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Phase Variation Mediates Reductions in Expression of Surface Proteins During Persistent Meningococcal Carriage

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Running title:- Genetic Variation During Meningococcal Carriage

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32 **Abstract**

33 Asymptomatic and persistent colonisation of the upper respiratory tract by *Neisseria*
34 *meningitidis* occurs despite elicitation of adaptive immune responses against surface
35 antigens. A putative mechanism for facilitating host persistence of this bacterial
36 commensal and pathogen is alterations in expression of surface antigens by simple
37 sequence repeat (SSR)-mediated phase variation. We investigated how often phase
38 variation occurs during persistent carriage by analysing the SSRs of eight loci in multiple
39 isolates from 21 carriers representative of 1-6 months carriage. Alterations in repeat
40 number were detected by a GeneScan analysis and occurred at 0.06
41 mutations/gene/month of carriage. The expression states were determined by Western
42 blotting and two genes, *fetA* and *nadA*, exhibited trends towards low expression states. A
43 critical finding from our unique examination of combinatorial expression states,
44 'phasotypes', was for significant reductions in expression of multiple phase-variable
45 surface proteins during persistent carriage of some strains. The immune responses in
46 these carriers were examined by measuring variant-specific PorA IgG antibodies, capsular
47 group Y IgG antibodies and serum bactericidal activity in concomitant serum samples.
48 Persistent carriage was associated with high levels of specific IgG antibodies and serum
49 bactericidal activity whilst recent strain acquisition correlated with a significant induction of
50 antibodies. We conclude that phase variable genes are driven into lower expression states
51 during long-term persistent meningococcal carriage, in part due to continuous exposure to
52 antibody-mediated selection, suggesting localised hypermutation has evolved to facilitate
53 host persistence.

54

55

56 **INTRODUCTION**

57 Heightened mutation or recombination rates in specific regions of bacterial genomes are a
58 feature of many bacterial commensals and pathogens (1-4). The mechanisms responsible
59 for this localised hypermutation are diverse (e.g. site-specific recombination and slippage
60 in repetitive DNA tracts), but generate high frequencies of variation in a stochastic manner
61 usually prior to exposure to a selective pressure (5). These hypermutable loci are termed
62 contingency loci and are speculated to have evolved as a mechanism for adaptation to
63 rapid, unpredictable and undetectable fluctuations in selective pressures. Localised
64 hypermutation is therefore likely to contribute to both host adaptation and the disease
65 phenotypes of bacteria but few studies have examined the frequency and types of events
66 occurring during natural infections by these organisms (6-9).

67 Infections by *Neisseria meningitidis* (Nm) can result in septicaemia and meningitis.
68 Disease is rapid in onset and has high rates of mortality and morbidity with infants
69 exhibiting the highest prevalence of disease. Vaccine-elicited serum bactericidal
70 antibodies (SBA) provide effective protection against Nm infections (10,11). Despite this,
71 Nm normally exists as a commensal on the naso/oropharyngeal surfaces of humans with
72 high frequencies of asymptomatic carriage in teenagers and young adults (12).
73 Meningococci can persist in individual hosts for 6-12 months with carriage eliciting
74 adaptive immune responses against surface determinants (13). The SBA of carriers
75 correlates with protection against disease by homologous strains (14,15) but is also
76 thought to mediate prevention of carriage of homologous strains. Evidence for the latter is
77 based on observations such as sequential carriage and replacement of one strain by
78 another strain with antigenically-mismatched outer membrane proteins (16) and herd
79 protection associated with meningococcal serogroup C (MenC) and serogroup A (MenA)
80 conjugate vaccination (17, 18). This raises a puzzling feature of meningococcal carriage,
81 namely, how does this bacterium persist in the face of an adaptive immune response
82 directed against surface antigens.

83 Microsatellites or simple sequence repeat (SSR) tracts are a major mechanism of
84 localised hypermutation being subject to high rates of insertions and deletions of repeats
85 during DNA replication (3). The reversible nature of SSR mutations has enabled evolution
86 of phase variation (PV), i.e. high frequency, reversible alterations in phenotypic
87 expression, for a diversity of surface molecules. In Nm, 40-60 genes per genome are
88 subject to SSR-mediated PV with these simple sequence contingency loci (SSCL)

89 encoding outer membrane proteins, enzymes involved in modulation of the surface
90 exposed-glycans of lipopolysaccharide and pilin, and restriction-modification systems
91 (9,19). The SSRs are present either within the reading frame, resulting in ON/OFF
92 switches in expression, or the promoter, driving modulations of gene expression. The
93 majority of Nm SSRs are polyC or polyG tracts, but there are also tetra-, penta-, hepta-
94 and longer repeat units. The outer membrane proteins encoded by Nm SSCLs include a
95 porin (PorA), iron-acquisition proteins (HmbR, HpuAB, FetA), adhesins and invasins (Opc,
96 Opa, NadA), and autotransporters (MspA/AusI, NalP/AspA, NadA). These proteins have
97 important primary functions – such as iron acquisition (e.g. HpuA, HmbR) and adhesion
98 (e.g. Opc, NadA, MspA) to host tissues - associated with their expression and hence
99 phase-variants in an ON or high expression state are likely to contribute to survival on
100 nasopharyngeal surfaces (20,21). Host persistence may be further facilitated by the
101 secondary functions of some of these proteins, for example, Opc contributes to
102 complement resistance (22,23). Selection for expression of the phenotypes associated
103 with these SSCLs is, therefore, potentially strong during host persistence. Conversely, an
104 opposing 'counter' selection for phase variants in an OFF or reduced expression state is
105 presumed to occur due to adaptive immune responses. The PorA and Opc proteins elicit
106 bactericidal antibodies during carriage (13,24) and *porA* phase variants, exhibiting reduced
107 expression of the PorA protein, can mediate escape of bactericidal antibodies *in vitro* (25).
108 PV of meningococcal SSCLs could therefore confer a major advantage as Nm persists on
109 mucosal surfaces during carriage, however, it is currently unclear whether phase variable
110 switches actually occur during carriage of Nm in native hosts nor whether there are
111 particular patterns of switching.

112 Characterisation of genetic variation during natural infections provides key
113 indications of how bacterial commensals and pathogens colonise and persist in their hosts.
114 Localised hypermutation is a phenomenon that is presumed to facilitate bacterial
115 adaptation to the fluctuating and opposing selective pressures (e.g. adherence versus
116 immune avoidance) encountered in host environments. In this study we set out to
117 determine how often SSR-mediated PV occurs in meningococci during natural infections
118 and to examine whether changes were driven by adaptive immune responses. Our studies
119 provide the first definitive information on the frequencies of localised hypermutation
120 occurring during long-term persistence of meningococci on the pharyngeal tissues of their
121 native hosts. Critically, our unique combinatorial investigation of multiple phase variable

122 loci indicates that host persistence is associated with a heightened prevalence of
123 'phasotypes' with lower expression states for multiple surface proteins. A simultaneous
124 evaluation of the immune responses in these carriers suggests that selection for low
125 expression 'phasotypes' is driven by continuous exposure to immune selective pressures.

126

127 **MATERIALS AND METHODS**

128 **Bacterial isolate growth and characterisation.** All meningococcal isolates were
129 obtained from a carriage study performed in Nottingham University between November
130 2008 and May 2009 as described previously (16). The study was approved by the
131 Nottingham University Medical School Ethics Committee, and written informed consent
132 was obtained from all volunteers. Up to twenty single colonies were re-streaked from initial
133 selective plates onto Columbia chocolate agar plates (Oxoid). After overnight growth at
134 37°C in 5% CO₂, sweeps of growth were used for preparation of glycerol stocks in BHI
135 broth plus 20% glycerol and genomic DNA by extraction with a DNAeasy Blood and Tissue
136 kit (Qiagen). For expression analyses, strains were initially grown overnight in BHI broth at
137 37°C and then diluted 10-fold in BHI broth followed by growth for 4-6 hours. Iron-repressed
138 genes were induced by addition of desferal at a working concentration of 30 µM to a mid-
139 log phase culture followed by incubation for an additional 1-2 hours.

140 Typing of two isolates per time point was described previously (16). Typing of
141 additional isolates was performed by PCR amplification with relevant capsule specific PCR
142 primers and primers specific for the relevant variable regions of the *porA* and *fetA* genes
143 as described previously (see (16) and Table A1).

144

145 **Enumeration of SSR repeat numbers.** The SSRs of each gene (i.e. *fetA*, *porA*, *opc*,
146 *hpuA*, *hmbR*, *nadA*, *mshA*, and *nalP*) for each strain were amplified and sequenced using
147 published or newly-designed primers spanning the repeat tract by previously described
148 methods (16, 25, 26, 27; Table A1). The number of repeats in SSRs of additional isolates
149 for each strain were determined by a GeneScan protocol as described elsewhere (28, 29).
150 Briefly, SSRs were amplified using two oligonucleotides, one of which was labelled on the
151 5' end with a fluorescent dye (FAM, 6-carboxyfluorescein), and subjected to
152 electrophoresis on an ABI3730 autosequencer in comparison to a GeneScan500LIZ size
153 standard (Applied Biosystems). Product sizes were calculated using PeakScanner v1.0

154 (Applied Biosystems) and repeat numbers determined by comparison to controls of known
155 size and repeat number from the same strain and hence presumed to have identical
156 flanking sequences. A sub-set of tracts were analysed by sequencing to confirm repeat
157 numbers (see Tables A2 and A3). Mononucleotide repeat tracts of ≥ 9 units produced two
158 or more labelled products due to slippage during PCR amplification. The ratio of the areas
159 of the primary and secondary peaks was determined and if the ratio was above 1.2 the
160 primary peak was utilised for determining repeat number otherwise the peak with the
161 largest size was selected.

162

163 **Comparative measurements of gene/protein expression levels.** A semi-quantitative
164 measure of protein expression levels for different phase variants of each strain was
165 performed by probing Western blots of meningococcal whole cell lysates with specific
166 antibodies (Table 1A). Meningococcal isolates were grown to mid-log phase either in the
167 presence or absence of an iron chelator in order to induce genes repressed by high iron
168 levels (i.e. *fetA* and *hpuA*). Cells were washed twice before being resuspended in
169 phosphate buffered saline (PBS). Cell numbers were adjusted to a constant OD550 and
170 then mixed with 2X SDS loading buffer at a 1:1 ratio. Cell lysates were electrophoresed on
171 8% polyacrylamide gels and transferred to PVDF membranes by application of fixed
172 current for 1 hour. Membranes were blocked overnight at 4°C in PBST-Milk (PBS/0.5%
173 Tween20 plus 5% skimmed milk) and subsequently probed with an appropriate primary
174 antibody diluted in PBST-Milk for 2 hours. Membranes were then washed three times with
175 PBST (PBS/0.5% Tween20) and then probed with either a 1:2000 dilution of an anti-
176 mouse or anti-rabbit IgG horseradish peroxidase conjugate for 1 hour. Bound antibodies
177 were detected with an ECL detection kit and X-ray film. Quantification of bands was
178 performed by scanning of blots and quantification using ImageJ.

179 Surface expression was analysed by flow cytometry as described previously (25).
180 Briefly, meningococcal cells were harvested from mid-log phase cultures, washed and
181 resuspended in assay buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM CaCl₂, 0.05%
182 Tween-20) containing primary antibody. Samples were incubated for 1 hr at room
183 temperature, washed thrice and incubated for 1 hour with a 1:100 dilution of an anti-mouse
184 IgG-fluorescein isocyanate conjugate (Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L), Life
185 Technologies™). After three washes, cells were resuspended in phosphate-buffered saline

186 containing 0.05% formalin. Samples were then analysed on a fluorescence-activated cell
187 sorter and mean fluorescent intensities were determined for each sample.

188 Expression of the *porA* gene was assessed by RT-qPCR. Meningococcal strains
189 were grown to mid-log phase in Mueller-Hinton broth before fixing in Bacteria Protect
190 Reagent (Qiagen). Total RNA was then extracted using an RNeasy Mini Kit with an
191 additional RNase-free DNase step (Qiagen) to remove DNA contamination. Custom
192 primers and probes used for gene expression assays are listed in Table A1. Reverse-
193 transcription PCR assays (RT-PCR) were completed in MicroAmp® Fast Optical 96-Well
194 Reaction Plates using TaqMan® RNA-to-CT™ 1-Step Kit (Applied Biosystems). Reactions
195 were set up in accordance with the Applied Biosystems protocol and analysed on Applied
196 Biosystems 7500. The house-keeping gene, glucose-6-phosphate 1-dehydrogenase (*gdh*)
197 was used as endogenous control gene and H44/76 total RNA as a standard positive
198 control. Fluorescence was recorded at the end of each extension step according to the
199 probes present in the reaction, at wavelengths determined by the Applied Biosystems
200 7500 Fast System Sequence Detection Software. Relative Quantity (RQ) values were
201 calculated by the using the $2^{-\Delta\Delta CT}$ method. The *gdh* reaction was used as an endogenous
202 control and all samples were calibrated to the positive control sample.

203

204 **Quantification of anti-PorA and anti-CapY antibodies in serum samples.** An
205 immunofluorescence bead-based immunoassay was utilised to quantify variant-specific
206 PorA antibodies in serum samples. Eight PorA variants (P1.5-1,2-2, P1.19,15, P1.19-1,15-
207 11, P1.5,2, P1.5-2,10, P1.7,16, P1.7-2,4, and P1.21,16) were cloned into an expression
208 vector enabling production of His-tagged recombinant PorA proteins. A ninth protein
209 (loopless, P1.-/-) was also produced from which the VR1 and VR2 regions had been
210 deleted. Proteins were produced as inclusion bodies, purified on a His-tag column in the
211 presence of urea, re-folded by droplet dilution and finally dialysed into an appropriate
212 buffer. Polystyrene microspheres labelled with fluorophores (Liquichip Carboxy beads,
213 BioRad) were coated with nickel-NTA (nitrilotriacetic acid). Each recombinant His-tagged
214 PorA variant was bound to a spectrally distinct microsphere.

215 PorA-coupled fluorescent beads were mixed with four dilutions of each serum
216 sample in Liquichip assay buffer (PBS/0.1% bovine serum albumin/0.05% Triton X-100) in
217 a 96-well filter plate and incubated for 30 minutes at room temperature on a rocking
218 platform. Samples were washed three times and then incubated with a 1:25 dilution of a
219 goat anti-human IgG R-phycoerythrin conjugate (ABD Serotec, Kidlington, UK) in Liquichip

220 assay buffer. Finally, samples were washed three times and analysed in a LiquiChip200
221 Workstation. Raw fluorescence data were converted into arbitrary units (AU) by
222 comparison to a standard curve generated using serial dilutions of pooled sera from
223 vaccinees who had been inoculated with either a MenBvac or MenNZB outer membrane
224 vesicle vaccine (derived from two clinical studies MNB1 and MNB2, respectively).
225 Separate standard curves were generated for each PorA variant hence comparisons
226 between AU for each variant are not possible. The quality of each data set was assessed
227 by statistical evaluation of the parallelism of the lines obtained for the standard curves
228 using CombiStats version 5 (European Directorate for the Quality of Medicines and
229 Healthcare, Council of Europe). The AU values for each variant were adjusted for non-
230 specific binding by subtraction of the AU value obtained for the loopless PorA variant, a
231 value of 0.05 was arbitrarily assigned when binding to this control protein exceeded that to
232 a particular PorA variant.

233 The amount of anti-CapY IgG antibodies in each serum samples were measured by
234 ELISA using purified serogroup Y capsular polysaccharide as a ligand as described in
235 Gheesling *et al.* (30).

236

237 **Serum bactericidal antibody assays.** Serum bactericidal antibody assays were
238 performed by standard techniques (31) in the Vaccine Evaluation Unit using a ST11
239 meningococcal strain expressing the serogroup Y antigen (M03-241125, CDC S1975,
240 Y:P1.5,2:ST11, serotype 2a, fHBP-Oxford 2.2, NadA 2.49, NHBA 29).

241

242 **Statistical analyses.** Each carrier was examined for significant changes in repeat tract
243 length between a pair of time points for every gene as follows:- repeat numbers were
244 determined for six or more colonies for each time point (apart from four time points where
245 less than six colonies were obtained); repeat numbers were binned into two non-
246 overlapping categories for the two time points; the repeat number of each isolate was
247 assigned to one of the two categories and placed into a contingency table; a difference
248 was then deemed significant if a comparison yielded $P < 0.05$ in a Fisher's exact test. Two-
249 tailed Fisher's exact tests were performed using GraphPad (graphpad.com/quickcalcs) or
250 Prism. Chi-squared tests were performed using www.physics.csbsju.edu/stats/. Geometric

251 mean concentrations were calculated using Prism. Wilcoxon rank signed tests were
252 performed in R using the COINS package.

253

254

255 RESULTS

256 Frequent changes occur in the SSRs of eight genes encoding outer membrane 257 proteins during persistent meningococcal carriage in the upper respiratory tracts of

258 humans. In order to examine the extent of localised hypermutation occurring during

259 persistent meningococcal carriage, bacterial samples were obtained from the

260 nasopharyngeal tissues of individuals colonised with the same meningococcal strain for up

261 to six months. These samples were from a longitudinal study of meningococcal carriage

262 performed with a cohort of 190 first year students attending six catered halls at the

263 University of Nottingham (16). This study was initiated in November 2008, five weeks after

264 the start of term, and involved four time points (1st, November; 2nd, December; 3rd,

265 February; 4th, May). We have previously described the identification of persistent carriers

266 and characterisation of the colonising strains (16). A sub-set of 21 persistent carriers were

267 selected for further analysis. These carriers were colonised with one of six strains:-

268 Y:P1.21,16:F3-7:ST1466(cc174, 7 carriers); Y:P1.21,16:F3-7:ST8510(cc174, 1 carrier);

269 E:P1.5,2:F1-7:ST1383(cc60, 5 carriers); Y:P1.5-1,10-1:F1-3:ST767(cc167, 3 carriers);

270 Y:P1.5-1,10-1:F4-1:ST1655(cc23, 4 carriers); B:P1.19,15:F5-1:ST5682(cc32, 1 carrier).

271 Ten of these carriers were persistently colonised for 5-6 months with the same strain,

272 whilst six exhibited either clearance or replacement of the initial strain by the 4th time point

273 and the remainder were only examined at early time points. As described previously,

274 oropharyngeal swabs were spread onto selective agar plates and then streaked to single

275 colonies prior to overnight growth. Up to 20 single isolates were obtained by re-streaking

276 single colonies onto non-selective plates for preparation of glycerol stocks and DNA. Thus

277 all isolates were subject to minimal *in vitro* passage to reduce the potential for alterations

278 in PV genotype. Where possible, six or more isolates were analysed as a statistically-

279 representative sample. The similarity of each set of isolates was confirmed by PCR with

280 primers specific for the relevant capsule, PorA and FetA type (data not shown).

281 A coherent group of eight genes (*porA*, *fetA*, *opc*, *hpuA*, *hmbR*, *nadA*, *mspA* and

282 *nalP*) encoding phase variable outer membrane proteins with known functional attributes

283 were chosen for analysis. Most of the other phase-variable meningococcal genes encode

284 glycosyltransferases, restriction-modification systems or proteins of unknown function (9,
 285 19). The repeat tract of each gene for each strain was amplified and sequenced. Two
 286 genes were not universally present with *hmbR* only in the cc60 and cc32 strains and *nadA*
 287 only in the cc174 and cc32 strains (data not shown). The SSR was located in the reading
 288 frame for four genes and exhibited a consistent association between the number of
 289 repeats and whether the reading frame was 'in-frame' or out-of-frame' and hence predicted
 290 to have an ON or OFF expression state, respectively. The predicted ON expression states
 291 for each gene were as follows:- 10G/13G, *hpuA*; 9G, *hmbR*; 10C/13C, *nalP*; and 6C/9C,
 292 *mspA*. For the other genes, the SSRs were either located upstream of (i.e. the 5'TAAA
 293 tract of *nadA*) or within the core promoter (polyC tracts in *fetA* and *opc* and a polyG tract in
 294 *porA*; see also Fig. 1A). For these genes, expression state could not be directly predicted
 295 from the sequence data. Flanking sequences and repeat tracts were conserved between
 296 strains (data not shown) with the exception of the cc60 strains, which contained an
 297 interrupted SSR in *fetA* and a 1 nt deletion in the putative -10 of *porA* (Fig. A1). A
 298 GeneScan assay was utilised to determine the repeat numbers for each gene in multiple
 299 isolates per time point (Tables A2 and A3). Low levels of variation in tract lengths were
 300 observed within time points (see Fig. 1 and Fig. A2 for examples). Shifts in tract length
 301 between time points were observed for at least one gene in every carrier except V185,
 302 with specific trends being evident for some genes. For example, the *fetA* SSR in cc174
 303 strains shifted from 9C to 8C or a mix of 10C and 11C variants in 5/8 carriers whilst the
 304 *nadA* SSR remained at or shifted to 9 or 12 5'TAAA in all 8 of the cc174 carriers.

305 Comparisons between genes indicated very low levels of alterations in the repeat
 306 tracts of *hmbR* and *mspA* whilst all the other genes experienced alterations in 30-40% of
 307 carriers (Fig. 2A). Both *fetA* and *nalP* exhibited significantly different frequencies of
 308 switching between the later as opposed to early time points ($P < 0.05$ for comparison of 1st-
 309 to-2nd versus 1st-to-4th switching events using a two-tailed Fisher's exact test), indicative of
 310 a correlation between length of carriage and propensity for PV. A correlation between
 311 mononucleotide repeat tract length and propensity to PV was also observed with no PV in
 312 short tracts (6 or 7 G/C repeats), very low levels in 8G/8C tracts, similar levels in tracts of 9
 313 to 11 repeats and a trend towards increasingly higher levels in longer tracts (Fig. 2B).

314 A switching rate per month of carriage was determined for a combination of the
315 eight phase variable genes with mononucleotide tracts of 9 or more repeats in the 1st/2nd
316 time point. A significant switch in repeat tract between the 1st/2nd and 4th time points for a
317 particular gene was calculated as described in the Materials and Methods. The number of
318 significant switches was divided by the total number of events analysed (i.e. 21/54) and by
319 the number of months of carriage (i.e. 5 or 6) resulting in an estimate of 0.06
320 mutations/gene/month of carriage. We conclude that there is a relatively high level of
321 alterations in the mononucleotide repeat tracts of Nm SSCL during carriage and that gene
322 type, repeat number and length of host persistence are important determinants of
323 mutability.

324

325 **Persistent meningococcal carriage is associated with accumulation of low**
326 **expression states for some phase variable genes.** Alterations in the SSRs provide an
327 indication of mutability but do not test whether there has been a change in phenotype.
328 Detection of any phenotypic variation associated with these SSCL required analysis of
329 whether alterations in SSRs mediated gene expression changes. Translational SSR-
330 mediated switching has a predictable correlation between tract length and expression
331 state. This was confirmed by analysis of SSRs predicted as 'in-frame' (i.e. ON) or 'out-of-
332 frame' (i.e. OFF) for all four genes (see Fig. 3 and Figs. A3-A7 for *hpuA*; Oldfield *et al.* (27)
333 for *mvpA* and *nalP*; Tauseef *et al.* (26), for *hmbR*). Transcriptional SSR-mediated
334 switching causes changes in gene expression through modulation of promoter activity and
335 hence is not readily predicted from repeat tract length. A series of Western blots were
336 performed for each strain generating associations between tract length and expression
337 state for *porA*, *fetA*, *nadA* and *opc* (Fig. 3; Fig. A3-A8; Tables A4-A6). All of the genes
338 apart from *porA* exhibited at least three clear expression states ranging from low (>5-fold
339 reduction) to intermediate to high expression (Table A5). For *porA*, high/intermediate
340 levels of expression were detected for multiple tracts whilst the lowest expression state
341 was only ~5-fold below the highest, larger differences but similar trends were detected in a
342 quantitative RT-PCR analysis of a sub-set of strains (Table A7). To correlate surface
343 expression with repeat number, six isolates were investigated by FACs. Variants of a cc32
344 strain with tracts of 10C or 9C exhibited 9-to-18-fold higher surface expression of FetA
345 than a variant with an 8C tract whilst similar levels of high expression of *porA* were
346 detected in variants of a cc174 with tracts of 11G, 12G or 13G (Fig. A8).

347 Expression state data were utilised to classify each SSR/gene combination into an
 348 expression code. Translational-SSR were coded into 0 and 2 for OFF and ON respectively
 349 and transcriptional-SSR into 0, 1 and 2 representing undetectable/low, intermediate and
 350 high expression. Low and intermediate expression were defined as a >5-fold and 1.5-to-5-
 351 fold, respectively, reduction relative to high expression as detected by Western blotting for
 352 *fetA*, *opc* and *nadA* or a combination of Western blotting and RT-PCR for *porA*. A
 353 combined overview of all carriers for all time points detected genic differences in the
 354 proportions of carriage isolates with the highest expression states:- PorA (91%); HpuA
 355 (66%); NalP (65%); FetA (42%); HmbR (24%); MspA (22%); Opc (18%); and NadA (7%)
 356 (Table A6). The largest temporal shift was observed for FetA, which started with 69% of
 357 isolates in the high state and ended with only 11% in this state by the 4th time point (i.e.
 358 after 5-6 months carriage). A significant reduction in FetA expression (P=0.008; Wilcoxon
 359 signed rank test with continuity correction) was observed for a comparison of the mean
 360 initial and final expression states observed across all carriers (Fig. A9). Non-significant
 361 trends were noted for the other genes with NadA and NalP also exhibiting reductions in
 362 expression state (Fig. A9). Expression of NadA was in the lowest state in all of the final
 363 time point samples with the absence of a trend towards reductions in expression being due
 364 to this gene being in the lowest expression state in many of the initial samples.

365

366 **Combinatorial reductions in phase variable gene expression occur during persistent**
 367 **meningococcal carriage.** A major advantage of our analysis of multiple phase variable
 368 genes is an ability to examine the combined expression states within an isolate. A
 369 combined code was generated for every isolate using the expression data for the 6 or 7
 370 phase variable genes present and analysed in each clonal group. These codes contain
 371 both genotypic and phenotypic information and hence are referred to as 'phasotypes'.
 372 Seven genes - three with two states and four with three states - could generate 648
 373 phasotypes. A total of 25 phasotypes was observed for the combined data for the 140
 374 isolates of cc174, indicating a limited exploration of the potential expression states (Table
 375 A8).

376 Each phasotype contains information on the combined expression states of the
 377 genes, which is a simple sum of the individual gene states (i.e. 2-2-2-2-2-2 has score 14,
 378 2-2-2-2-2-0 is 12, etc). The phasotypes were grouped by 'expression score' and the

379 patterns of change between time points were examined for each carrier (Fig. 4A-C). The
380 mean phasotype score was also calculated for each time point of every carrier (data not
381 shown) and the change in this score plotted against months of carriage (Fig. 4D). The
382 cc167 and cc23 strains were combined as they have identical PorA sub-types (P1.5-1,10-
383 1) and gene combinations (i.e. absence of *nadA* and *hmbR*). Only one carrier exhibited no
384 change in expression score (V185; persistent carrier of a serogroup E, cc60 strain) whilst
385 varying patterns of changes were observed in other carriers. A reduction in phasotype
386 score was observed in 11 of the 21 carriers between the initial and final time point of
387 observed carriage but was variable between strains with a shift towards lower expression
388 phasotypes in 6/8, 4/7, 0/5 and 1/1 of the cc174, cc167/cc23, cc60 and cc32 strains,
389 respectively. A significant change towards a lower mean phasotype score was detected for
390 the cc174 carriers ($P = 0.03$ in a Wilcoxon rank test with continuity correction) but not for
391 the cc167/cc23 or cc60 strains. The absence of a shift towards lower expression scores in
392 the cc60 strains could be due to only one of these carriers exhibiting more than three
393 months observed carriage and three of the genes having short repeat tracts (*fetA*, 6G;
394 *nalP*, 7C; *mspA*, 8C). Overall, persistent carriage is associated with a shift towards lower
395 combined phasotype scores but exhibits a strain bias.

396 The effect of persistence length on the reduction in phasotype score was examined
397 for all 21 carriers. A trend was observed for reductions in phasotype score as a function of
398 persistent carriage such that 44% (8/18), 50% (8/16) and 70% (7/10) of carriers exhibited
399 reduced scores relative to the initial time point after 1, 2-3 or 5-6 months carriage. Analysis
400 of the phasotype scores for isolates provided further evidence of the effect of prolonged
401 carriage (Table 1). Thus for the cc174 isolates, there was a significant shift from 85% with
402 a score of ≥ 7 in the initial time point to 100% with ≤ 6 after 5-6 months carriage ($P > 0.001$ in
403 a Chi-squared test). Similarly, there was a reduction in expression score from 65% with ≥ 7
404 to 62% with ≤ 6 for the cc167/cc23 isolates ($P = 0.002$). Conversely, the cc60 strains
405 exhibited a significant trend towards rising phasotype scores ($P = 0.002$) however, as
406 discussed above, there was a lack of data for more than three months carriage with this
407 clonal complex. Thus, prolonged carriage is associated with reductions in phasotype score
408 and the accumulation of phasotypes with lower expression states.
409

410 **Variant-specific PorA antibodies can be detected using a multiplex microserology**
411 **assay in sera from persistent meningococcal carriers.** In order to understand why
412 switches in the phase-variable genes are occurring it is important to characterise the
413 selective pressures acting on the products of these genes. Adaptive immune responses
414 exert a strong selective pressure on the surface antigens of meningococci with a
415 significant potential impact on PV expression status. The phase variable PorA protein is a
416 major component of the outer membrane and contains two variable regions, VR1 and
417 VR2, which are the main targets for bactericidal meningococcal antibodies and are also
418 utilised for strain typing with VR specific mAbs (32). We tested for the presence of VR
419 region specific PorA antibodies in serum samples collected concomitantly with
420 nasopharyngeal swabs from the 21 persistent carriers. These sera were complemented
421 with additional sera from the same carriage study (16), this included a set of sera from
422 persistent non-carriers as controls and sera from volunteers exhibiting acquisition of
423 carriage as a test of whether gain-of-carriage was associated with induction of
424 meningococcal specific antibodies.

425 The levels of PorA-specific IgG antibodies were analysed using a multiplex
426 fluorescent-microsphere protocol and a Lighchip workstation (Qiagen). A single
427 combination of seven PorA variants was utilised for the majority of the assays, which
428 included PorA variants with VRs similar/identical to those present in the carriage isolates
429 and a modified PorA, lacking VR1 and VR2, used as a control for background reactivity
430 (Patel, Chan and Feavers, unpublished data). Antibody levels were measured in arbitrary
431 units (AU) relative to pooled meningococcal vaccinee sera and hence quantitative
432 comparisons of reactivity between variants was not possible.

433 The specificity and reproducibility of the multiplex PorA assay was previously
434 validated utilising variant-specific PorA monoclonal antibodies but has not been
435 extensively tested with sera from carriers of known PorA variant type (Patel, Chan,
436 Findlow, Borrow, Trotter and Feavers, unpublished data). We, therefore, analysed the
437 results for evidence of specificity and utility in detection of variant specific antibodies
438 (Table A9, Table A10 and data not shown). Importantly, general reactivity to all PorA
439 variants was noted as being very low, and probably due to non-specific background, in
440 persistent non-carriers (Table 2; Fig. 5). High levels of monospecific activity were detected
441 in at least one of the time points for five carriers (V51, V54, V64, V69 and V185) against
442 the PorA variant protein containing one or both VRs matching the carriage isolate. Sera
443 from other carriers exhibited a high level of reactivity mainly to the homologous PorA

444 variant whilst three carriers (V59, V88 and V124) had a pan-response against all variants
445 and three (V93, V138 and V176) had non-carrier levels of reactivity. Thus some carriers
446 exhibit monospecific responses whilst others elicit varying degrees of cross-reactivity,
447 which could be due to cross-reactive epitopes, recent carriage of other strains or a
448 'bystander' induction of responses to all previously-encountered PorA variants. The
449 specificity of these responses was confirmed for a selection of samples by using VR
450 specific mAbs to block binding of antibodies to one of the VRs (Table A10). Both VR1 and
451 VR2 specific mAb antibodies reduced binding of serum antibodies but VR2 antibodies
452 were generally more effective at blocking reactivity. Overall, the multiplex assay exhibited
453 utility for detection of variant-specific IgG PorA antibodies in sera from meningococcal
454 carriers.

455

456 **Persistent meningococcal carriage is associated high levels of adaptive immune**
457 **responses against surface determinants.** The above studies indicated that variant-
458 specific PorA antibodies could be readily detected in the sera of these carriers. However,
459 as variability in the antigenic regions of PorA prevented direct comparisons between
460 strains, antibody responses against the serogroup Y (MenY) capsular antigen were also
461 investigated to facilitate comparisons between volunteers and due to the high prevalence
462 of MenY strains among our persistent carrier samples. Immune responses to the
463 serogroup Y capsular antigen were assessed by a CapY-IgG specific ELISA (i.e. with
464 purified capsular polysaccharide). The sera were also tested for bactericidal antibodies
465 using an ST-11 strain expressing a serogroup Y capsule and a P1.5,2 PorA variant. As the
466 PorA protein was mismatched in either both VRs (cc174) or VR2 (cc167/cc23), this assay
467 will mainly detect bactericidal IgM and IgG anti-CapY antibodies. The expression states
468 and/or sequence variation, as compared to test strains, in the other phase-variable genes
469 were however unknown and may make a minor contribution to bactericidal titre.

470 Sera from carriers subject to different types of carriage (acquisition, clearance and
471 clonal replacement, and persistent) were analysed to determine the levels and temporal
472 behaviour of antibody responses (Table A9; Fig. 5). As observed for PorA, most of the
473 non-carriers had low levels of CapY IgG antibodies and low SBA titres apart from three
474 who had with either high CapY IgG (V135 and V150) or high SBA (V97) titres, which could
475 be due to carriage of homologous meningococcal strains prior to enrolment in the study or
476 vaccination with a CapY-conjugate vaccine. Acquisition of carriage was associated with a

477 significant rise in variant-specific PorA antibodies and SBA titre and a non-significant
478 increase in anti-CapY IgG. Antibody levels and SBA titres increased as a function of time
479 post-colonisation. Replacement or clearance of the initial strain was associated with high
480 PorA and CapY IgG antibody concentrations and high SBA titres similar to persistent
481 carriers with no significant overall decrease following loss of carriage, suggesting
482 persistence of antibodies in the absence of on-going stimulation. Significantly higher levels
483 of reactivity were detected in sera from persistent carriers as compared to non-carriers to
484 the homologous PorA type and CapY at all time points (Fig. 5A and 5B). Similarly, SBA
485 titres were higher in persistent carriers than non-carriers although a small but significant
486 drop in SBA titre was found in the fourth time point possibly due to waning of a specific
487 IgM response (Fig. 5C). A major increase between the November (i.e. first) and later time
488 points in both anti-PorA and anti-CapY antibodies was observed in two carriers, V88 and
489 V43, whilst two others exhibited increases in PorA IgG (V64) or CapY IgG (V51). These
490 rises were indicative of recent colonisation and commensurate with the timing of sample
491 collection, which was performed near to the start of term when high levels of spread of
492 meningococcal strains is anticipated among this typical group of university students.

493 Relative amounts of antibodies were assessed for the 21 carriers subject to PV
494 analysis in groups comparable to those utilised for the phasotype scores (Table 2). The
495 cc174 and cc167/cc23 strains exhibited higher concentrations of CapY IgG and SBA titres
496 than non-carriers at all time points ($P < 0.05$ with an unpaired T test) apart from a non-
497 significant difference in CapY IgG at the 1st time point in cc174 carriers probably due to
498 recent colonisation in three of these carriers (V43, V51 and V88) and hence insufficient
499 time for elicitation of antibodies. The levels of variant-specific PorA antibodies were
500 significantly higher in the cc167/23 and cc60 carriers than non-carriers ($P < 0.05$). Six of the
501 cc174 carriers had 10-fold higher levels of P1.21,16 specific PorA antibodies levels than
502 non carriers at two or more time points but specific antibodies weren't detectable in two
503 (V59 and V138) carriers.

504 In nine of the eleven carriers exhibiting a drop in average phasotype score, high
505 variant-specific PorA antibodies, CapY responses and SBA titres were detected at multiple
506 time points during persistence of the meningococcal clone. Of the others, one (V88)
507 exhibited a late rise in PorA levels at the third time point, correlating with a major reduction
508 in phasotype score, whilst in V54 the CapY IgG and SBA response were weak but the
509 PorA specific responses were very high. The exception was for carrier V176 wherein no

510 PorA antibodies could be detected. Collectively, we detected high levels of surface-antigen
 511 specific and bactericidal antibodies in the majority of persistent carriers in which phase
 512 variable reductions in OMP expression were observed.

513

514 **DISCUSSION**

515 Contingency loci are present in many pathogenic and commensal bacteria and are thought
 516 to generate high levels of genetic variation enabling adaptation to fluctuations in the
 517 stringent selective pressures exerted by the host milieu. A significant gap in our
 518 understanding of contingency loci is the extent of their contributions to natural infections
 519 whether asymptomatic or disease-associated. This study has determined the frequencies
 520 and patterns of SSR-mediated PV occurring during asymptomatic, persistent carriage of
 521 Nm, a pathogen/commensal with multiple contingency loci, in the upper respiratory tract of
 522 humans - their only hosts - and detected trends towards lower expression states for
 523 specific and combinations of phase variable surface proteins (herein termed 'phasotypes').
 524 Our report highlights the importance of examining bacterial isolates from natural
 525 environments and implies a role for PV in facilitating persistent carriage of a bacterial
 526 pathogen.

527 By measuring variation in eight contingency loci, we estimate that the SSR-
 528 mediated PV rate for polyG/C tracts of 9 or more repeats is 0.06 mutations/gene/month of
 529 carriage for Nm in the upper respiratory tract of humans. In contrast, genetic variation in
 530 the VRs of PorA was only detected in one of these 21 carriers and no variation was
 531 detected in the FetA VRs (16 and data not shown) indicating an antigenic variation rate of
 532 0.006/gene/month of carriage (1 mutation in 172 months of carriage). Thus changes in the
 533 structure of an antigen by point mutation or recombination following lateral gene transfer
 534 are infrequent as compared to phase-variable alterations in expression mediated by
 535 mutations in SSR tracts. There are 30-40 phase variable genes in each Nm strain most of
 536 which modify OMPs or the structures of surface determinants. Thus localised
 537 hypermutation is the major source of genetic variability occurring during asymptomatic
 538 carriage of a meningococcal clone within an individual person.

539 Temporal fluctuations in the selective pressures acting on the different expression
 540 states of phase-variable loci is likely to be the major force driving any temporal patterns in
 541 alterations to the SSR tracts. Thus selection for the ON state may be balanced and
 542 exceeded by selection for the OFF state during long-term host persistence of a
 543 meningococcal strain resulting in an observation of ON-to-OFF switching. A key finding

544 from our phenotypic analysis of SSCL was for switches to lower expression states of
545 specific OMPs – namely NadA, FetA and NalP - during persistent carriage. The other
546 phase variable OMPs showed either variability in their expression states or, in the case of
547 PorA, a continuous high expression state. It should be noted that all our results pertain to
548 events on the mucosal surface as sampling of carriers was by swabbing of epithelial
549 surfaces. One implication of the observed accumulation of OFF variants as a function of
550 persistent carriage is that once colonisation has been established within an individual,
551 selection for high/ON expression states of phase variable OMPs is reduced and selection
552 for OFF expression states can drive phase variable loci into minimal expression states.
553 The *nadA* gene was found in a low expression state in all isolates except a few from early
554 time points (Fig. 1A; Table A2). As this gene encodes an adhesin (33,34), our results
555 suggest that NadA is only required for initial colonisation and is rapidly subject to selection
556 for loss of expression as Nm persists in a host. Similar requirements during colonisation
557 may be ascribed to Opc and MspA, which encode known and putative adhesins (35,36)
558 and were also found mainly in low expression states (52% for Opc; 78% for MspA; Table
559 A6). The FetA OMP showed a significant trend towards lower expression states as a
560 function of persistence (Fig. A10) and a high prevalence of ON variants during early time
561 points (Table A6). The prevalence of high-expressing variants of FetA was partially due to
562 clonal expansion of the cc174 strains in one hall of residence (16). However the
563 siderophore-binding iron-uptake attributes of FetA (37) may mean that siderophores are a
564 potent source of iron during initial colonization by Nm but are replaced by other sources
565 (transferrin and haemoglobin) as bacterial numbers increase and perturb the normal
566 mucosal surface. Finally, NalP showed a trend towards an OFF expression state possibly
567 connected with a growing requirement for establishment of a biofilm, which NalP
568 antagonizes by cleavage of other surface meningococcal proteins (38,39).

569 The above discussion indicates how selection for the functions of phase variable
570 OMPs could result in an elevated prevalence of high/ON expression at specific times
571 during persistent carriage, but, apart from NalP, has not elaborated on how selection for
572 low/OFF expression states is exerted. A novel approach pioneered for *C. jejuni* isolates
573 (40) and utilised in this study was to examine isolates for alterations in the combined
574 expression states of multiple phase-variable loci. The term 'phasotypes' has been adopted
575 herein (see also 41) to convey the idea that these types are based on conversion of non-
576 arbitrary genotypic information (i.e. SSR repeat number) into a potential phenotypic state.
577 Phasotype does not indicate an actual *in vivo* phenotypic state as expression of some of

578 these phase-variable genes may be controlled by external signals (i.e. *fetA* is iron-
579 regulated). Nevertheless changes in the 'phasotype' are indicative of how the bacterial
580 cells are responding to selective forces. We note that the 'phasotype' system is portable
581 and could be utilised by other laboratories for comparisons of the expression states of
582 phase-variable genes.

583 A novel finding from our analysis of phasotypes is for an overall decrease in surface
584 expression of multiple OMPs during persistent mucosal carriage. This observation implies
585 that selection is acting on the combination of genes present on the Nm surface. One
586 explanation for this finding is that meningococcal cells expressing lower amounts of OMPs
587 have a growth advantage and replace high OMP expressers. This selective pressure is,
588 however, probably very weak and easily counteracted by any selection for expression of a
589 gene. A second explanation is that adaptive immune responses against surface OMPs
590 select for antigenic variation and reduced expression states.

591 Multiple studies have shown that Nm carriage elicits serum IgG and bactericidal
592 antibodies against a range of OMPs, including PorA, Opc, PilE and NadA, and other
593 surface molecules such as the capsule and lipopolysaccharide (13,42). Mucosal IgA
594 responses to whole Nm cells and to PorA have also been detected (43,44). We
595 demonstrated the presence of high levels of IgG antibodies specific for the homologous
596 PorA variant in the majority of persistent carriers and high levels of anti-CapY specific IgG
597 antibodies (Table 2; Fig. 5). Furthermore we show that these high levels persist through
598 six months continuous carriage, that specific antibodies are rapidly elicited upon
599 acquisition of carriage and that there is also a strong serum bactericidal activity response.
600 Whilst we have yet to confirm whether antibody responses are elicited against all nine
601 OMPs, our results suggest that a robust anti-OMP response is generated as observed in
602 other Nm carriers. As selection of low-expression PorA phase variants has been observed
603 *in vitro* (25), the absence of an effect of the specific antibodies on PorA expression levels
604 is a contraindication for a role of the adaptive immune response. However, adaptive
605 immune responses may exert only a weak effect that could be counteracted by a stronger
606 selection for expression of this OMP. Variability in the strength of selection for the high/ON
607 states of other OMPs may permit weak immune selection to drive these proteins into lower
608 expression states. Variability in the patterns of switching between OMPs may also
609 emanate from another source. Reduction in the combination of surface expressed OMPs
610 implies an effect on the complexity of the bacterial surface rather than the amount of each
611 protein. This could be due to the observed synergistic effects of bactericidal antibodies

612 against multiple minor OMPs (45) or cross-linking and neutralization of bacterial cells by a
613 polyclonal secretory IgA response (46), resulting in variability in which OMP is down-
614 regulated between isolates and carriers. Overall, our findings are supportive of the
615 hypothesis that prolonged exposure to antibody-mediated selection drives Nm cells
616 towards reduced expression states for phase variable OMPs during persistent carriage in
617 their natural hosts. Thus PV may facilitate host persistence by mediating escape of
618 adaptive immune responses whilst simultaneously rendering the resident Nm strain more
619 sensitive to clearance by innate immune effectors or to competition and replacement by an
620 antigenically-divergent Nm strain.

621 The reductions in expression of OMPs have potential implications for
622 meningococcal protein vaccines. Two of these proteins, NadA and PorA, are present
623 within the recently-licensed Bexsero® vaccine whilst NaIP modifies NHBA another
624 component of this vaccine (47,48). The FetA protein has also been proposed as a
625 potential vaccine candidate (49). The accumulation of low expression variants of NadA
626 and FetA indicates that vaccines including these antigens would not prevent persistent
627 carriage but would be most likely to act during the initial stages of host colonization.
628 Testing for herd immunity by meningococcal vaccines will therefore require a focus on
629 prevention of acquisition of carriage in naïve individuals and a careful design of vaccine
630 trials to monitor the relative times of strain acquisition and vaccine-induced antibody
631 responses.

632 This study has defined the frequency and patterns of alterations in SSCL encoding
633 eight OMPs during persistent Nm carriage. Our results indicate persistent carriage of Nm
634 populations is associated with reductions in expression of single and combinations of
635 SSCLs with evidence for adaptive immune responses being one of the major selective
636 pressures driving the population into this state. Comparisons of the phasotypes of these
637 carriage isolates with disease isolates of similar strain types can now be performed to
638 determine whether particular phasotypes are required for meningococci to cause disease
639 and if the lack of disease associated with long-term carriage of meningococci is due simply
640 to the specific immune responses or is also prevented by the accumulation of non-invasive
641 phasotypes.

642

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651

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829

830 Figure 1. Changes in repeat tract length of five genes during persistent carriage of cc174
 831 strains. Multiple meningococcal isolates of the same strain were collected from eight
 832 volunteers (labelled as V in the figure) persistently colonised with a cc174 serogroup Y
 833 strain either ST1466 (V51, V52, V58, V59, V88, V138) or ST8510 (V54; V43 exhibited
 834 replacement of ST8510 with ST1466 between the first and second time points). Up to six
 835 isolates per time point were analysed for up to four time points (1st-4th which were
 836 separated by 1, 2, or 3 months respectively) for the number of simple sequence repeats in
 837 five phase variable genes as follows:- panel (A), *fetA* (polyG tract, open circles), *nadA*
 838 (tetranucleotide 5'TAAA tract, open triangles), *porA* (polyG tract, filled triangles); panel (B),
 839 *hpuA* (poly G tract, filled diamonds) and *opc* (polyG tract, open squares).

840

841 Figure 2. Effect of persistent carriage on changes in the repeat tracts of phase variable
 842 meningococcal genes. Each gene was examined for significant changes in repeat tract
 843 length between a pair of time points for carriers persistently colonised with the same
 844 meningococcal strain and plotted as the percentage of carriers with no significant changes.
 845 The total number of carrier samples examined for each of the four pairs of time points
 846 were:- *fetA* (18, 16, 7, 10); *porA* (18, 16, 7, 10); *opc* (18, 16, 7, 10); *nadA* (9, 8, 4, 5); *hpuA*
 847 (18, 16, 7, 10); *hmbR* (5, 3, 1, 2); *nalP* (18, 16, 7, 10); *mshA* (18, 16, 7, 10). Time points
 848 were:- 1st to 2nd (1 month), black bars; 2nd to 3rd, (2 months), dark grey bars; 3rd to 4th,
 849 (3 months), light grey bars; 1st-4th or 2nd-4th (5 or 6 months respectively), white bars.
 850 Panel (A) shows changes per gene. Panel (B) shows changes as a function of the repeat
 851 tract length relative to the tract length in the initial time point of each pair.

852

853 Figure 3. Comparison of protein expression levels for cc174 phase variants with different
 854 tract lengths. Whole cells lysates were prepared from meningococcal cells grown to mid-
 855 log phase with (lanes 1-7) or without (lanes 8-14) induction of iron-repressed genes.
 856 Western blots were probed with 1:1,000 or 1:2,000 dilutions of primary antibodies/antisera
 857 (see Table 1A) followed by an appropriate secondary antibody. Note that the an anti-F1-3
 858 FetA variant mouse polyclonal and an anti-meningococcal serotype P1.16 mouse mAb
 859 were used to detect FetA and PorA, respectively, whereas the other antisera recognise a
 860 wide range of antigenic types of the relevant protein. Repeat numbers are indicated as
 861 either the number of 'G's in a polyG tract or, for *nadA*, the number of 5'TAAA repeats.
 862 Lanes 1 and 8, N54.1; lanes 2 and 9, N343.5; lanes 3 and 10, N369.1; lanes 4 and 11,
 863 N352.3; lanes 5 and 12, N288.5; lanes 6 and 13, N343.2; lanes 7 and 14, N438.3. These

864 isolates were from two different cc174 ST-types – ST8510 (N54, N343) and ST1466
 865 (N288, N352, N369, N438).

866

867 Figure 4. Longitudinal alterations in the multiplex phase variation expression states during
 868 persistent carriage of meningococcal strains. The expression states of phase variable
 869 genes were determined from a combination of repeat number and direct assessments of
 870 expression state by Western blotting. The expression states of phase variable loci were
 871 coded as 0 (OFF/low), 1 (intermediate) and 2 (ON/high) - see text. The combined pattern
 872 of expression states (i.e. phasotypes) for six or seven genes (as indicated in each panel)
 873 were determined for up to six isolates per time point. A total score was assigned to each
 874 phasotype by combining the expression scores of individual genes (i.e. seven genes in
 875 their maximum expression state scores 14) and then phasotypes with similar scores were
 876 colour-coded and plotted in panels A-C:- red (10-14); orange (8-9), magenta (7); yellow
 877 (6); green (4-5); and blue (0-3). A mean score for each time point of each volunteer was
 878 calculated from these total phasotype scores. Panel (A), eight cc174 carriers and one cc32
 879 carrier (V176); panel (B), five cc60 carriers; panel (C), three cc167 and four cc23 carriers.
 880 Panel (D), graphs for three groups of strains, cc174, cc60 and cc167/cc23, showing the
 881 change in mean score relative to the initial time point when carriage was first detected.

882

883 Figure 5. Antigen-specific immune responses in serum samples from meningococcal
 884 carriers. Volunteers were grouped into four categories according to the type of
 885 meningococcal carriage detected by nasopharyngeal swabbing:- Persistent Carriers, same
 886 strains detected at all time points; Acquisition, progression from absence to presence of
 887 carriage with time points separated into pre-colonisation (Pre) and less than or more than
 888 three months after colonisation (i.e. Post(<3) and Post(>3) respectively);
 889 Replacement/Clearance, initial strain either replaced by antigenically-different strain or not
 890 detected in the 4th time point; Non-carriers, no meningococcal carriage detected at any
 891 time point. One to four sera were analysed for each volunteer. Panel A, anti-PorA IgG
 892 antibodies detected by a multiplex fluorescence-bead assay (AU, arbitrary units). Panel
 893 B, anti-CapY IgG antibodies detected by ELISA against purified capsular antigen. Panel C,
 894 serum bactericidal activity against an ST-11 meningococcal strain expressing a serogroup
 895 Y capsular antigen. Values represent the dilution providing 50% killing.

896

897

898 Table 1. Longitudinal changes in expression states of phase variable meningococcal genes

Phasotype Score ¹	Number of Isolates Per Observed Months of Carriage (%)			
	Initial	1 month	2-3 months	5-6 months
cc174 (FetA-Opc-NadA-HpuA-PorA-NalP-MspA)				
10	5 (10%)	2 (5%)	0	0
9	4 (8%)	0	0	0
8	24 (50%)	22 (55%)	14 (38%)	0
7	8 (17%)	1 (3%)	7 (19%)	0
6	7 (15%)	15 (38%)	7 (19%)	15 (63%)
5	0	0	1 (3%)	2 (8%)
0-4	0	0	8 (21%)	7 (29%)
Total	48	40	37	24
cc60 (FetA-Opc-HmbR-HpuA-PorA-NalP-MspA)				
8	6 (20%)	17 (57%)	7 (58%)	6 (100%)
7	5 (17%)	5 (17%)	1 (8%)	0
6	11 (37%)	8 (27%)	4 (33%)	0
5	8 (27%)	0	0	0
Total	30	30	12	6
cc167/cc23 (FetA-Opc-Hpua-PorA-NalP-MspA)				
10-12	8 (21%)	5 (21%)	5 (14%)	0
9	6 (16%)	8 (33%)	11 (31%)	2 (8%)
8	2 (5%)	5 (21%)	8 (22%)	4 (17%)
7	5 (13%)	0	0	3 (13%)
6	5 (13%)	0	1 (3%)	2 (8%)
5	12 (32%)	6 (25%)	8 (22%)	8 (33%)
0-4	0	0	3 (8%)	5 (21%)
Total	38	24	36	24
cc32 (FetA-Opc-NadA-HpuA-PorA-NalP-MspA)				
10-12	6 (100%)	5 (83%)	4 (67%)	3 (50%)
9	0	0	2 (33%)	3 (50%)
6	0	1 (17%)	0	0
Total	6	6	6	6

899 ¹Genotype score was determined from a combination of the expression states for 6-7 genes as
 900 outlined in brackets for each clonal complex and Table A8.
 901

902 Table 2. Levels of anti-PorA, anti-CapY and bactericidal antibodies in serum of meningococcal
 903 carriers

Antigen/Antibody type or activity ¹	Geometric Mean Antibody Concentration or Titre at Each Time Point (range, number of samples)			
	1st (Nov)	2nd (Dec)	3rd (March)	4th (May)
cc174 (Y:P1.21,16)				
P1.21,16/IgG	1.4 (0.05-40.5; 8)	1.5 (0.05-40.7; 8)	3.4 (0.05-76.1; 8)	1.7 (0.05-25.07; 7)
CapY/IgG	1.8 (0.3-20.5; 5)	ND ²	3.8 (0.4-17.1; 4)	4.6 (0.6-27.5; 7)
CapY/SBA	1077 (3-8192; 8)	6889 (4096-16384; 4)	2656 (128-8192; 8)	939 (32-4096; 8)
cc60 (E:P1.5,2)				
P1.5,2/IgG	1.5 (0.2-4.1; 3)	2.7 (0.5-7.8; 3)	1.5 (0.4-8.0; 3)	ND
cc167/cc23 (Y:P1.5-1,10-1)				
P1.5-2,10/IgG	1.4 (0.5-6.8; 6)	1.2 (0.3-2.7; 7)	3.2 (0.3-17.2; 6)	3.9 (2.8-8.0; 6)
CapY/IgG	15.2 (8.6-38.5; 4)	9.2 (7.5-11.2; 2)	20.5 (6.4-29.8; 4)	14.9 (4.4-67.3)
CapY/SBA	1465 (3-16384; 5)	2353 (128-16384; 5)	4598 (2048-16384; 6)	1783 (1024-8192; 5)
cc32 (B:P1.19,15)				
P1.19,15	0.1 (NR; 1)	0.1 (NR; 1)	0.2 (NR; 1)	0.3 (NR; 1)
Non-carriers				
PorA/IgG	0.2 (0.1-0.5; 8)	0.1 (0.1-0.6; 6)	0.2 (0.1-0.6; 5)	0.4 (0.2-0.9; 2)
CapY/IgG	0.2 (0.1-0.3; 5)	ND	0.2 (0.1-0.2; 4)	
CapY/SBA	5 (3-128; 8)	7 (3-256; 5)	3 (3-8; 3)	9 (3-512; 8)

904 ¹Antibody concentrations:- PorA (P1) variants, arbitrary units; serogroup Y capsular antigen
 905 (CapY), ug/ml; serum bactericidal activity (SBA) for an ST-11/Y strain, highest dilution resulting in
 906 ≥50% killing; ²ND, no data; NR, not relevant.









