* locus, the 5' end of these alleles differed in nine nucleotides resulting in four amino acid differences and the only locus containing identical alleles in the two strains was *opaJ*.

Epitope localization

Previous studies showed that mAbs which distinguished among Opa proteins recognized epitopes which were encoded within the HV regions and exposed on the bacterial cell surface (Connell *et al.*, 1988; Robinson *et al.*, 1988; Aho *et al.*, 1991; Hobbs *et al.*, 1994). To map the epitopes recognized by the mAbs in Table 2 more precisely, we tested antibody binding to overlapping peptides, synthesized according to the sequences of the HV regions of the Opa proteins from Mali. 12-mers overlapping by nine amino acids were synthesized on pins and tested for reactivity with all the mAbs. Each mAb reacted specifically with a single HV region which corresponded to its specificity for Opa proteins. None of the mAbs reacted with peptides derived from the HV1 region of *opaA5200* or *opaD5201*, whereas at least one antibody reacted with all other HV regions tested. Synthetic 10-mers overlapping by nine amino acids were then made to cover the reacting regions and tested again to determine the minimal binding sites (Fig. 3c, Fig. 5). Each of the minimal binding sites was contained within the region that reacted in the first set of peptides. Five minimal peptide sequences were recognized by the nine mAbs and formed the basis of their assignment to the classes in Table 2. Within an epitope class in Table 2, different hybridoma cell lines had the same minimal binding sites, but sometimes differed in detailed specificity as indicated by the intensities of reactions with individual peptides (data not shown).

MAb 15-1-P5.5 recognized the HV2 region from the same Opa proteins (Opa540, Opa1800, Opa5000, Opa5100) as H.22, AG108 or AF313, but reacted with a different, non-overlapping minimal sequence (IIQ versus TATSP) (Fig. 3c, Fig. 5b).

DISCUSSION

According to results from MLEE, *N. meningitidis* is characterized by great genetic diversity (Caugant *et al.*, 1987). DNA transformation often leads to recombination and genetic shuffling (Spratt *et al.*, 1995), and similarity of isolates based on MLEE does not necessarily indicate clonal relationships within the pathogenic neisseriae (O'Rourke & Spratt, 1994). However, serogroup A (Wang *et al.*, 1992) and some serogroup B and C bacteria, including those of the ET-37 complex (Wang *et al.*, 1993), do belong to well-defined clonal groupings which are repeatedly isolated from cases of disease in different countries (Achtman, 1995). Even within these clonal groups, limited variation of certain variable antigens such as porins, LOS, pilin and Opa proteins has been noted (Achtman *et al.*, 1992; Wang *et al.*, 1992, 1993; Suker *et al.*, 1994; Morelli *et al.*, 1997). It is likely that recombination and/or horizontal genetic exchange events have been responsible for generating the 26 distinguishable Opa proteins among isolates of the ET-37 complex.

We previously demonstrated diversity due to recombinational reassortment of HV regions in the *opa* genes of serogroup A sub-group IV-1 meningococci isolated

from a single epidemic (Hobbs *et al.*, 1994). Subsequent analysis of a larger group of isolates has shown a predominant single Opa repertoire among these bacteria (unpublished data) as well as within sub-group III bacteria from different epidemics (Morelli *et al.*, 1997). However, changes in *opa* alleles accumulate over decades, leading to major differences between the repertoires of older and younger isolates (Hobbs *et al.*, 1994; Morelli *et al.*, 1997). Many of the observed changes in the Opa repertoire reflect gene conversion resulting in duplication of parts or of complete *opa* alleles. Such recombination can result in new combinations of individual antigenic determinants encoded by HV regions which could then potentially be transmitted to other strains by horizontal genetic exchange. The present analysis demonstrates that similar recombinational events have occurred between *opa* genes during microevolution of isolates belonging to the ET-37 complex, resulting in two somewhat different Opa repertoires being present in FAM18 and the isolates from Mali. The results show that *opa* gene duplication by translocation occurred within a strain that was ancestral to the Mali isolates and that horizontal genetic exchange resulted in the presence of different HV1 regions in the two repertoires. Nevertheless, four different HV-encoded antigenic determinants are shared in both repertoires (Fig. 4). The mAbs H.22, H.21, 9-1-P5.4 and 7-24-D9 react with HV-encoded Opa epitopes in numerous additional strains of the ET-37 complex from geographically diverse sources (Wang *et al.*, 1993). Thus, it seems likely that *opa* sequences related to those in FAM18 and the Mali isolates are present in ET-37 bacteria from diverse sources.

Horizontal genetic exchange can result in identical or almost identical genes in unrelated meningococci and such observations have been summarized within the concept of a global gene pool (Maiden *et al.*, 1996). The HV1 region of the *opaD1700*, *opaD5201* and *opaA5200* alleles differ by only one nucleotide from that of the *opaB133* allele of a serogroup A, sub-group IV-1 strain (Hobbs *et al.*, 1994; Morelli *et al.*, 1997). Similarly, the *opaB95* allele in a serogroup I strain differs by only a few nucleotides from the *opaB94* allele in serogroup A, sub-group III bacteria (Maiden *et al.*, 1996; Morelli *et al.*, 1997). The data presented here reveal another example of this phenomenon: *opaJ5200* from the Mali bacteria differs by only one nucleotide from *opaB5202*, found in a serogroup A, sub-group III strain isolated in Sweden in 1978 (accession number AF001203, Morelli *et al.*, 1997). These observations suggest that the *opa* genes described here will also be occasionally found in other genetically unrelated neisseriae.

Opa antigenic determinants have largely been defined on the basis of binding of murine mAbs. The extent to which these epitopes resemble the regions of Opa proteins that generate humoral immune responses in humans is not known. Human antibodies to PorA porin, Opc protein and IgA1 protease can recognize epitopes that overlap with those recognized by murine mAbs (de Cossio *et al.*, 1992; Delvig *et al.*, 1994; Morelli *et al.*,

1994), although the detailed epitopes recognized differed between the human and murine antibodies. The information presented here on the minimal binding sites recognized by murine mAbs may facilitate analysis of epitopes on Opa proteins recognized by sera from convalescent patients in Mali.

The functional significance of Opa antigenic variation is not understood. Virji *et al.* (1993) demonstrated differences in *in vitro* adherence and invasion between meningococci expressing different Opa proteins. There may be selective pressures maintaining *opa* sequences required for bacterial interactions with host cells *in vivo*; because HV regions encode exposed portions of Opa proteins, they may well be involved in such interactions. It seems unlikely that the sequence of individual HV regions suffices to account for the efficiency of these interactions, because Opa proteins sharing HV1 or HV2 regions can differ in efficiency of adherence and invasion (Virji *et al.*, 1993), and conformational interactions within the folded protein are probably crucial for bacteria–host interactions. Our comparison of the *opa* genes of FAM18 and the Mali isolates demonstrates that the Opa900 protein of FAM18 is not essential for infection; nor is the HV1-encoded determinant of Opa1800 (FAM18), because meningococci from Mali lacking these *opa* gene sequences were capable of causing disease. Similarly, the HV1 region of Opa5100 from the Mali bacteria cannot be essential because FAM18 lacked this sequence. The overall antigenic diversity of meningococcal Opa proteins from unrelated bacteria suggests that a variety of primary amino acid sequences are capable of fulfilling the structural requirements for Opa function(s).

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