



Short Paper

Phase variable DNA repeats in *Neisseria gonorrhoeae* influence transcription, translation, and protein sequence variation

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DOI: 10.1099/mgen.0.000078

There are many types of repeated DNA sequences in the genomes of the species of the genus *Neisseria*, from homopolymeric tracts to tandem repeats of hundreds of bases. Some of these have roles in the phase-variable expression of genes. When a repeat mediates phase variation, reversible switching between tract lengths occurs, which in the species of the genus *Neisseria* most often causes the gene to switch between on and off states through frame shifting of the open reading frame. Changes in repeat tract lengths may also influence the strength of transcription from a promoter. For phenotypes that can be readily observed, such as expression of the surface-expressed Opa proteins or pili, verification that repeats are mediating phase variation is relatively straightforward. For other genes, particularly those where the function has not been identified, gathering evidence of repeat tract changes can be more difficult. Here we present analysis of the repetitive sequences that could mediate phase variation in the *Neisseria gonorrhoeae* strain NCCP11945 genome sequence and compare these results with other gonococcal genome sequences. Evidence is presented for an updated phase-variable gene repertoire in this species, including a class of phase variation that causes amino acid changes at the C-terminus of the protein, not previously described in *N. gonorrhoeae*.

Keywords: gonococcus; phase variation; C-terminal variation; homopolymeric tract; simple sequence repeats.

Data statement: All supporting data, code and protocols have been provided within the article or through supplementary data files.

Data Summary

1. Sequence data for *Neisseria gonorrhoeae* strains investigated are available in GenBank under the following accession numbers: FA1090 (NC_002946.2; url - http://www.ncbi.nlm.nih.gov/nuccore/NC_002946.2); NCCP11945 (NC_011035.1; url - http://www.ncbi.nlm.nih.gov/nuccore/NC_011035.1; & CP001050.1; url - <http://www.ncbi.nlm.nih.gov/nuccore/CP001050.1>); MS11 (NC_022240.1; url - http://www.ncbi.nlm.nih.gov/nuccore/NC_022240.1); FA19 (NZ_CP012026.1; url -

http://www.ncbi.nlm.nih.gov/nuccore/NZ_CP012026.1); FA6140 (NZ_CP012027.1; url - http://www.ncbi.nlm.nih.gov/nuccore/NZ_CP012027.1); 35/02 (NZ_CP012028.1; url - http://www.ncbi.nlm.nih.gov/nuccore/NZ_CP012028.1). These were accessed on the 15th of April 2016 for use in this study.

2. Sequence data were assessed via BLAST interrogation of the nr database restricted to the *N. gonorrhoeae* species (url - <http://www.ncbi.nlm.nih.gov/>).
3. Genome resequencing data for *N. gonorrhoeae* strain NCCP11945 has been deposited under BioProject PRJNA322254 (url - <http://www.ncbi.nlm.nih.gov/bio-project/322254>).
4. Genome resequencing data was assessed via Galaxy (url - usegalaxy.org).

Received 23 May 2016; Accepted 08 July 2016

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Introduction

In *Neisseria gonorrhoeae*, the causative agent of gonorrhoea, DNA repeats are intimately linked to the biology of the organism. *N. gonorrhoeae*, and the closely related bacterial species *Neisseria meningitidis*, undergo phase-variable stochastic switching of gene expression for several surface structures, contributing to antigenic variation and immune evasion as well as niche adaptation in the course of infection (Bhat *et al.*, 1991; Moxon *et al.*, 2006; Carbone *et al.*, 2009; Srikhanta *et al.*, 2009; Omer *et al.*, 2011). Phase variation is mediated by simple sequence repeats associated with genes. In the species of the genus *Neisseria* the vast majority contain homopolymeric tracts within the coding sequences (Snyder *et al.*, 2001).

Comparative sequence analysis between a single *N. gonorrhoeae* and several *N. meningitidis* genome sequences identified over 100 potentially phase-variable genes (Snyder *et al.*, 2001), some of which have later been demonstrated to be phase-variable experimentally (Jordan *et al.*, 2005). Transcriptional and translational phase variation have been extensively studied in the species of the genus *Neisseria*, however an additional class of simple sequence repeat-mediated phase variation has been described in *Helicobacter canadensis* following whole-genome analysis (Snyder *et al.*, 2010). Simple sequence repeat-mediated changes in the presence or absence of C-terminal cell wall attachment motifs has also been described in *Streptococcus agalactiae* (Janulczyk *et al.*, 2010). In *N. meningitidis*, a gene fusion between *pglB2* and the downstream phosphoglycosyltransferase gene appears to be mediated by a poly-A repeat tract (Viburiene *et al.*, 2013).

With the availability of additional gonococcal genome sequences, the gonococcal phase-variable repertoire has here been re-assessed. As a result, phase variation in which repeats at the 3' ends of genes mediate changes in the C-terminal sequence of the proteins is described as part of a refined phase-variable gene repertoire.

Methods

Identification of phase-variable genes. Using the previous phase-variable gene repertoires reported for *N. gonorrhoeae* and *N. meningitidis* (Snyder *et al.*, 2001; Martin *et al.*, 2003; Jordan *et al.*, 2005), the homologues in *N. gonorrhoeae* strain NCCP11945 were sought (CP00150.1; Chung *et al.*, 2008). In addition, pattern search in xBASE (Chaudhuri *et al.*, 2008) was used to identify other repeats, based on previous evidence of phase variation in the species of the genus *Neisseria*: \geq (G)₈; \geq (C)₈; \geq (CAAACAC)₃; \geq (CAAATAC)₃; \geq (CCCAA)₃; \geq (GCCA)₃; \geq (A)₉; \geq (T)₉; \geq (AAGC)₃; \geq (TTCC)₃; and \geq (CTTCT)₃. No other repeats have been demonstrated to cause phase variation in this species. Genome sequences for *N. gonorrhoeae* strains NCCP11945 (NC_011035.1), FA1090 (NC_002946.2), FA19 (NZ_CP012026.1), FA6140 (NZ_CP012027.1), 35/02 (NZ_CP012028.1), and MS11 (NC_022240.1) were downloaded on 15th April 2016 and compared using progressive Mauve v2.3.1 (Darling *et al.*, 2004) to identify orthologues (Table S1, available in the online Supplementary Material).

Impact Statement

Phase variation plays a vital role in the ability of *Neisseria gonorrhoeae* to adapt to the various niche environments encountered. Through stochastic switching in the expression of key genes and regulatory systems, mediated by simple sequence repeats, the population of bacteria are diverse and readily able to survive in the face of selective pressures. Not all simple sequence repeats within the genome mediate phase variation. Previous investigations have sought to define the phase-variable repertoire of the species of the genus *Neisseria* and have identified a large number of candidates using a small number of genome sequences. With the availability of more genome sequence data and additional experimental data, we have refined the original repertoire to include those most likely to be phase-variable in *N. gonorrhoeae*. As these genes are important for survival, their definition as phase-variable is important for understanding pathogenesis and for potential future therapies. The advent of high-throughput sequencing has the potential to reveal additional cases of within-strain variations in repeat tracts, supporting phase-variable candidacy of genes.

Identification of repeat variation within *N. gonorrhoeae* strain NCCP11945. *N. gonorrhoeae* strain NCCP11945 was grown on GC agar (Oxoid) with Kellogg's (Kellogg *et al.*, 1963) and 5 % Fe(NO₃)₃ supplements at 37 °C in a candle tin for a period of 8 weeks with passages to fresh agar plates every 2 days or at 37 °C 5 % CO₂ for a period of 20 weeks with passages to fresh agar plates every 2–3 days. At each passage, cells were scraped from the plate and resuspended in 1 ml of GC broth to a turbidity equivalent to a 0.5 McFarland standard before inoculation onto fresh plates using a sterile cotton swab. DNA was extracted from such resuspensions using the Puregene Yeast/Bacterial kit (Qiagen). A sample (1 µg or 100 ng) of the DNA was genome sequenced using the Ion Personal Genome Machine, Ion Express Fragment Library kit, Ion Express Template kit, and Ion Sequencing kit (Life Technologies) or using the Illumina-based methods of the MicrobesNG service (microbesng.uk). Sequence read data was interpreted using Galaxy on usegalaxy.org (Afgan *et al.*, 2016). Briefly, the reference sequence (NC_011035.1), Ion Torrent data for eight-week passages (KU1-4, KU1-45), Ion Torrent data for 20-week passages (KU1-95, KU1-96), and Illumina data for 20-week passages (2928-NS1_1 & 2929-NS1_2 and 2929-NS2_1 & 2929-NS2_2) were uploaded to Galaxy. The Ion Torrent bam format files were converted to fastq format using BAMTools Convert (Barnett *et al.*, 2011). FASTQ Groomer was used on all NGS data (Blankenberg *et al.*, 2010). Bowtie2 was used to map the reads against the reference (Langmead *et al.*, 2009; Langmead *et al.*, 2012) before visualisation using the Integrated Genomics Viewer (Robinson *et al.*, 2011; Thorvaldsdóttir *et al.*, 2013).

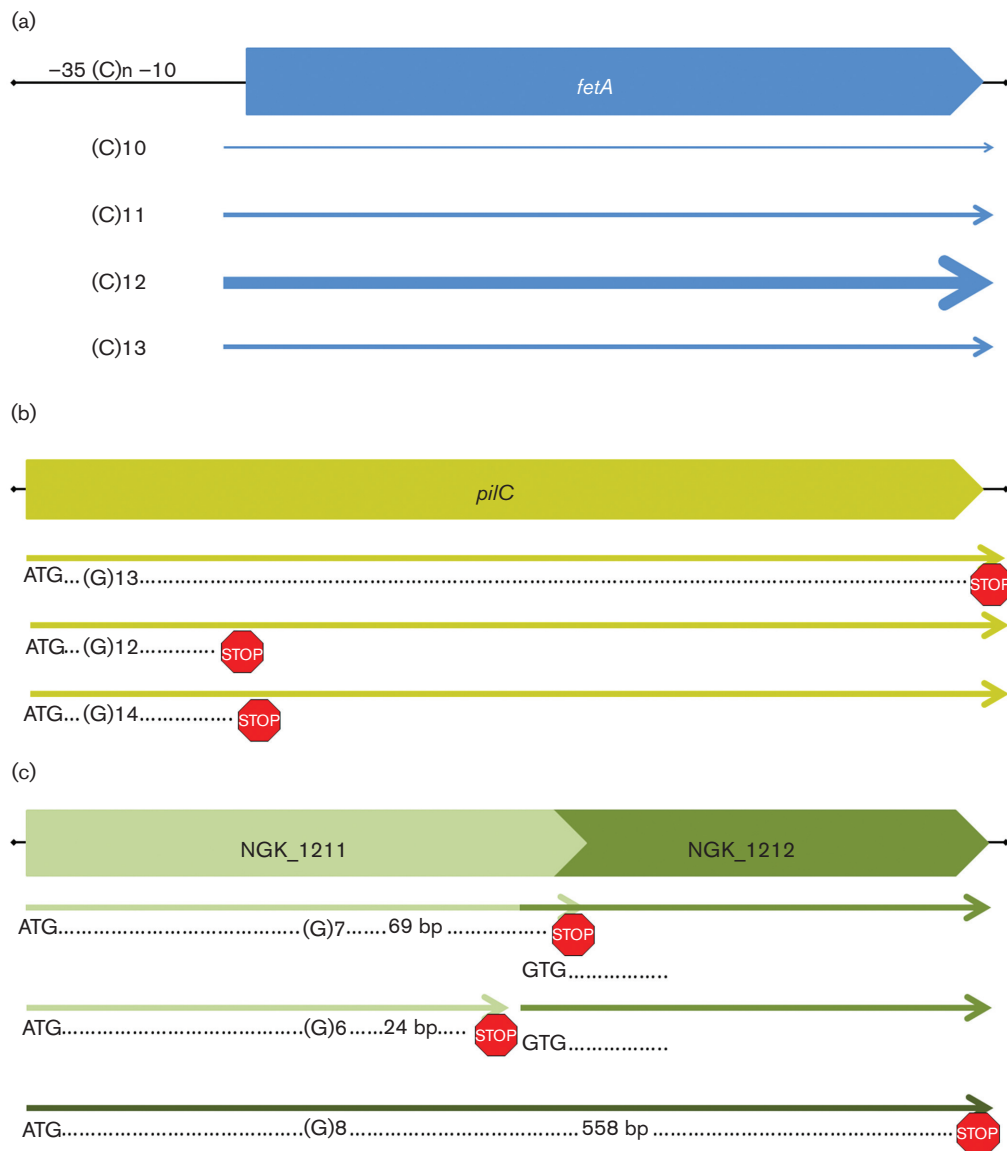


Fig. 1. Illustrations of the types of phase variation in *N. gonorrhoeae*. (a): Transcriptional phase variation, in which changes in a repeat tract alter the facing and spacing of the -10 and -35 promoter elements and the level of transcription of the gene. Phase variation of *fetA* is used as an example, where it has been shown that differences in spacing of the -10 and -35 elements due to changes in the poly-C repeat tract alter expression levels, represented by the widths of the arrows (Carson *et al.*, 2000). (b): Translational phase variation, in which changes in a repeat tract towards the 5' end of the coding sequence alter the reading frame of a coding region and switch expression on and off due to frame-shift. Phase variation of *pilC* is used as an example, where it has been shown that changes in the poly-G repeat tract generate frame-shifts which switch protein expression on and off (Jonsson *et al.*, 1991). (c): C-terminal phase variation, in which changes in a repeat tract towards the 3' end of the coding sequence alter the reading frame of a coding region and switch the encoded C-terminal amino acids between the three reading frames. In the example NGK_1211, two of the reading frames result in different C-terminal ends to the protein, while the third generates a fusion with the downstream coding sequence, NGK_1212. Only some examples of C-terminal phase variation result in this type of fusion (Table 3).

Results and Discussion

Phase variable genes

The phase-variable gene repertoire of *N. gonorrhoeae* strain NCCP11945 was investigated and compared against gonococcal strains FA1090, FA19, FA6140, 35/02, and MS11 to

assess the presence of similar repeat tracts across the species and variations in repeat tract lengths between strains.

Transcriptional phase variation is mediated by repeats within or associated with the promoter region (Fig. 1a). Changes in the repeat alters the level of transcription of the gene, as in *fetA* (*frpB*; NGK_2557) where differences in the

Table 1. Transcriptional phase variable genes in *N. gonorrhoeae*

Gene	FA1090 locus*	Repeat in FA1090*	NCCP11945 locus†	Repeat in NCCP11945†	Repeat in FA19‡	Repeat in FA6140§	Repeat in 35/02	Repeat in MS11¶	<i>N. gonorrhoeae</i> candidacy#	Reference
<i>porA</i>	NGO_04715	(T)11C(G) 6T	NGK_0906 & NGK_0907**	(T)9C(G)6T	(T)8C(G) 8T	(T)9C(G)6T	(T)9C(G)6T	(T)9C(G) 6TT	Known	van der Ende <i>et al.</i> (1995)
Lipoprotein	NGO2047	(A)9	NGK_2186††	(A)8	(A)8	(A)9	(A)9	(A)8	Yes	
<i>fetA / ffpB</i>	NGO2093	(C)13	NGK_2557	(C)14	(C)10	(C)11	(C)11	(C)11	Known	Carson <i>et al.</i> (2000)

*From the *N. gonorrhoeae* strain FA1090 genome sequence (NC_002946.2).

†From the *N. gonorrhoeae* strain NCCP11945 genome sequence (CP00150.1).

‡From the *N. gonorrhoeae* strain FA19 genome sequence (NZ_CP012026.1).

§From the *N. gonorrhoeae* strain FA6140 genome sequence (NZ_CP012027.1).

||From the *N. gonorrhoeae* strain 35/02 genome sequence (NZ_CP012028.1).

¶From the *N. gonorrhoeae* strain MS11 genome sequence (NC_022240.1).

#Gene phase variation candidacy in *N. gonorrhoeae*. Known, phase variation has been reported in the literature. Yes, there is evidence of repeat tract variation between strains supporting phase variation.

**This coding sequence appears to be frame-shifted and annotated as two coding sequences.

††NGK_2186 and NGO2047 annotations are on opposite strands.

Table 2. Translational phase-variable genes in *N. gonorrhoeae*

Gene	FA1090 locus*	Repeat in FA1090*	NCCP11945 locus†	Repeat in NCCP11945†	Repeat in FA19‡	Repeat in FA6140§	Repeat in 35/02	Repeat in MS11¶	N. gonorrhoeae candidacy#	Reference
<i>pilC2</i>	NGO0055	(G)13	NGK_0074	(G)9	(G)10	(G)13	(G)9CAGG	(G)12	Known	Jonsson <i>et al.</i> , 1991
<i>opa</i>	NGO0066a	(CTTCT)13CTTCG	NGK_0096	(CTTCT)8CTTCC	(CTTCT)4CTTCC	CTT(CTTCT)10CTTCC	(CTTCT)6(CTTCC)2	(CTTCT)8CTT CC	Known	Stern & Meyer, 1987
<i>opa</i>	NGO0070	(CTTCT)9CTTCG	NGK_0102	(CTTCT)7CTTCC	(CTTCT)8CTTCC	(CTTCT)17CTTCC	(CTTCT)7CTT CC	(CTTCT)7CTT CC	Known	Stern & Meyer, 1987
<i>pglH</i>	NGO0086	(C)10	NP	NP	no repeat	no repeat	no repeat	NP	Known	Power <i>et al.</i> , 2003
<i>pglG</i>	NGO0087	A(C)7	NP	NP	A(C)9	A(C)6	A(C)6	NP	Known	Power <i>et al.</i> , 2003
<i>pglE</i>	NGO0207	(CAAACAC)4	NGK_0339	(CAAACAC)8 (CAAAATAC)3	(CAAACAC)6CAAA TACC AAAACCCAAA TAC	(CAAACAC)10CAAAA TACC AAAACCCAAA TACC AAAACAC	(CAAACAC)24 CAAAATAC	(CAAACAC)15(CAAAATAC)3	Known	Power <i>et al.</i> , 2003
<i>hsdS</i>	NGO_02155	(G)7	NGK_0571	(G)7	(G)8	(CAAAATAC)2 (G)9	(G)7	(G)7	Known	Adamczyk-Poplawska <i>et al.</i> , 2011
Hypothetical	NGO0527	(C)6A(C)9GC	NGK_1405	(C)6A(C)8GC	(C)7A(C)4T(C)3GC	(C)7A(C)4T(C)3GC	(C)11A(C)8GC GC	(C)6A(C)14GC C	Yes	
<i>modB</i>	NGO0545	(CCCAA)12	NGK_1384	(CCCAA)11	(CCCAA)12	(CCCAA)4	(CCCAA)11	(CCCAA)7	Known	Srikhanta <i>et al.</i> , 2009
Replication initiation factor	NGO_06135	(C)8TTATCTAACA (G)7	NGK_1957	(C)11TTATCTAACA (G)8	(C)7TTATCTAACA (G)7	(C)6TTATCT AACA(G)5	(C)11TTATCT AACA(G)8	(C)10TTATCT AACA(G)7	Yes	
<i>modA</i>	NGO0641	(GCCA)37	NGK_1272	(GCCA)18	(GCCA)24GTCA	(GCCA)24	(GCCA)19	(GCCA)24	Known	Srikhanta <i>et al.</i> , 2009
Replication initiation factor	NGO_06695	(C)8TTATCTAACA (G)7	NGK_1486	(C)9TTATCTAACA (G)7	(C)7TTATCTAACA (G)7	(C)9TTATCT AACA(G)6	(C)11TTATCT AACA(G)8	(C)9TTATCTA ACA(G)7	Yes	
<i>opa</i>	NGO0950a	(CTTCT)16CTTCC	NGK_0847	(CTTCT)19CTTCC	(CTTCT)13CTTCC	(CTTCT)16CTTCC	(CTTCT)8CTT CC	(CTTCT)4CTT CC	Known	Stern & Meyer, 1987
Hypothetical	NGO0964	(AAGC)4	NGK_0831a	(AAGC)8	(AAGC)15	(AAGC)7	(AAGC)9	(AAGC)7	Yes	
<i>virG</i>	NGO0985	(AAGC)3	NGK_0804	(AAGC)3	(AAGC)2	(AAGC)3	(AAGC)3	(AAGC)3	Yes	
<i>opa</i>	NGO1040a	(CTTCT)20CTTCC	NGK_0749	(CTTCT)20CTTCC	(CTTCT)10CTTCC	(CTTCT)7CTTCC	(CTTCT)12CTTCC	(CTTCT)13CTTCC)2	Known	Stern & Meyer, 1987
<i>opa</i>	NGO1073a	(CTTCT)2CTTCC	NGK_0693	(CTTCT)10CTTCC	(CTTCT)11CTTCC	(CTTCT)12CTTCC	(CTTCT)18CTTCC	(CTTCT)7CTT CC	Known	Stern & Meyer, 1987
<i>opa</i>	NGO1277a	(CTTCT)11CTTCC	NGK_1495	(CTTCT)7CTTCC	CTT(CTTCT)11CTTCC	(CTTCT)11CTTCC	(CTTCT)7CTT CC	(CTTCT)8CTT CC	Known	Stern & Meyer, 1987
Adhesion	NGO1445	(CAAG)3	NGK_1705	(CAAG)12CAAA	(CAAG)12CAAA	(CAAG)6CAAA	(CAAG)3	(CAAG)3	Yes	

Table 2. cont.

Gene	FA1090 locus*	Repeat in FA1090*	NCCP11945 locus†	Repeat in NCCP11945†	Repeat in FA19‡	Repeat in FA6140§	Repeat in 35/02	Repeat in MS11¶	N. gonorrhoeae candidacy#	Reference
<i>opa</i>	NGO1463a	20CAAA (CTTCT) 10CTTCC	NGK_1729	(CTTCT) 7CTTCC	(CTTCT)11CTTCC	(CTTCT)12CTTCC	9CAAA (CTTCT)12CT TCC	6CAAA (CTTCT)10CT TCC	Known	Stern & Meyer, 1987
<i>opa</i>	NGO1513	(CTTCT) 12CTTCC	NGK_1799	(CTTCT) 14CTTCC	CTT(CTTCT) 10CTTCC	NP	(CTTCT) 6CTT CG	(CTTCT) 7CTT CG	Known	Stern & Meyer, 1987
<i>opa</i>	NGO1553a	(CTTCT) 4CTTCC	NGK_1847	(CTTCT)9CT TCC	(CTTCT)17CTTCC	(CTTCT)8CTTCC	(CTTCT) 8CTT CC	(CTTCT)14CT TCC	Known	Stern & Meyer, 1987
<i>autA</i>	NGO1689	(AAGO)3	NGK_2082	(AAGC)3	(AAGC)14	(AAGC)3	(AAGC)3	(AAGC)3	Known	Peak et al., 1999 ; Arenas et al., 2015
<i>pgtA</i>	NGO1765	(G)11	NGK_2516	GGGAGCGGG	(G)19	(G)19	GGGAGCGGG	GGGAGCGGG	Known	Banerjee et al., 2002
Repetitive large surface lipoprotein	NGO_09875 & NGO_09870**	(G)8	NGK_2422 & NGK_2423**	(G)7	(G)7	(G)7	(G)7	(G)7	Yes	
<i>opa</i>	NGO1861a	(CTTCT) 13CTTCC	NGK_2410	(CTTCT) 11CTTCC	(CTTCT)13CTTCC	(CTTCT)2CTTCC	(CTTCT)13CT TCC	(CTTCT)30CT TCC	Known	Stern & Meyer, 1987
<i>pilI</i>	NGO1912	(G)11	NGK_2342	(G)13	(G)11	GGGC(G)11	(G)15	(G)11	Known	Jonsson et al., 1991
Hypothetical	NGO1953	(C)8	NGK_2297	(C)8	(C)8	(C)8	(C)9	(C)8	Yes	
Pyrimidine 5'-nucleotidase	NGO2055 & NGO2054**	(C)6	NGK_2176	CAAAACCCC	CAAAACCCC	CAAAACCCC	(C)9	(C)10	Yes	
<i>opa</i>	NGO2060a	(CTTCT) 10CTTCC	NP	(CTTCT) 14CTTCC	NP	NP	(CTTCT) 6CTT CC	(CTTCT) 7CTT CC	Known	Stern & Meyer, 1987
<i>opa</i>	NP		NGK_2170	(CTTCT) 14CTTCC	NP	NP	NP	NP	Known	Stern & Meyer, 1987
<i>igtG</i>	NGO2072	(C)11	NGK_2534 & NGK_2533**	(C)12	(C)10	(C)10	(C)10	(C)10	Known	Mackinnon et al., 2002
<i>hpaA</i>	NGO2110	(G)9	NGK_2581	(G)10	(G)9	(G)9	(G)8	(G)8	Known	Chen et al., 1998
<i>igtA</i>	NGO11610	(G)11	NGK_2630	(G)11	(G)14A	(G)17A	(G)20A	(G)10A	Known	Erwin et al., 1996
<i>igtC</i>	NGO2156	(G)14	NGK_2632	(G)13	(G)13	(G)10	(G)16	(G)8	Known	Shafer et al., 2002
<i>igtD</i>	NGO2158	A(G)14	NGK_2634	A(G)16	(G)13	A(G)12	A(G)18	A(G)13	Known	Shafer et al., 2002

*From the *N. gonorrhoeae* strain FA1090 genome sequence (NC_002946.2).†From the *N. gonorrhoeae* strain NCCP11945 genome sequence (CP00150.1).

‡From the *N. gonorrhoeae* strain FA19 genome sequence (NZ_CP012026.1).
 §From the *N. gonorrhoeae* strain FA6140 genome sequence (NZ_CP012027.1).
 ||From the *N. gonorrhoeae* strain 35/02 genome sequence (NZ_CP012028.1).
 ¶From the *N. gonorrhoeae* strain MS11 genome sequence (NC_022240.1).
 #Gene phase variation candidacy in *N. gonorrhoeae*. Known , phase variation has been reported in the literature. Yes, there is evidence of repeat tract variation between strains supporting phase variation.

**This coding sequence appears to be frame-shifted and annotated as two coding sequences.

NP, The coding sequence is not present in this strain.

length of the poly-C homopolymeric tract between the –10 and –35 promoter regions alters expression (Carson *et al.*, 2000). There are three transcriptional phase-variable genes in *N. gonorrhoeae* strain NCCP11945 (Table 1), *fetA* (NGK_2557), a lipoprotein (NGK_2186), and *porA* (NGK_0906/NGK_0907), yet in gonococci *porA* does not have an intact coding region. Variation in the repeats between gonococcal strains is found for all three transcriptional phase-variable genes (Table 1).

Most common in the species of the genus *Neisseria* is translational phase-variation where, as in *pilC*, the repeat is within the 5' portion of the coding region of the gene (Fig. 1b). Changes in the repeat tract generate frame-shift mutations in two of the three open reading frames, with the gene only being translated into protein when the repeat tract length puts the gene in-frame. Whilst many phase-variable genes in the species of the genus *Neisseria* contain homopolymeric tracts, some experience copy number changes in repetitive sequences, such as the CTTCT repeat in *opa* (Muralidharan *et al.*, 1987; Bhat *et al.*, 1991) or the AAGC repeat in *autA* (Peak *et al.*, 1999; Arenas *et al.*, 2015). In the *N. gonorrhoeae* strains examined here, the AAGC repeat in *virG* (NGK_0804) is only present in two or three copies (Table 2), rather than several copies as in NGK_0831a and *autA* (NGK_2082). Although *virG* has low copy number for the repeat, variations between strains are observed and strains with many copies may yet be identified [there are currently none >(AAGC)₃ in the NCBI nr/nt or wgs databases], therefore it is placed amongst the phase-variable genes even though this may be at low frequency or be a strain-specific effect. There are 36 translational phase-variable genes in *N. gonorrhoeae* based on the species examined (Table 2).

In addition, a third class of repeat-mediated phase-variable gene was identified (Snyder *et al.*, 2010). In these C-terminal phase-variable genes, a repeat tract towards the 3' of the coding region is able to alter the sequence at the C-terminus of the encoded protein (Fig. 1c). In *N. gonorrhoeae* strain NCCP11945, four of these C-terminal phase-variable genes were identified (Table 3). It is likely that in the case of the pilin sequence (NGK_2161), changes in the repeat are causing pilus protein changes, mediating antigenic variation through a phase-variable mechanism. Comparisons also show repeat tract variation in a membrane protein (NGK_1211) and *mafB* cassette (NGK_1624), supporting C-terminal phase variation in the species of the genus *Neisseria*. Variations in the products of *mafB* cassettes are believed to contribute to competition between species within the niche (Jamet *et al.*, 2015). Although no variation was observed in these strains in *ispH* (NGK_0106), (G)₈ repeats are known to vary in *lgtC* (NGK_1632), *hpuA* (NGK_2581), and *hsdS* (NGK_0571) (Table 2), therefore it is highly likely that the repeat in *ispH* also has the capacity to vary.

A number of previously reported candidates are not supported by evidence of phase variation, based on the absence

Table 3. C-terminal phase-variable genes in *N. gonorrhoeae*

Gene	FA1090 locus*	Repeat at in FA1090*	NCCP11945 locus†	Repeat in NCCP11945†	Amino acids after the repeat in each frame of	NCCP11945‡‡
FA19§	FA6140	35/02 ¶	MA11#	<i>N. gonorrhoeae</i> candidacy**		
<i>ispH</i>	NGO0072	(G)8	NGK_0106	(G)8	26 50 61	(G)8 (G)8 (G)8 (Yes)
Membrane protein	NGO0691	(G)6	NGK_1211	(G)7	23 8 186	(G)7 (G)6 (G)7 (Yes)
<i>m afB</i> cassette	WX61_RS02820	(C)7	NGK_1624	(C)9	46 2 37	deletion†† (C)10 (C)8 (C)10 (Yes)
Pilin cassette	WX61_RS02820	CCGC	NGK_2161	(C)8	20 8 91¶	(C)5GCC CCGCC (C)5 CCT (C)5 (Yes)

*From the *N. gonorrhoeae* strain FA1090 genome sequence (NC_002946.2).

†From the *N. gonorrhoeae* strain NCCP11945 genome sequence (CP00150.1).

‡In each column are the number of amino acids encoded 3' of the repeat before the closest termination codon in each of the three reading frames.

§From the *N. gonorrhoeae* strain FA19 genome sequence (NZ_CP012026.1).

||From the *N. gonorrhoeae* strain FA6140 genome sequence (NZ_CP012027.1).

¶From the *N. gonorrhoeae* strain 35/02 genome sequence (NZ_CP012028.1).

#From the *N. gonorrhoeae* strain MS11 genome sequence (NC_022240.1).

**Gene phase variation candidacy in *N. gonorrhoeae*. Yes, there is evidence of repeat tract variation between strains supporting phase variation. (Yes), although there is no variation between strains investigated here, tracts of this length vary in other genes (Chen *et al.*, 1998; Shafer *et al.*, 2002; Adamczyk-Poplawska *et al.*, 2011).

††There is a 400 bp deletion in this strain encompassing the region that would contain this repeat.

Table 4. Genes for which there is no evidence of phase variation in *N. gonorrhoeae*

Gene	FA1090 locus*	Repeat in FA1090*	NCCP1945 locus†	Repeat in NCCP1945†	Repeat in FA19#	Repeat in FA6140\$	Repeat in 35/02	Repeat in MS11¶	<i>N. gonorrhoeae</i> candidacy#
Prolyl endopeptidase	NGO0026	GGGGGGG	NGK_0034	GGGGGGG	GGGGGGG	GGGGGGG	GGGGGGG	GGGGGGG	No. Replacement tract
<i>pill/wbpC</i>	NGO0065	C(G)6	NGK_0089**	C(G)6	C(G)6	C(G)6	C(G)6	C(G)6	No. No variation.
Phosphoesterase	NGO0081	(C)7	NGK_0 n 9	(C)7	(C)7	(C)7	(C)7	(C)7	No. No variation.
Hypothetical	NGO0121	(A)6	NGK_0167	(A)6	(A)6	(A)6	(A)6	(A)6	No. No variation.
<i>cvaA</i>	NGO0123	(C)4	NGK_0168	(C)4	(C)4	(C)4	(C)4	(C)4	No. No variation.
<i>potD -2</i>	NGO0206	AA(C)5	NGK_0338	AA(C)5	AA(C)5	AA(C)5	AA(C)5	AA(C)5	No. No variation.
Hypothetical	NGO0532	AACGGCAAACA	NGK_1400	AACGGCAAACA	AACGGCAAACA	AACGGCAAACA	AACGGCAAACA	AACGGCAAACA	No. Replacement tract
<i>nifS</i>	NGO0636	CCACACCC	NGK_1278	CCACACCC	CCACACCC	CCACACCC	CCACACCC	CCACACCC	No. Replacement tract
<i>ltdD</i>	NGO0639	(G)7	NGK_1275	(G)7	(G)7	(G)7	(G)7	(G)7	No. No variation.
Methylase	NGO0676	(A)9	NGK_1230	(A)9	(A)9	(A)9	(A)9	(A)9	No. No variation.
NlaIV									
<i>dnaX</i>	NGO0743	(C)7	NGK_1135	(C)7	(C)7	(C)7	(C)7	(C)7	No. No variation.
<i>mobA</i>	NGO0754	GGAAGG	NGK_1123	GGAAGG	GGAAGG	GGAAGG	GGAAGG	GGAAGG	No. Replacement tract
<i>ppx</i>	NGO1041	(C)7	NGK_0745	(C)7	(C)7	(C)7	(C)7	(07	No. No variation.
<i>fxp/ccoP</i>	NGO1371	(AT)5	NGK_1607	(AT)5	(AT)5	(AT)5	(AT)5	(AT)5	No. No variation.
Hypothetical	NGO1384	G(A)7	NGK_1622	(A)8	(A)8	(A)8	(A)8	G(A)7	No. No variation in length.
<i>pntA</i>	NGO1470	CCCTGCTGG	NGK_1735	CCCTGCTGG	CCCTGCTGG	CCCTGCTGG	CCCTGCTGG	CCCTGCTGG	No. Replacement tract
<i>amiC</i>	NGO1501	TTCGCC	NGK_1783	TTCGCC	TTCGCC	TTCGCC	TTCGCC	TTCGCC	No. Replacement tract
<i>dca</i>	NGO1540	TGTGGGGG	NGK_1830	TGTGGGGG	TGTGGGGG	TGTGGGGG	TGTGGGGG	TGTGGGGG	No. Replacement tract
<i>anmK</i>	NGO1583	(C)7	NGK_1884	(C)7	(C)7	(C)7	(C)7	(C)7	No. No variation.
<i>dinG</i>	NGO1708	(C)4T CC	NGK_2106	(C)4TCC	(C)4TCC	(C)4TCC	(C)4TCC	(C)4TCC	No. Replacement tract
<i>rplK</i>	NGO1855	(C)7	NGK_2416	(C)7	(C)7	(C)7	(C)7	(C)7	No. No variation.
Hypothetical	NGO1970	(TA)5	NGK_2274	(TA)5	(TA)5	(TA)5	(TA)5	(TA)5	No. No variation.
<i>mafA -3</i>	NGO1972	(G)5	NGK_2270	(G)5	(G)5	(G)5	(G)5	(G)5	No. No variation.
<i>map</i>	NGO1983	(C)6	NGK_2258	(C)6	(C)6	(C)6	(C)6	(C)6	No. No variation.
<i>plsX</i>	NGO2171	(TTCC)3	NGK_2652	(TTCC)3	(TTCC)3	(TTCC)3	(TTCC)3	(TTCC)3	No. No variation.
<i>lbpA</i>	NGO0260a	NR	NGK_0401	GGGGGGG	GGGGGGG	GGGGGGG	NR	TGAAACGG	No. Replacement tract

*From the *N. gonorrhoeae* strain FA1090 genome sequence (NC_002946.2).†From the *N. gonorrhoeae* strain NCCP1945 genome sequence (CP00150.1).

‡From the *N. gonorrhoeae* strain FA19 genome sequence (NZ_CP012026.1).

§From the *N. gonorrhoeae* strain FA6140 genome sequence (NZ_CP012027.1).

||From the *N. gonorrhoeae* strain 35/02 genome sequence (NZ_CP012028.1).

¶From the *N. gonorrhoeae* strain MS11 genome sequence (NC_022240.1).

#Gene phase variation candidacy in *N. gonorrhoeae*. No. Replacement tract: due to the replacement of the repeat tract with other nucleotides, this is not phase-variable. No. No variation: due to no observed variation in the repeat tract, this is not phase-variable. No. No variation in length: due to the equal length tract in all strains, this is not phase-variable.

**This coding sequence contains a point mutation, which generates a premature termination codon.

NR, The region of the coding sequence containing the repeat tract does not have homology to the aligned region in these strains.

of tract length changes between the strains (Table 4). For example, although tract variation was reported for *cvaA* (NGK_0168), *mafA*-3 (NGK_2270), and *dca* (NGK_1830) in *N. meningitidis* (Martin *et al.*, 2003), there are no changes observed in the short (C)4 and (G)5 tracts in these genes in *N. gonorrhoeae* (Table 4). They are therefore unlikely to be phase-variable in this species. Likewise, neither of the dinucleotide-repeat-containing genes (NGK_1607 and NGK_2274) show variations (Table 4); dinucleotides are not likely to be phase-variable in the species of the genus *Neisseria* (Martin *et al.*, 2003). All of these genes contain short repeats that do not vary or alternative nucleotide sequences in the strains investigated (Table 4).

This analysis identified 29 genes that are known to be phase variable (Tables 1, 2), either in *N. gonorrhoeae* or *N. meningitidis* including 12 paralogues of *opa* (11 in each strain; Muralidharan *et al.*, 1987; Bhat *et al.*, 1991) and 17 other known phase-variable genes (Stern *et al.*, 1987; Jonsson *et al.*, 1991; Van der Ende *et al.*, 1995; Erwin *et al.*, 1996; Chen *et al.*, 1998; Peak *et al.*, 1999; Carson *et al.*, 2000; Banerjee *et al.*, 2002; Mackinnon *et al.*, 2002; Shafer *et al.*, 2002; Power *et al.*, 2003; Srikhanta *et al.*, 2009; Adamczyk-Poplawska *et al.*, 2011; Arenas *et al.*, 2015). Thirteen additional genes have variations in the repeat tracts when the six *N. gonorrhoeae* genome sequences are compared, one transcriptional, nine translational, and three C-terminal repeats. Based on homology and presence of conserved domains, these genes are believed to encode two replication initiation factors, an adhesion protein, a pyrimidine 5'-nucleotidase, two lipoproteins, two membrane proteins, two secreted proteins, and three hypothetical proteins (Tables 1–3).

Combined with the previous data on repeat variation within and between gonococcal strains and demonstration of phase variation (Sparling *et al.*, 1986; Yang *et al.*, 1996; Lewis *et al.*, 1999; Snyder *et al.*, 2001; Power *et al.*, 2003; Jordan *et al.*, 2005; Srikhanta *et al.*, 2009), a revised repertoire of 43 transcriptional (Table 1), translational (Table 2), and C-terminal (Table 3) phase-variable genes is proposed for *N. gonorrhoeae* as a species. This is fewer than previous predictions (76 in Jordan *et al.*, 2005) and thus far two-thirds (67 %, 29 out of 43) have been experimentally demonstrated to be phase-variable (Tables 1, 2). The additional 14 genes, 13 of which show strain-to-strain repeat variation, require additional investigation.

Phase variable repeat copy number variation *in vitro*

Previously, for *H. canadensis*, 454 and Illumina genome sequence read data was used to support candidacy of phase-variable genes (Snyder *et al.*, 2010). In the present study, Ion Torrent and Illumina genome sequence read data from *N. gonorrhoeae* strain NCCP11945 that had been passaged in the laboratory for 8 weeks or for 20 weeks was analysed for changes to phase-variable repeats for the 14 genes for which there is no within-strain evidence of phase variation (Tables 1–3). Changes were observed in known phase-

Table 5. Genes for which there is sequencing-based evidence of phase variation in *N. gonorrhoeae* strain NCCP11945

Gene	FA1090 locus*	Repeat in FA1090*	NCCP11945 locus†	Repeat in NCCP11945†	Repeat in FA19‡	Repeat in FA6140§	Repeat in 35/02	Repeat in MS115	<i>N. gonorrhoeae</i> candidacy#	Ion Torrent**	Illumina††
<i>ispH</i>	NGO0072	(G)8	NGK_0106	(G)8	(G)8	(G)8	(G)8	(G)8	(yes)	Repeat varies	Repeat does not vary
<i>virG</i>	NGO0985	(AAGC)3	NGK_0804	(AAGC)3	(AAGC)2	(AAGC)3	(AAGC)3	(AAAG)3	Yes	Repeat does not vary	Repeat varies
Hypothetical	NGO0964	(AAGC)4	NGK_0831a	(AAGC)8	(AAGC)15	(AAGC)7	(AAGC)9	(AAGC)7	Yes	Repeat varies	Repeat varies
Membrane protein	NGO0691	(G)6	NGK_1211	(G)7	(G)7	(G)6	(G)7	(G)7	Yes	Repeat varies	Repeat does not vary
Hypothetical	NGO0527	(C)6A(C)9CC	NGK_1405	(C)6A(C)8GC	(C)7A(C)4T(C)3GC	(C)7A(C)4T(C)3GC	(C)11A(C)8GGC	(C)6A(C)14GCC	Yes	Repeat varies	Repeat does not vary
Replication initiation factor	NGO_06695	(C)8TTATCTAACA	NGK1486	(C)9TTATCTAACA	(C)7TTATCTAACA	(C)9TTATCTAACA	(C)11TTATCTAACA	(C)9T T AT CT AACAG(G)7	Yes	Repeat varies	Repeat varies
<i>mjfB</i> cassette	NGO1386	(C)7	NGK_1624	(C)9	{deletion}	(C)10	(C)8	(C)10	Yes	Repeat varies	Repeat varies
Adhesion	NGO1445	(CAAG)20CAA	NGK_1705	(CAAG)12CAA	(CAAG)12CAA	(CAAG)6CAA	(CAAG)9CAA	(CAAG)6CAA	Yes	Repeat varies	Repeat varies
Replication initiation factor	NGO_06135	(C)8TTATCTAACA	NGK_1957	(C)11TTATCTAACA	(C)7TTATCTAACA	(C)56TTATCTAACA	(C)11TTATCTAACA	(C)10T TAT C TAACA(G)7	Yes	Repeat varies	Repeat varies
Pilin cassette	NGO_11140	(C)6CCG	NGK_2161	(C)8	(C)5GCC	(C)8CGCC	(C)5CCT	(C)5	Yes	Repeat varies	Repeat varies
Pyrimidine 5'-nucleotidase	NGO2055 & NGO2054II	(C)6	NGK_2176	CAAAACCC	CAAAACCC	CAAAACCC	(C)9	(C)10	Yes	No repeat	No repeat
Lipoprotein	NGO2047	(A)9	NGK_2186A\$A\$	(A)8	(A)8	(A)9	(A)9	(A)8	Yes	Repeat varies	Repeat varies
Hypothetical	NGO1953	(C)8	NGK_2297	(C)8	(C)8	(C)8	(C)9	(C)8	Yes	Repeat varies	Repeat does not vary
Repetitive large surface lipoprotein	NGO_09875 & NGO2093	(G)8	NGK_2422 & NGK_2423	(G)7	(G)7	(G)7	(G)7	(G)7	Yes	Repeat varies	Repeat does not vary
<i>feaA</i> / <i>frpB</i>	NGO09870II	(C)13	NGK_2557	(C)14	(C)10	(C)11	(C)11	(C)11	Known	Repeat varies	Repeat varies
<i>pilC</i> 1	NGO1912	(G)11	NGK_2342	(G)13	(G)11	GGGC(G)11	(G)15	(G)11	Known	Repeat varies	Repeat varies
<i>opa</i>	NGO0950a	(CTTCT)16CTTCC	NGK_0847	(CTTCT)19CTTCC	(CTTCT)13CTTCC	(CTTCT)16CTTCC	(CTTCT)8CTTCC	(CT T CT) 4CT TCC	Known	No reads through repeat	Repeat varies

*From the *N. gonorrhoeae* strain FA1090 genome sequence (NC_002946.2).†From the *N. gonorrhoeae* strain NCCP11945 genome sequence (CP00150.1).

‡From the *N. gonorrhoeae* strain FA19 genome sequence (NZ_CP012026.1).

§From the *N. gonorrhoeae* strain FA6140 genome sequence (NZ_CP012027.1).

||From the *N. gonorrhoeae* strain 35/02 genome sequence (NZ_CP012028.1).

¶From the *N. gonorrhoeae* strain MS11 genome sequence (NC_022240.1).

#Gene phase variation candidacy in *N. gonorrhoeae*. Known, phase variation has been reported in the literature. Yes, there is evidence of repeat tract variation between strains supporting phase variation. (Yes), although there is no variation between strains investigated here, tracts of this length vary on other genes (Chen *et al.*, 1998; Shafer *et al.*, 2002; Adamczyk-Poplawska *et al.*, 2011).

**Based on Ion Torrent sequencing data from cultures grown with passage for 8 weeks and from cultures grown with passage for 20 weeks (accession numbers SRR3547950, SRR3547951, SRR3547952, SRR3547953).

††Based on Illumina sequencing data from cultures grown with passage for 20 weeks (accession numbers SRR3547954, SRR3547955, SRR3547956, SRR3547957).

‡‡There is a 400 bp deletion in this strain encompassing the region that would contain this repeat.

§§NGK_2186 and NGO2047 annotations are on opposite strands.

||||This coding sequence appears to be frame-shifted and annotated as two coding sequences.

variable genes *pilC1*, *opa*, and *fetA*, suggesting that read data can support phase variability by demonstrating within-strain variation in tracts (Table 5). Of the 14 genes, only *virG* (NGK_0804) and the pyrimidine 5'-nucleotidase (NGK_2176) showed no changes in repeats (Table 5). Probably, the *virG* (AAGC)₃ copy number is too low to vary, however there may be strains with greater copy number in which it would. Likewise, the poly-C repeat in NGK_2176 has been replaced with CAAACCCC in strain NCCP11945 and therefore would not be expected to phase vary in this strain, however phase variation is likely in strain MS11, for example.

The Ion Torrent sequencing technology has been criticised for generating homopolymer-associated indels (Loman *et al.*, 2012) and that the tracts can be incorrect at more than eight bases (Quail *et al.*, 2012), the optimal length for phase variation. Homopolymeric tracts in Illumina data are believed to be less error prone (Schirmer *et al.*, 2015). However, repeat sequence data from Illumina often agreed with Ion Torrent on the presence of variation (9 of 14 genes with variation in Ion Torrent, Table 5). When the Illumina data did not show repeat variation, this often corresponded to relatively low read coverage of the region compared to the Ion Torrent data (Table S2).

It is currently impossible to differentiate genuine biologically induced indels from sequencing errors (Narzisi & Schatz, 2015). We may find that what we ascribe to errors can also be subtle changes that are constantly being generated within the bacterial population. From this data, the expected biological variation supporting phase variation appears to be present in *N. gonorrhoeae* strain NCCP11945 for 12 as yet unexplored genes.

Conclusion

In conclusion, *N. gonorrhoeae* possesses three different mechanisms for phase variation: transcriptional; translational; and C-terminal. Stochastic systems obviously play important roles in the biology of the organism given the variety and number of genes involved. The functions of previously unexplored phase-variable genes, including one transcriptional phase-variable gene, nine translational phase-variable genes, and four C-terminal phase-variable genes require further investigation.

Acknowledgements

C. P. C. was supported by a Sparks project grant 11KIN01 and 13KIN01. Illumina genome sequencing was provided by MicrobesNG (<http://www.microbesng.uk>), which is supported by the BBSRC (grant number BB/L024209/1).

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