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RESEARCH ARTICLE

Lack of antigenic diversification of major outer membrane proteins during clonal waves of *Neisseria meningitidis* serogroup A colonization and disease

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This very interesting study compares outer membrane protein sequences in a number of *Neisseria meningitidis* serogroup. A disease and carriage isolates from a defined region or subsaharan Africa between 2002 and 2008. It concludes that there is a remarkable antigenic stability in this population and that herd immunity is not a strong driving force for antigenic diversification. The study is well conceived and carried out, the results are clearly presented and the conclusions pertinent. This is important and useful information

Keywords
Neisseria meningitidis; outer membrane protein; herd immunity.

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Abstract

In particular in the 'meningitis belt' of sub-Saharan Africa, epidemic meningococcal meningitis is a severe public health problem. In the past decades, serogroup A lineages have been the dominant etiologic agents, but also other serogroups have caused outbreaks. A comprehensive vaccine based on subcapsular outer membrane proteins (OMPs) is not available. Here, we have investigated whether meningococcal populations overcome herd immunity by changing antigenic properties of their OMPs. Meningococcal isolates were collected in the context of longitudinal studies in Ghana between 2002 and 2008 and in Burkina Faso between 2006 and 2007. Serogroup A strains isolated during two clonal waves of colonization and disease showed no diversification in the genes encoding their PorA, PorB, and FetA proteins. However, we detected occasional allelic exchange of *opa* genes, as well as wide variation in the number of intragenic tandem repeats, showing that phase variation of Opa protein expression is a frequent event. Altogether we observed a remarkable antigenic stability of the PorA, PorB and FetA proteins over years. Our results indicate that while herd immunity may be responsible for the disappearance of meningococcal clones over time, it is not a strong driving force for antigenic diversification of the major OMPs analyzed here.

Introduction

Neisseria meningitidis, a Gram-negative diplococcus, is an obligate commensal of humans, usually carried in the upper airways of healthy individuals. The bacterium is transmitted to close contacts by the spread of respiratory secretions. Occasionally, *N. meningitidis* strains might penetrate the mucosal membrane, multiply in the bloodstream, and gain access to the cerebrospinal fluid. The overall incidence rate of meningococcal disease in Europe and North America is 1–3 per 100 000 person-years. The highest incidence rates are found in countries of sub-Saharan Africa, in the so-called 'meningitis belt', which extends from Ethiopia to Senegal. In these countries, annual incidence rates of as high as 1000 per 100 000 person-years are recorded during the most severe epidemics (Caugant, 2008). The savannah climate of the meningitis belt with an annual precipitation index of 300–1100 mm and extremely dry and hot winter seasons seems to be optimal for transmission of invasive meningococcal disease. Epidemics classically occur in the dry season, between December and April. They nearly always start in the early part of the dry season and then stop abruptly at the onset of rains. High temperature, low absolute humidity and the harmattan (a dusty wind that

blows from the Sahara) at the end of the dry season may favor the occurrence of meningococcal disease by damaging the local mucosal defenses (Greenwood, 1999).

Based on different capsular polysaccharide structures, 13 serogroups of *N. meningitidis* can be distinguished. The vast majority of invasive meningococcal disease is caused by six of these serogroups (A, B, C, W-135, X, and Y) (Stephens *et al.*, 2007). Most of the large epidemics in Africa in the past 100 years were associated with serogroup A (Caugant, 2008). Until recently, reactive immunization campaigns with polysaccharide vaccines initiated after the onset of meningitis epidemics have been used to limit epidemics. Now a monovalent conjugate vaccine against serogroup A meningococci, the most common cause of large epidemics, is being introduced stepwise in the countries of the African meningitis belt. In contrast to the unconjugated polysaccharides, conjugate vaccines will not only protect the vaccinated from invasive disease, but are also expected to block transmission of the meningococci expressing the vaccine serogroup.

Lack of a stable nasopharyngeal population of apathogenic meningococci may contribute to the vulnerability of the local population of the meningitis belt to major epidemics (Leimkugel *et al.*, 2007b). These are caused by hypervirulent clones of *N. meningitidis*, which may travel through the entire meningitis belt, but seem to remain only for a few years associated with a particular population (Leimkugel *et al.*, 2007a, b). Herd immunity may lead to the disappearance of individual clonal complexes, but new clones with a different antigenic make-up may subsequently spread. Clonal waves of colonization and disease are therefore a characteristic feature of the epidemiology of meningococcal meningitis in the African meningitis belt (Leimkugel *et al.*, 2007a, b). *Neisseria meningitidis* serogroup A strains of the clonal complex 5 that are associated with the sequence types (STs) 5, 7, and 2859 have been responsible for outbreaks in the last two decades (Nicolas *et al.*, 2005; Teyssou & Muros-Le, 2007; Sie *et al.*, 2008). Molecular typing approaches based on DNA sequencing allow meningococci to be distinguished and tracked (Jolley *et al.*, 2007). During the last decade, multi-locus sequence typing (MLST) based on the identification of the alleles of fragments of seven meningococcal housekeeping genes has developed into the 'gold standard' for typing these bacterial pathogens (Maiden *et al.*, 1998; Brehony *et al.*, 2007).

Meningococci are naturally competent for transformation by exogenous DNA, and high rates of recombination have been observed (Jolley *et al.*, 2005). However, most genetic exchange appears to takes place between closely related meningococci, and it has been suggested that recombination may be primarily a mechanism for genome repair that will only occasionally result in generation of diversity (Caugant & Maiden, 2009). Nevertheless, *N. meningitidis* is genetically and antigenically highly diverse (http://pubmlst.org/neisseria/). On the other hand, hyper-invasive lineages seem to be surprisingly stable over decades and during global spread (Caugant, 2008). This may facilitate development of a comprehensive protein-based vaccine, effective against a broad range of hyper-virulent meningococci. Cross-reactivity could be achieved by targeting antigenically invariant subcapsular structures or by combining a cocktail of vaccine antigens selected on the basis of molecular epidemiological studies. A number of meningococcal surface structures are thought to play a role in mucosal colonization, hematogenous spread and crossing of the blood brain barrier (Hill *et al.*, 2010). To escape immune surveillance, meningococci have developed a range of mechanisms to change surface components. We assume that development of herd immunity is responsible for the complete disappearance of meningococcal clones after a few years of colonization of populations in the African meningitis belt (Leimkugel *et al.*, 2007a, b). Here we have investigated whether meningococcal populations escape from immune detection by varying their outer membrane proteins (OMPs) PorA, PorB, FetA, and Opa. The serogroup A ST7 and ST 2859 meningococci analyzed have been collected between March 2002 and April 2008 in the course of longitudinal meningococcal colonization and disease surveys in Ghana and Burkina Faso (Leimkugel *et al.*, 2007a, b; Sie *et al.*, 2008).

Materials and methods

Bacterial isolates

The *N. meningitidis* isolates investigated in this study had been collected in the Kassena-Nankana District (KND) and the neighboring district of Bawku in Ghana and in the Nouna Health District (NHD) in the Kossi region of Burkina Faso. Case strains were isolated from the cerebrospinal fluid of meningitis patients, and carriage strains were isolated from throat swabs collected in the context of longitudinal carriage surveys. Isolation and characterization of strains has been described previously (Gagneux, 2000; Leimkugel *et al.*, 2007a, b; Sie *et al.*, 2008). For the analysis of genetic diversification, serogroup A ST7 and ST2859 strains isolated from cases and carriers at different times during clonal colonization and disease waves were selected from our strain collection. Included were nine ST7 carriage and seven ST7 case isolates collected between March 2002 and March 2005 in the KND of Ghana. In the case of ST2859 strains, we analyzed six case and seven carriage isolates collected between March 2006 and March 2007 in the NHD of Burkina Faso, three case and 15 carriage isolates from the KND, as well as three case isolates collected between March 2007 and April 2008 in the neighboring district of Bawku.

Genetic analysis

DNA was extracted from bacterial pellets using the Wizard® Genomic DNA Purification Kit (Promega AG, Duebendorf, Switzerland). The DNA concentration was measured using a Nano drop Spectrophotometer (Witec Ag, Littau, Switzerland). PCR was performed using 5 μL of 10× BD buffer and 1 μL of FirePol Taq polymerase, 1.25 mM $MgCl_2$ (Solis BioDyne, Tartu, Estonia), 100 ng of genomic DNA or the equivalent volume of nuclease-free water as a negative

control, a 0.2 μM concentration of each forward and reverse primer, and a 0.2 mM concentration of each deoxynucleoside triphosphate in a total volume of 50 μL. PCRs were run in a T Professional Basic PCR machine (Biometra GmbH, Göttingen, Germany). The thermal profile for PCR amplification included an initial denaturation step of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 1 min 20 s up to 2 min. The PCRs were finalized by an extension step at 72 °C for 10 min. PCR products were analyzed on 1% agarose gels by gel electrophoresis using ethidium bromide staining and the AlphaImager illuminator and AlphaImager software (Alpha Innotech, San Leandro, CA). PCR products were purified using a NucleoSpin purification kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) and subjected to direct sequencing or cloned using a TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA), transformed into Novablue competent cells (Merck, Darmstadt, Germany), and sequenced after DNA preparation (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Sequencing was performed by Macrogen Inc, Seoul, Korea. For PorA, PorB, and FetA typing, primers were used as previously described (Sacchi *et al.*, 1998; Thompson *et al.*, 2003; Russell *et al.*, 2004). Primers used for amplification and sequencing of the *opa* genes and their flanking regions are listed in Table 1.

Design of the primers used for amplification of opaA, opaB, and opaD was based on the serogroup A strain Z2491 genome sequence (Parkhill *et al.*, 2000) and the PRIMER3 program (http://frodo.wi.mit.edu/). The sequences obtained were analyzed making use of the *N. meningitidis* homepage (http://neisseria.org/nm/) and the *Neisseria* sequence typing homepage (http://pubmlst.org/neisseria/). The multiple sequence alignment websites Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) and CLUSTALW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) were used for comparisons between *Opa* gene sequences and their flanking regions. The EMBOSS TRANSEQ tool was used for the translation of the retrieved *opa* gene sequences (http://www.ebi.ac.uk/Tools/emboss/transeq/).

Results and discussion

Humans are the only natural host for *N. meningitidis*, and nasopharyngeal carriage rates are known to be much higher than disease rates. In a longitudinal study in the northern Ghana, we have observed waves of colonization and disease with hyper-virulent clones of serogroup A meningococci. About 4 years after their local emergence, these clones disappeared completely (Leimkugel *et al.*, 2007a, b). This may be attributed to the development of herd immunity against the colonizing clone in the local population. During their spread from country to country, meningococcal clones undergo microevolution with recombination as major driving force for genetic variation (Achtman, 1995). As a consequence, clones that, based on MLST, appear to be closely related, may differ – due to multiple recombination events –

Table 1 Amplification and sequencing primers for *opa* genes and their flanking regions

OpaA	Amplification primers	VNTR15af	TCATCCGCTACATTGTGTTGA
		OpaA2r	TCGTCATTCCCACGGAAGT
	Sequencing primers	VNTR15af	TCATCCGCTACATTGTGTTGA
		OpaA4r	TTTCCTGATTTTCCGTCTTCA
		OpaA5r	ATGACGGTTCGGGTATTTCC
		OpaA4f	GCGGCAGATTATGCCAGTTA
OpaB	Amplification primers	OpaB2f	CAGGACAAGGCGACGAG
		OpaB5r	TGTCTGGACGGGGATGT
	Sequencing primers	VNTR15br	GCACACCGATATAGGGTTTGAA
		OpaBf	GTGTTGAAACATCGCCACAA
		OpaBr1	GGCATTTTTCCATGCGTTT
		OpaBff	GCGAGAACTGAAGACGGAAA
OpaD	Amplification primers	OpaD5f	TCTCCGTAGAGGAAATGATGC
		OpaD3r	AAGTGGGAATCTAGGACGTAAAA
	Sequencing primers	OpDf	TCATCCGCTATATTGTGTTGA
		Opa26f	TGGGTCTTGGTGTCATCG
		OpA26r	GAATAATTACTTTCTTTCCATTTTCTG
		OpD2f	CGCCCCAAACCTGATATAGT
		OpDr2	GAAACGGTGGGAATTGTGTAA
OpaJ	Amplification primers	Opaj5f	CGCCCCAAACCTGATATAGT
		Opaj1r	ATCTAGAACGTGGGGTTTGG
	Sequencing primers	Opaj5f	CGCCCCAAACCTGATATAGT
		Opaj7f	TGATATAGTCCGCTCCTGCAA
		Opaj8f	CGGTGCAGACAAAGACAAAA
		Opaj9f	GTCGCCGGTGCTGCTA
		Opaj10r	TAGCAGCACCGGCGAC
		Opaj3r	TTTGGGCAACTGTTTTTATCC

Table 2 Origin and characteristics of serogroup A meningococci and their OMPs analyzed

				MLST	PorA	PorB	FetA	OpaA		OpaB		OpaD		OpaJ	
N	Source	Origin	Time	ST	Allele	Allele	Allele	Allele	Repeats	Allele	Repeats	Allele	Repeats	Allele	Repeats
1396	Carriage	KND	March 2002	7	20, 9	3-47	F3-1	242	14	253	[12]	296	[9]	213	7
1577	Carriage	KND	March 2003	7	20, 9	3-47	F3-1	242	13	253	[12]	296	10	213	8
1808	Case	KND	January 2004	7	20, 9	3-47	F3-1	242	[9]	253	8	296	[12]	213	8
1813	Case	KND	February 2004	7	20, 9	3-47	F3-1	242	[9]	253	8	296	[12]	213	8
1812	Case	KND	February 2004	7	20, 9	3-47	F3-1	242	10	253	[9]	296	10	213	[9]
1822	Case	KND	February 2004	7	20, 9	3-47	F3-1	242	14	253	[12]	296	10	213	[9]
1838	Case	KND	March 2004	7	20, 9	3-47	F3-1	242	16	253	[12]	296	10	213	[9]
1902	Carriage	KND	March 2004	7	20, 9	3-47	F3-1	242	14	253	11	296	[9]	213	[9]
1990	Carriage	KND	March 2004	7	20, 9	3-47	F3-1	242	[15]	253	5	296	[12]	213	10
1991	Carriage	KND	November 2004	7	20, 9	3-47	F3-1	242	[15]	253	5	296	[12]	213	10
2008	Case	KND	February 2005	7	20, 9	3-47	F3-1	242	11	253	[9]	296	13	213	[9]
2009	Case	KND	February 2005	7	20, 9	3-47	F3-1	242	11	253	10	296	14	213	[9]
2018	Carriage	KND	March 2005	7	20, 9	3-47	F3-1	242	8	253	8	296	10	213	[9]
2019	Carriage	KND	March 2005	7	20, 9	3-47	F3-1	242	8	253	11	296	10	213	[9]
2020	Carriage	KND	March 2005	7	20, 9	3-47	F3-1	242	11	253	8	296	13	213	[9]
2021	Carriage	KND	March 2005	7	20, 9	3-47	F3-1	242	11	253	8	296	12	213	[9]
2173	Case	NHD	March 2006	2859	20, 9	3-47	F3-1	242	8	253	8	296	10	127	[12]
2171	Case	NHD	March 2006	2859	20, 9	3-47	F3-1	242	8	253	8	296	[9]	127	[12]
2172	Case	NHD	March 2006	2859	20, 9	3-47	F3-1	242	8	253	8	296	[9]	127	[12]
2174	Case	NHD	March 2006	2859	20, 9	3-47	F3-1	242	8	253	8	296	[9]	127	[12]
2175	Case	NHD	March 2006	2859	20, 9	3-47	F3-1	242	8	253	8	296	[9]	127	[12]
2176	Case	NHD	March 2006	2859	20, 9	3-47	F3-1	242	8	253	8	296	[9]	127	11
2202	Carriage	NHD	March 2006	2859	20, 9	3-47	F3-1	242	[9]	253	8	296	10	127	13
2243	Carriage	NHD	March 2006	2859	20, 9	3-47	F3-1	242	8	253	8	296	[9]	127	11
2365	Carriage	NHD	November 2006	2859	20, 9	3-47	F3-1	242	7	253	9	296	7	127	13
2378	Carriage	NHD	November 2006	2859	20, 9	3-47	F3-1	242	14	253	8	296	[9]	127	17
2554	Carriage	NHD	March 2007	2859	20, 9	3-47	F3-1	242	10	253	11	296	[9]	127	[9]
2560	Carriage	NHD	March 2007	2859	20, 9	3-47	F3-1	242	8	253	8	296	[12]	127	[12]
2587	Carriage	NHD	March 2007	2859	20, 9	3-47	F3-1	242	8	253	8	296	[12]	127	8
2537	Case	Bawku	March 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	11
2539	Case	Bawku	March 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2541	Case	Bawku	March 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2545	Case	KND	April 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2502	Carriage	KND	April 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2620	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2622	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2624	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2626	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2628	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2630	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2632	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]

Table 2 (continued)

N	Source	Origin	Time	MLST ST	PorA Allele	PorB Allele	FetA Allele	OpaA		OpaB		OpaD		OpaJ	
								Allele	Repeats	Allele	Repeats	Allele	Repeats	Allele	Repeats
2635	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2669	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2671	Carriage	KND	November 2007	2859	20, 9	3-47	F3-1	253	8	253	8	296	10	127	[12]
2699	Carriage	KND	April 2008	2859	20, 9	3-47	F3-1	253	10	253	7	296	[12]	127	[12]
2700	Carriage	KND	April 2008	2859	20, 9	3-47	F3-1	253	10	253	7	296	[12]	127	[12]
2701	Carriage	KND	April 2008	2859	20, 9	3-47	F3-1	253	10	253	7	296	[12]	127	[12]
2703	Carriage	KND	April 2008	2859	20, 9	3-47	F3-1	253	10	253	7	296	[12]	127	[12]
2708	Case	KND	April 2008	2859	20, 9	3-47	F3-1	253	10	253	7	296	[12]	127	[12]
2707	Case	KND	February 2008	2859	20, 9	3-47	F3-1	253	8	253	8	296	[12]	127	10

The *opa* genes that were found to be in frame are framed.

very substantially in their antigenic profiles A. Lamelas and G. Pluschke, Swiss Tropical and Public Health Institute.

Antibodies directed against OMPs have been implicated in the development of natural immunity against meningococci. In particular, antibodies directed against the PorA and PorB proteins seem to provide serosubtype-specific protection (Jordens *et al.*, 2004). This prompted us to investigate whether immune selection pressure building up in the population during colonization waves leads to an antigenic diversification of OMPs in the colonizing meningococcal population.

The strains included in the sequence analysis of OMP-encoding genes have been isolated from cerebrospinal fluid (CSF) of meningitis patients or from the pharynx of healthy carriers during two sequential colonization and disease waves in northern Ghana and during a meningococcal disease outbreak in Burkina Faso. Serogroup A ST7 strains have been isolated from the start (2002) till the end (2005) of a colonization wave in northern Ghana (Leimkugel *et al.*, 2007a, b). In the case of serogroup A ST 2859, isolates from an outbreak in Burkina Faso (2006–2007) (Sie *et al.*, 2008) and in northern Ghana (2007–2008) were compared.

It has been proposed that the propensity of *N. meningitidis* to accumulate mutations increases dramatically toward the end of an epidemic, presumably due to immune pressure, and that with time, variants arise that are founders of new clonal complexes (Achtman, 2004). However, in none of the strains investigated here a single mutational change in the genes encoding the surface proteins PorA, PorB, or FetA was found. All PorA sequences had the VR1, VR2: 20, 9 variable regions. The Por B sequences were invariably of class 3 and had the allele 47. The variable region of Fet A was F3-1. In spite of microevolution of the epidemic clones, as detected by pulsed field gel electrophoresis (Leimkugel *et al.*, 2007a, b), the sequence of the analyzed OMP-encoding genes thus was strikingly stable. No mutations were detected in the variable regions of the surface proteins PorA, PorB, and FetA in any of the strains analyzed. The benefit of accumulating mutations to escape herd immunity may be dispossessed by the fitness cost that such mutations confer (Achtman, 2004). Furthermore, the lack of a genetically diverse pharyngeal flora of *N. meningitidis*, in the study population (Leimkugel *et al.*, 2007a, b), limiting the extent of horizontal genetic exchange (Achtman, 1995) may contribute to this striking lack of diversification. On the other hand, immune selection may act more strongly on exposed antigens other than the major OMPs analyzed here.

While the Ghanian meningococcal isolates analyzed here have been collected over a longer period of time (between 2002 and 2008), conclusions on the stability of OMPs for the strains from Burkina Faso are less strong, as the analyzed strains were isolated over a period of only 2 years. Another very important point to consider is that, although we did not detect any diversification in the genes encoding PorA, Por B, and FetA, expression levels of PorA and FetA are subject to variation. Antibodies elicited against these proteins in the host population may give selective advantage to meningococcal strains with decreased expression levels

of PorA and FetA (Crowe *et al.*, 1989; Carson *et al.*, 2000). PorB expression levels have been described to be constant, however (Abad *et al.*, 2006).

The multiple copies of genes encoding the Opa proteins can be turned on and off during chromosomal replication by slipped-strand mispairing of tandem CTTCT repeats present in their open reading frames. Here, we have used the published genome sequence of the serogroup A ST4 strain Z2491 to design primers specific for the flanking regions of the *opaA*, *opaB*, and *opaD* genes. In contrast to strain Z2491, the ST7 and ST2859 strains analyzed here all contained in addition an *opa*J gene, as has been described for other ST5-complex/subgroup III strains (Callaghan *et al.*, 2006). While all PorA, PorB, and FetA had the same alleles irrespective of the time point and location of isolation as well as the ST of the strains examined (Table 2), two of the four *opa* genes analyzed were subject to some variation. While all the ST7 strains from Ghana and the ST2859 strains from Burkina Faso had the *opaA* allele 242, all Ghanaian ST2859 strains had the allele 253. No variation was observed within the three individual groups of epidemiologically related isolates. ST7 and ST2859 are MLST single-locus variants, and the ST2859 genocloud may have developed from ST7 meningococci in Africa. The change in the *opaA* allele in the ST 2859 strains that emerged in Ghana in 2008 may have been due to homologous recombination and immune selection against the 242 allele (Callaghan *et al.*, 2008; Levin & Cornejo, 2009). In the case of the *opaJ* gene, all ST7 isolates had the allele 213, whereas the ST2859 strains both from Ghana and from Burkina Faso had the allele 127.

The numbers of pentanucleotide repeats within the coding sequences of the *opa* genes were subject to much wider variation. However, strains isolated at the same location and a similar time point tended to be relatively uniform. For example, all Ghanaian ST2859 isolates from 2007 had 8 *opaA*, 8 *opaB*, and 10 *opaD* repeats, while 5/6 isolates from 2008 had 10 *opaA*, 7 *opaB*, and 12 *opaD* repeats. This variation in numbers of tandem repeats is due to slipped-strand mispairing and results in phase variation involving on/off expression of the respective *opa* gene (Murphy *et al.*, 1989). Alleles with a number of pentanucleotide repeats are a multiple of three represent functional genes (Table 2). Antigenic variation in the Opa genes has been suggested to mediate immune evasion (Davidsen & Tonjum, 2006; Callaghan *et al.*, 2008). Functional open reading frames were found for all four *opa* genes, but no more than two functional *opa* genes were found in any of the strains. A Poisson regression analysis was performed (http://www.fisherstat.com/Pages/default.aspx), and disease and colonization isolates did not differ significantly in the Opa protein expression patterns (Table 2). While the invasive bacteria may represent rare genetic variants of the colonizing meningococcal population, a change in the Opa protein expression pattern thus does not seem to be relevant for an adaptation for multiplication in the blood stream and the CSF.

Control of meningitis epidemics has relied so far on reactive vaccination strategies with polysaccharide vaccines, and a serogroup A conjugate vaccine is currently being introduced in the meningitis belt. In our study, we have observed an apparent lack of diversification of PorA, PorB, and FetA, and a tailor-made vaccine targeting those OMPs may be able to control the epidemic strains investigated. However, to develop a comprehensive vaccine based on subcapsular OMPs, whole-genome analyses of epidemiologically well-defined isolate collections may be needed. Comparative genomics may give insight into the driving forces behind the microevolution of *N. meningitidis*, allowing a more comprehensive analysis of antigen variability, as well as the identification of new vaccine targets. Results of such analyses may thus help identifying antigens suitable for inclusion into a multivalent protein subunit vaccine (Tan *et al.*, 2010).

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