Brief communication

Phase variation of Opa proteins of *Neisseria meningitidis* and the effects of bacterial transformation

MANISH SADARANGANI[1],*, J CLAIRE HOE[1], KATHERINE MAKEPEACE[1], PETER VAN DER LEY[2] and ANDREW J POLLARD[1]

[1]*Oxford Vaccine Group, Department of Paediatrics, University of Oxford, Centre for Clinical Vaccinology and Tropical Medicine, Churchill Hospital, Oxford OX3 7LE, UK*

[2]*Institute for Translational Vaccinology, Antonie van Leeuwenhoeklaan 9, 3721 MA, Bilthoven, The Netherlands*

**Corresponding author (Fax, +44 (0)1865 234235; Email, manish.sadarangani@paediatrics.ox.ac.uk)*

Opa proteins are major proteins involved in meningococcal colonization of the nasopharynx and immune interactions. Opa proteins undergo phase variation (PV) due to the presence of the 5′-CTCTT-3′ coding repeat (CR) sequence. The dynamics of PV of meningococcal Opa proteins is unknown. Opa PV, including the effect of transformation on PV, was assessed using a panel of Opa-deficient strains of *Neisseria meningitidis*. Analysis of Opa expression from UK disease-causing isolates was undertaken. Different *opa* genes demonstrated variable rates of PV, between 6.4×10^{-4} and 6.9×10^{-3} per cell per generation. *opa* genes with a longer CR tract had a higher rate of PV (r^2=0.77, p=0.1212). Bacterial transformation resulted in a 180-fold increase in PV rate. The majority of *opa* genes in UK disease isolates (315/463, 68.0%) were in the 'on' phase, suggesting the importance of Opa proteins during invasive disease. These data provide valuable information for the first time regarding meningococcal Opa PV. The presence of Opa PV in meningococcal populations and high expression of Opa among invasive strains likely indicates the importance of this protein in bacterial colonization in the human nasopharynx. These findings have potential implications for development of vaccines derived from meningococcal outer membranes.

[Sadarangani M, Hoe JC, Makepeace K, van der Ley P and Pollard AJ 2016 Phase variation of Opa proteins of *Neisseria meningitidis* and the effects of bacterial transformation. *J. Biosci.*] **DOI** 10.1007/s12038-016-9588-y

1. Introduction

Neisseria meningitidis causes approximately 500,000 cases of meningitis and septicemia worldwide annually, with a case-fatality rate of approximately 10% (World Health Organization 1998). Most disease in temperate countries is caused by capsular group B organisms (Halperin *et al.* 2012). A number of group B vaccines based on different combinations of subcapsular antigens have been developed, including several outer membrane vesicle (OMV) vaccines (Sadarangani and Pollard 2010). Many OMVs in vaccines used in clinical trials have been derived from strains which have been genetically modified by bacterial transformation to alter the expression of specific surface components in order to improve immunogenicity. Therefore, cellular events influenced by transformation are highly relevant to vaccine development.

The Opacity-associated (Opa) adhesin proteins are major phase-variable proteins found in the outer membrane of *N. meningitidis*. Each bacterium contains four *opa* genes (*opaA*, *opaB*, *opaD* and *opaJ*), which may encode identical or different Opa proteins, and can therefore express up to four different Opa variants at any one time (Tettelin *et al.* 2000). Opa proteins are critical in meningococcal pathogenesis, mediating bacterial adherence to the nasopharynx and modulating human cellular immunity via interactions with T cells and neutrophils (Virji *et al.* 1993; Gray-Owen 2003).

Keywords. Colonization; meningococcus; Opa protein; phase variation; transformation

Phase variation (PV) describes the phenomenon whereby pathogens can genetically switch 'on' and 'off', or vary, the expression level of specific components, and is a major mechanism of variability for *N. meningitidis* (Snyder *et al.* 2001). Several meningococcal surface structures exhibit PV, including the capsule, pili and outer membrane proteins PorA, NadA, Opc and Opa. PV allows the meningococcus to adapt to the different host environments (i.e. nasopharynx, blood, meninges) encountered during infection. PV is believed to be vital for the organism to evade the host immune response by regular alteration of its surface, which is especially important for *N. meningitidis* since it is usually carried asymptomatically in the nasopharynx for up to several months. Understanding the dynamics of meningococcal PV will therefore enable the appropriate development of vaccines targeting subcapsular antigens, such as OMVs. PV of Opa proteins is thought to predominantly occur through slipped-strand mispairing within a pentanucleotide coding repeat (CR) sequence 5′-CTCTT-3′ present within the *opa* open reading frame (within the signal peptide), resulting in frame shifting during DNA replication and the translation of non-functional, truncated proteins (Stern *et al.* 1986).

We have previously described the construction of Opa-deficient derivatives of *N. meningitidis* strain H44/76, produced by sequential disruption of *opa* genes using antibiotic resistance cassettes (Sadarangani *et al.* 2012). In this study, strains from this unique library were used to analyse the PV of different Opa proteins to identify factors which may influence Opa PV rates.

2. Materials and methods

2.1 *Bacterial strains and growth conditions*

Wild type *N. meningitidis* strain H44/76 and derivative Opa-deficient strains, which have previously been described, were used (table 1) (Sadarangani *et al.* 2012). *N. meningitidis* was grown on brain heart infusion (BHI) agar (Merck, Darmstadt, Germany) supplemented with Levinthal's base (10% v/v) at 37°C in a humidified 5% CO_2 atmosphere for 16–18 h. Selective media was supplemented with kanamycin (100 µg/mL) (Sigma-Aldrich, Gillingham, UK).

2.2 *Transformation of Neisseria meningitidis*

N. meningitidis was transformed using the spot transformation technique, as previously described (Sadarangani *et al.* 2012). Briefly, approximately 10^8 colony forming units (cfu) of bacterial suspension from overnight growth was incubated with approximately 1 µg of pT7-E2-F2-kan plasmid DNA, plated over a 1–2 cm diameter region on BHI agar. pT7-E2F2-kan contains the LPS biosynthesis gene *lpt3* disrupted with a kanamycin resistance cassette (Mackinnon *et al.* 2002). After incubation at 37°C, 5% CO_2 for 4–8 h bacteria were plated onto selective and non-selective BHI agar and incubated for a further 16–18 h. Experiments were performed in duplicate.

2.3 *Colony blotting*

Colony blotting with anti-Opa monoclonal antibodies (mAbs) 15-1-P5.5 and MN20E12.70 (de Jonge *et al.* 2003) was performed to identify the expression of surface-expressed Opa proteins. mAb 15-1-P5.5 targeted OpaA and OpaJ of H44/76 and mAb MN20E12.70 bound to OpaB and OpaD. Prior to blotting, bacteria were diluted in phosphate buffered saline (PBS) and plated onto BHI agar to achieve 1500–3000 colonies per plate. Plates were incubated at 37°C, 5% CO_2 for 16–18 h, following which a nitrocellulose membrane (pore size 0.45 µm, GE Healthcare, Buckinghamshire, UK) was used to adsorb the colonies. The nitrocellulose membrane was air-dried, then blocked for 1 h with 5% skimmed milk in PBST (PBS containing 0.1% Tween 20 and 7.7 mM sodium azide). The membrane was then washed several times with wash buffer (154 mM NaCl, 0.05% Tween 20) and the mAb added after dilution to 1:50,000 in PBST containing 1% bovine serum albumin (BSA). After 1–2 h, the membrane was washed several times prior to incubation with goat anti-mouse IgG (Fab specific)-alkaline phosphatase antibody (Sigma-Aldrich), diluted 1:10,000 in PBST (without sodium azide) containing 1% BSA, for 1–2 h. Following further wash steps, alkaline phosphatase activity was detected by the addition of 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) (Sigma-Aldrich). The membrane was incubated for 10 min and the reaction stopped by rinsing with water.

2.4 *Estimation of opa mutation rates*

While PV describes the alteration in Opa surface expression, the term 'mutation rate' is used here to describe the genetic changes occurring in the cell which underlie PV. The mutation rates of *opa* genes relevant to PV (i.e. the mutation rate of the CR sequences) were estimated using the following equation:

$$\alpha = \frac{2}{1+\mathbf{x}}\left[1-\sqrt[n]{1-(1+x)p_n}\right],$$

where α represents the mutation rate per cell per generation, the back-variation rate is x times the forward rate,

n is the number of generations of bacterial cell division and p_n is the proportion of mutants at n generations (Saunders *et al.* 2003). This equation assumes no fitness advantage between the two phase-variable states, which was assumed in this study given that it was performed *in vitro* without any selection pressures. Therefore, x=1 was used in this equation. In an initial experiment, colonies were plated out using 10-fold serial dilutions between 10^{-2} and 10^{-4}, which demonstrated that each colony contained approximately 20 generations (n=20). To estimate the mutation rates from Opa expression experiments, it was assumed that the mutation rate was double the PV rate because for an *opa* gene in the 'off' state, 50% of alterations in the length of the CR tract would result in a detectable 'on' state, while the other 50% would not be detected by colony blotting as the expression would remain 'off'.

2.5 *Opa expression in UK meningococcal disease isolates*

The Meningitis Research Foundation (MRF) Meningococcus Genome Library (MGL) was used to analyse the expression of Opa proteins in UK disease-causing meningococci (*http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_neisseria_mrfgenomes*). The library contained whole genome sequence data from UK disease-causing isolates (predominantly from blood and cerebrospinal fluid (CSF)) of *N. meningitidis* which had caused infections between 2010 and 2013.

3. Results

3.1 *opa mutation rate*

To assess the *opa* mutation rate, analysis was performed using anti-Opa mAbs 15-1-P5.5 and MN20E12.70. To circumvent the partial cross-reactivity of the mAbs and specifically assess individual Opa proteins, mutant strains were utilised – each strain was derived from H44/76 and had a single *opa* gene disrupted. In a given experiment, therefore, the mAb used would only be able to bind to one of the Opa proteins – for example, to assess PV of OpaA, strain M001/02 (Δ*opaJ*) was used with mAb 15-1-P5.5. In addition the Opa protein being assessed was not expressed at the start of the experiment due to the number of CR sequences present (table 1). *opaD* had the highest mutation rate of 6.9×10^{-3} per cell per generation, followed by *opaA* (1.9×10^{-3} per cell per generation) (table 2). *opaB* and *opaJ* had lower mutation rates of 6.4×10^{-4} and 7.9×10^{-4} per cell per generation, respectively. A longer CR tract was associated with an increased mutation rate (r^2=0.77, p=0.1212), although this did not achieve statistical significance.

3.2 *The effect of transformation on opa mutation rate*

To assess the effect of transformation on *opa* mutation rate, strain M001/02, which expressed no Opa and had *opaJ* deleted (table 1), was transformed with pT7-E2F2-kan, and OpaA expression determined by colony blotting with mAb 15-1-P5.5. When the total number of bacteria were

Table 1. Strains of Neisseria meningitidis used in this study

Strain	Number of CR sequences in *opa* gene				Opa expression*			
	opaA	*opaB*	*opaD*	*opaJ*	OpaA	OpaB	OpaD	OpaJ
H44/76	10	8	14	11	Off	Off	Off	Off
M001/02	10	8	14	Δ	Off	Off	Off	Del
M002/02	Δ	8	14	11	Del	Off	Off	Off
M003/01	10	8	Δ	11	Off	Off	Del	Off
M004/01	10	Δ	14	11	Off	Del	Off	Off
M001/01	9	8	15	Δ	On	Off	On	Del
M007/01	Δ	8	15	Δ	Del	Off	On	Del

*Expression state of the majority of bacteria of each strain.

All strains were derived from the H44/76 wild-type strain as previously described (Sadarangani *et al.* 2012). Opa expression is phase-variable and is 'On' only when the number of CR sequences is a multiple of three, otherwise it is 'Off'. Expression state and number of CR sequences at the start of experiments are shown in this table. M001/02 and M002/02 are targeted by monoclonal antibody (mAb) 15-1-P5.5 when OpaA (M001/02) or OpaJ (M002/02) are 'On'. M003/01 and M004/01 are targeted by mAb MN20E12.70 when OpaB (M003/01) or OpaD (M004/01) are 'On'. Δ indicates the *opa* gene has been disrupted, so protein expression is not possible (indicated by 'Del'). M001/01 was used as a positive control due to the expression of OpaA and OpaD and therefore binding of both mAbs 15-1-P5.5 and MN20E12.70. M007/01 was used as a negative control since both *opaA* and *opaJ* have been disrupted this strain is unable to bind mAb 15-1-P5.5.

Table 2. Mutation rates of *opa* genes by nucleotide sequence analysis and colony blotting

	opaA	*opaB*	*opaD*	*opaJ*
Strain used	M001/02	M003/01	M004/01	M002/02
Monoclonal antibody used	15-1-P5.5	MN20E12.70	MN20E12.70	15-1-P5.5
Number of colonies assessed	2617	2830	2690	2279
Number with modified Opa expression	25	9	90	9
Mutation rate per cell per generation (95% CI)**	1.9×10^{-3} (1.5×10^{-3} to 2.3×10^{-3})	6.4×10^{-4} (4.3×10^{-4} to 8.5×10^{-4})	6.9×10^{-3} (6.2×10^{-3} to 7.7×10^{-3})	7.9×10^{-4} ($5.3\times10^{-}$ to 1.1×10^{-3})

CI = confidence interval; CR=coding repeat (5′-CTCTT-3′).
*Limit of detection using this method and number of colonies.
**To estimate mutation rates from colony blotting, it was assumed that the mutation rate was double the PV rate because for an *opa* gene in the 'off' state, 50% of alterations in the length of the CR tract would result in a detectable 'on' state, while the other 50% would not be detected by colony blotting as the expression would remain 'off' - only when the number of CR sequences is a multiple of three is Opa expressed on the bacterial surface.

considered, the *opaA* mutation rate was 5.6×10^{-4} per cell per generation (95% CI 4.1×10^{-4} to 7.1×10^{-4}). In successfully transformed bacteria (transformation rate 1.5×10^{-7}), the mutation rate was 180 times higher at 1.0×10^{-1} per cell per generation (95% CI 8.4×10^{-2} to 1.2×10^{-1}). Overall, 32.1% of bacteria had switched from the 'off' state to the 'on' state. Given that only 50% of alterations in the length of the CR tract result in a switch from an 'off' state to a detectable 'on' state, it can be estimated that 64% (95% CI 62.1 to 66.4) of the transformants were therefore hypermutable, at least transiently.

3.3 *Opa expression in UK meningococcal disease isolates*

To investigate the influence of Opa PV at a population level, we interrogated a database of UK meningococcal disease isolates to assess Opa expression. There were a total of 1,381 isolates in the MRF MGL database, of which 451 contained sequence data for a total of 463 *opa* genes (annotated as *NEIS1719*). Overall, 315/463 (68.0%) *opa* genes would result in expression of Opa on the bacterial surface. There were a total of 82 unique *opa* sequences when the variability of the 5′ sequence upstream of the sequence encoding the mature surface-expressed protein was excluded, of which 52 were found once only and 30 were present on multiple occasions. Further analysis of the duplicates among these confirmed that variability between genes encoding the same Opa protein in different isolates was almost entirely due to differences in the length of the CTCTT repeat.

4. Discussion

Our finding that transformation of *N. meningitidis* with unrelated plasmid DNA resulted in a 180-fold increase in Opa PV rate has potential significance for meningococcal pathogenesis. Increased PV following transformation is likely to confer a survival advantage to bacteria during natural infection. Transformation is a powerful mechanism for generating genetic diversity, spreading advantageous allelic variants, and mediating some forms of antigenic variation. During colonisation of the human nasopharynx multiple bacterial species co-exist, resulting in relatively large amounts of extracellular DNA and consequently frequent transformation events, although transformation is most likely to occur using DNA from the same species. Increased population diversity aids immune evasion by modification of potential targets on the bacterial surface, and may occur due to increased PV in a single organism following DNA uptake and/or by selection of 'hypermutable' strains at the population level (Alexander *et al.* 2004; Bayliss *et al.* 2008). The former is supported by a previous study which

demonstrated a 24- to 73-fold increase in PV following transformation with chromosomal neisserial DNA due to inhibition of the mismatch repair (MMR) genes by single stranded DNA (Alexander *et al.* 2004). Mutations in the MMR genes *mutS* or *mutL* can produce 'hypermutable' strains, which have a 10-fold increase in basal mutation rates and a 1000-fold increase in PV rates of genes containing mononucleotide repeat tracts (Bayliss *et al.* 2008; Martin *et al.* 2004; Richardson and Stojiljkovic 2001). In our study we were unable to confirm whether these loci were mutated by the transformation process and therefore the mechanism of the increased PV rate.

The PV rates we found are similar to those previously determined for gonococcal Opa proteins (Mayer 1982). The data suggested a relationship between repeat tract length and PV rate, which has previously been described for the polyG tract of *N. meningitidis hmbR* and in *Haemophilus influenzae*, and is thought to be due to increased instability of the region of DNA (De Bolle *et al.* 2000; Richardson *et al.* 2002). Other important factors in determining PV rates include the type of repeat sequence (poly-G/C tracts are less stable than poly-A/T (Warmlander *et al.* 2002)), the location of a gene within the chromosome (in particular repetitive DNA in close proximity and transcription levels of that region of the chromosome) and gene promoter strength. To more fully assess Opa PV it would be ideal to assess different Opa proteins in a range of *N. meningitidis* strains, and directly measure both forward and backward switching rates. The presence of multiple Opa proteins per organism and availability of only the specific monoclonal antibodies described means we were limited to using H44/76 and the derivative strains we have constructed. While it is possible that these findings are specific to this strain, our results are consistent with previous studies.

Our finding of increased Opa PV following transformation has potential implications for development of meningococcal OMV vaccines because a number of such vaccines are based on strains which have been genetically modified via transformation (Cartwright *et al.* 1999; de Kleijn *et al.* 2000; Pettersson *et al.* 2006; Finney *et al.* 2007; van den Dobbelsteen *et al.* 2007; Weynants *et al.* 2007, 2009; Koeberling *et al.* 2009; Bonvehi *et al.* 2010; Zollinger *et al.* 2010; Keiser *et al.* 2010, 2011). Characterisation of the modified bacteria and resulting OMVs usually focuses on the vaccine antigens, without a detailed assessment of other antigens. To confirm this phenomenon of transformation-induced increased PV, it would be ideal to conduct these transformation experiments with multiple plasmids, as well as whole genomic DNA, and assess several phase variable proteins. It is possible that disruption of the LPS biosynthesis gene *lpt3* had a direct effect on transformation rate, although this has not been described previously. We have previously shown that transformation with *opa*-containing plasmid DNA did not modify the expression of other major outer membrane proteins PorA, PorB, RmpM or factor H binding protein (fHbp) (Sadarangani *et al.* 2012), but the phenomenon of increased PV following transformation has been previously described (Alexander *et al.* 2004) and the narrow confidence intervals we found suggest this effect also occurs for Opa proteins.

Results of the analysis of the MRF MGL suggested there may be a population level fitness advantage conferred by Opa expression in blood and CSF, which could be related to increased adhesion and colonization by Opa+ variants, or the immunomodulatory effects of Opa contributing to survival of the organism during invasive disease (Sadarangani *et al.* 2011). An important role for Opa during infection with all pathogenic *Neisseria* is supported by the observation that Opa expressing bacteria are recovered during natural gonococcal infection (James and Swanson 1978) and following inoculation of humans with Opa non-expressing bacteria (Swanson *et al.* 1988; Jerse *et al.* 1994). A major limitation of analysis of *opa* genes in the MRF MGL is that coverage of *opa* using whole genome sequencing is relatively poor due to it being a multi-copy gene, and being surrounded by significant repetitive DNA. However there is no clear explanation why the successfully sequenced genes would result in a biased dataset and biological plausibility exists as to the importance of Opa in meningococcal pathogenesis. In addition the treatment and *in vitro* culture of these clinical strains between isolation and genome sequencing is unknown and may vary between strains - it is therefore possible that there may be *in vitro* selection with respect to Opa expression, although this would be minimised due to the absence of specific selection pressure during *in vitro* culture.

PV of Opa proteins is of particular importance for a number of reasons. Opa PV may be a key mechanism of immune evasion by the meningococcus, since Opa proteins induce bactericidal antibodies following meningococcal infection and after immunization with serogroup B OMV vaccines (Mandrell and Zollinger 1989). PV of other outer membrane proteins included in meningococcal vaccines have been shown to mediate escape from bactericidal antibodies, and further investigation of this phenomenon will aid the future design of such vaccines (Bayliss *et al.* 2008; Tauseef *et al.* 2013; Alamro *et al.* 2014). Many OMV vaccines in clinical trials have been derived from genetically modified strains (created by transformation) and characterization of the modified bacteria and resulting OMVs usually focuses on the vaccine antigens, without a detailed assessment of other antigens. The process of transformation to construct these vaccine strains may result in changes in expression of other phase variable meningococcal proteins, leading to the possibility that modification of just one or two proteins may significantly alter the immunogenicity or reactogenicity of the resulting OMV, or have an effect on bacterial growth and therefore influence vaccine production.

Further study of the major phase variable proteins during vaccine development may therefore aid the development of more refined products with better immunogenicity and reactogenicity profiles, which may also be more easily produced. Future studies of meningococcal Opa PV would ideally include the examination of a number of diverse strains, including those from invasive disease and carriage.

Acknowledgements

This study was funded by Action Medical Research through a Research Training Fellowship awarded to MS (RTF1263) and supported by the Oxford Partnership Comprehensive Biomedical Research Centre with funding from the Department of Health's National Institute of Health Research Biomedical Research Centres funding scheme. This publication made use of the Meningitis Research Foundation Meningococcus Genome Library (*http://www.meningitis.org/research/genome*) developed by Public Health England, the Wellcome Trust Sanger Institute and the University of Oxford as a collaboration with funding from the Meningitis Research Foundation.

AJP is a Jenner Institute Investigator and James Martin Senior Fellow. AJP was previously named as an inventor on a patent for the use of Opa proteins in meningococcal vaccines. AJP has previously conducted clinical trials of meningococcal vaccines on behalf of the University of Oxford, but receives no personal payments from them. AJP is chair of the UK Department of Health's Joint Committee on Vaccines and Immunisation and chair of the European Medicine Agency's Scientific Advisory Group on Vaccines. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health. Other authors have no conflicts of interest.

References

Alamro M, Bidmos FA, Chan H, Oldfield NJ, Newton E, Bai X, Aidley J, Care R, *et al*. 2014 Phase variation mediates reductions in expression of surface proteins during persistent meningococcal carriage. *Infect. Immun.* **82** 2472–2484

Alexander HL, Richardson AR and Stojiljkovic I 2004 Natural transformation and phase variation modulation in Neisseria meningitidis. *Mol. Microbiol.* **52** 771–783

Bayliss CD, Hoe JC, Makepeace K, Martin P, Hood DW and Moxon ER 2008 *Neisseria meningitidis* escape from the bactericidal activity of a monoclonal antibody is mediated by phase variation of lgtG and enhanced by a mutator phenotype. *Infect. Immun.* **76** 5038–5048

Bonvehi P, Boutriau D, Casellas J, Weynants V, Feron C and Poolman J 2010 Three doses of an experimental detoxified L3-derived lipooligosaccharide meningococcal vaccine offer good safety but low immunogenicity in healthy young adults. *Clin. Vaccine Immunol.* **17** 1460–1466

Cartwright K, Morris R, Rumke H, Fox A, Borrow R, Begg N, Richmond P and Poolman J 1999 Immunogenicity and reactogenicity in UK infants of a novel meningococcal vesicle vaccine containing multiple class 1 (PorA) outer membrane proteins. *Vaccine* **17** 2612–2619

De Bolle X, Bayliss CD, Field D, van de Ven T, Saunders NJ, Hood DW and Moxon ER 2000 The length of a tetranucleotide repeat tract in Haemophilus influenzae determines the phase variation rate of a gene with homology to type III DNA methyltransferases. *Mol. Microbiol.* **35** 211–222

de Jonge MI, Vidarsson G, van Dijken HH, Hoogerhout P, van Alphen L, Dankert J and van der Ley P 2003 Functional activity of antibodies against the recombinant OpaJ protein from *Neisseria meningitidis*. *Infect. Immun.* **71** 2331–2340

de Kleijn ED, de Groot R, Labadie J, Lafeber AB, van den Dobbelsteen G, van Alphen L, van Dijken H, Kuipers B, *et al*. 2000 Immunogenicity and safety of a hexavalent meningococcal outer-membrane-vesicle vaccine in children of 2-3 and 7-8 years of age. *Vaccine* **18** 1456–1466

Finney M, Vaughan T, Taylor S, Hudson MJ, Pratt C, Wheeler JX, Vipond C, Feavers I, *et al*. 2007 Characterization of the key antigenic components and pre-clinical immune responses to a meningococcal disease vaccine based on neisseria lactamica outer membrane vesicles. *Hum. Vaccin.* **4** 23–30

Gray-Owen SD 2003 Neisserial Opa proteins: impact on colonization, dissemination and immunity. *Scand. J. Infect. Dis.* **35** 614–618

Halperin SA, Bettinger JA, Greenwood B, Harrison LH, Jelfs J, Ladhani SN, McIntyre P, Ramsay ME, *et al*. 2012 The changing and dynamic epidemiology of meningococcal disease. *Vaccine* **30** B26–B36

James JF and Swanson J 1978 Studies on gonococcus infection. XIII. Occurrence of color/opacity colonial variants in clinical cultures. *Infect. Immun.* **19** 332–340

Jerse AE, Cohen MS, Drown PM, Whicker LG, Isbey SF, Seifert HS and Cannon JG 1994 Multiple gonococcal opacity proteins are expressed during experimental urethral infection in the male. *J. Exp. Med.* **179** 911–920

Keiser PB, Biggs-Cicatelli S, Moran EE, Schmiel DH, Pinto VB, Burden RE, Miller LB, Moon JE, *et al*. 2011 A phase 1 study of a meningococcal native outer membrane vesicle vaccine made from a group B strain with deleted lpxL1 and synX, over-expressed factor H binding protein, two PorAs and stabilized OpcA expression. *Vaccine* **29** 1413–1420

Keiser PB, Gibbs BT, Coster TS, Moran EE, Stoddard MB, Labrie JE 3rd, Schmiel DH, Pinto V, *et al*. 2010 A phase 1 study of a group B meningococcal native outer membrane vesicle vaccine made from a strain with deleted lpxL2 and synX and stable expression of opcA. *Vaccine* **28** 6970–6976

Koeberling O, Giuntini S, Seubert A and Granoff DM 2009 Meningococcal outer membrane vesicle vaccines derived from mutant strains engineered to express factor H binding proteins from antigenic variant groups 1 and 2. *Clin. Vaccine Immunol.* **16** 156–162

Mackinnon FG, Cox AD, Plested JS, Tang CM, Makepeace K, Coull PA, Wright JC, Chalmers R, *et al*. 2002 Identification of a

gene (lpt-3) required for the addition of phosphoethanolamine to the lipopolysaccharide inner core of *Neisseria meningitidis* and its role in mediating susceptibility to bactericidal killing and opsonophagocytosis. *Mol. Microbiol.* **43** 931–943

Mandrell RE and Zollinger WD 1989 Human immune response to meningococcal outer membrane protein epitopes after natural infection or vaccination. *Infect. Immun.* **57** 1590–1598

Martin P, Sun L, Hood DW and Moxon ER 2004 Involvement of genes of genome maintenance in the regulation of phase variation frequencies in *Neisseria meningitidis*. *Microbiology* **150** 3001–3012

Mayer LW 1982 Rates in vitro changes of gonococcal colony opacity phenotypes. *Infect. Immun.* **37** 481–485

Pettersson A, Kortekaas J, Weynants VE, Voet P, Poolman JT, Bos MP and Tommassen J 2006 Vaccine potential of the Neisseria meningitidis lactoferrin-binding proteins LbpA and LbpB. *Vaccine* **24** 3545–3557

Richardson AR and Stojiljkovic I 2001 Mismatch repair and the regulation of phase variation in *Neisseria meningitidis*. *Mol. Microbiol.* **40** 645–655

Richardson AR, Yu Z, Popovic T and Stojiljkovic I 2002 Mutator clones of *Neisseria meningitidis* in epidemic serogroup A disease. *Proc. Natl. Acad. Sci. USA* **99** 6103–6107

Sadarangani M, Hoe JC, Callaghan MJ, Jones C, Chan H, Makepeace K, Daniels-Treffandier H, Deadman ME, *et al*. 2012 Construction of Opa-positive and Opa-negative strains of *Neisseria meningitidis* to evaluate a novel meningococcal vaccine. *PLoS One* **7** e51045

Sadarangani M and Pollard AJ 2010 Serogroup B meningococcal vaccines-an unfinished story. *Lancet Infect. Dis.* **10** 112–124

Sadarangani M, Pollard AJ and Gray-Owen SD 2011 Opa proteins and CEACAMs: pathways of immune engagement for pathogenic Neisseria. *FEMS Microbiol. Rev.* **35** 498–514

Saunders NJ, Moxon ER and Gravenor MB 2003 Mutation rates: estimating phase variation rates when fitness differences are present and their impact on population structure. *Microbiology* **149** 485–495

Snyder LA, Butcher SA and Saunders NJ 2001 Comparative whole-genome analyses reveal over 100 putative phase-variable genes in the pathogenic Neisseria spp. *Microbiology* **147** 2321–2332

Stern A, Brown M, Nickel P and Meyer TF 1986 Opacity genes in Neisseria gonorrhoeae: control of phase and antigenic variation. *Cell* **47** 61–71

Swanson J, Barrera O, Sola J and Boslego J 1988 Expression of outer membrane protein II by gonococci in experimental gonorrhea. *J. Exp. Med.* **168** 2121–2129

Tauseef I, Ali YM and Bayliss CD 2013 Phase variation of PorA, a major outer membrane protein, mediates escape of bactericidal antibodies by *Neisseria meningitidis*. *Infect. Immun.* **81** 1374–1380

Tettelin H, Saunders NJ, Heidelberg J, Jeffries AC, Nelson KE, Eisen JA, Ketchum KA, Hood DW, *et al*. 2000 Complete genome sequence of *Neisseria meningitidis* serogroup B strain MC58. *Science* **287** 1809–1815

van den Dobbelsteen GP, van Dijken HH, Pillai S and van Alphen L 2007 Immunogenicity of a combination vaccine containing pneumococcal conjugates and meningococcal PorA OMVs. *Vaccine* **25** 2491–2496

Virji M, Makepeace K, Ferguson DJ, Achtman M and Moxon ER 1993 Meningococcal Opa and Opc proteins: their role in colonization and invasion of human epithelial and endothelial cells. *Mol. Microbiol.* **10** 499–510

Warmlander S, Sponer JE, Sponer J and Leijon M 2002 The influence of the thymine C5 methyl group on spontaneous base pair breathing in DNA. *J. Biol. Chem.* **277** 28491–28497

Weynants V, Denoel P, Devos N, Janssens D, Feron C, Goraj K, Momin P, Monnom D, *et al*. 2009 Genetically modified L3,7 and L2 lipooligosaccharides from *Neisseria meningitidis* serogroup B confer a broad cross-bactericidal response. *Infect. Immun.* **77** 2084–2093

Weynants VE, Feron CM, Goraj KK, Bos MP, Denoel PA, Verlant VG, Tommassen J, Peak IR, *et al*. 2007 Additive and synergistic bactericidal activity of antibodies directed against minor outer membrane proteins of *Neisseria meningitidis*. *Infect. Immun.* **75** 5434–5442

World Health Organization 1998 *Control of epidemic meningococcal disease. WHO practical guidelines* 2nd edition (Geneva: World Health Organization)

Zollinger WD, Donets MA, Schmiel DH, Pinto VB, Labrie JE 3rd, Moran EE, Brandt BL, Ionin B, *et al*. 2010 Design and evaluation in mice of a broadly protective meningococcal group B native outer membrane vesicle vaccine. *Vaccine* **28** 5057–5067

MS received 14 June 2015; accepted 16 December 2015

Corresponding editor: B JAGADEESHWAR RAO