Complete and variant forms of the 'gonococcal genetic island' in *Neisseria meningitidis*

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Comparative genome hybridization using the pan-*Neisseria* microarray identified genes from the gonococcal genetic island (GGI) within *Neisseria meningitidis* strains of serogroups W-135, H, and Z. While some of these strains contain nearly all of the genes of the GGI, there are differences in the presence of some of these genes between the strains, including between those of the same serogroup. Attempts were then made to determine the location of the GGI in these meningococci. Sequencing of *Neisseria gonorrhoeae* strain MS11 revealed that the GGI is a conjugative plasmid that can be chromosomally integrated at the *dif* sites near *ung* and can also be present in its circularized form. In *N. meningitidis*, a *dif* site is present in this location and also serves as the point of chromosomal integration of the GGI in this species.

INTRODUCTION

In *Neisseria gonorrhoeae* strain MS11, a large section of sequence has been identified that is not present in the *N. gonorrhoeae* genome sequence of strain FA1090 (Dillard & Seifert, 2001; Hamilton *et al.*, 2005). This was identified following the investigation of a strain-specific serum resistance gene (McShan *et al.*, 1987), an associated peptidoglycan hydrolase (Dillard & Seifert, 1997), and sequencing of the associated 57 kb flanking sequences, which are present in most, but not all, gonococcal strains (Dillard & Hamilton, 2002; Hamilton *et al.*, 2005). Due to this element's previously reported absence from *Neisseria meningitidis*, it has been called the 'gonococcal genetic island' (GGI) (Dillard & Seifert, 2001; Dillard & Hamilton, 2002). Our annotation of this sequence, provided by Joe Dillard for use in the construction of the pan-*Neisseria* microarray (Snyder *et al.*, 2004), includes 57 potential coding sequences. The sequence has many features that indicate horizontal acquisition of the region, including atypical G+C content, and flanking sequences similar to the *dif* sites recognized by XerCD recombinases in *Escherichia coli* and *Haemophilus influenzae* (Dillard & Hamilton, 2002).

The original pan-*Neisseria* microarray (Snyder *et al.*, 2004) was designed, constructed, and tested by an international consortium and contains probes representing all coding

regions from three neisserial genomes (*N. gonorrhoeae* strain FA1090, and *N. meningitidis* strains MC58 and Z2491) and our predicted gene sequences from the GGI of *N. gonorrhoeae* strain MS11. Probes were designed based upon a comparative analysis of the genomes, so that common probes could be used to assess orthologous genes between the neisserial strains and species. The use of the pan-*Neisseria* microarray to assess gene complements in strains other than those used for its design has been published previously for *N. gonorrhoeae* (Snyder *et al.*, 2004). An extended version of the pan-*Neisseria* microarray (-v2) has been constructed, which includes additional gene probes based on the *N. meningitidis* strain FAM18 genome sequence, neisserial genes from GenBank/EMBL that are not in any of the genome sequences currently available, and genes identified through our ongoing investigations of minimal mobile elements (MMEs) (Saunders & Snyder, 2002; Snyder *et al.*, 2004).

We previously reported the presence of the GGI in *N. gonorrhoeae* strain FA19, identified by comparative genome hybridization (CGH) using the pan-*Neisseria* microarray (Snyder *et al.*, 2004). During a test of the ability of the pan-*Neisseria* microarray-v2 to generate CGH data from diverse strains of *N. meningitidis*, hybridization to the GGI-associated probes was identified in serogroup H strain 98/250521. Due to previous reports suggesting that GGI genes were restricted to *N. gonorrhoeae* (Dillard & Seifert, 2001; Dillard & Hamilton, 2002), these results were repeated and expanded to include other serogroup H strains, as well as strains from other serogroups. The microarray hybridization data suggest that these different *N. meningitidis* capsular serogroup strains contain both complete and partial versions of the GGI.

Abbreviations: CDS, coding sequence; CGH, comparative genome hybridization; GGI, gonococcal genetic island; MLST, multilocus sequence typing; MME, minimal mobile element.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ171942, DQ171943, DQ171944, and DQ171945.

METHODS

The pan-*Neisseria* microarray-v2. The revised and expanded pan-*Neisseria* microarray (-v2) was designed and generated by using the same strategy as and similar protocols to those described previously for the original pan-*Neisseria* microarray (Snyder *et al.*, 2004). The pan-*Neisseria* microarray-v2 contains probes directed to the most conserved regions of the annotated potential coding sequences of *N. gonorrhoeae* strain FA1090 (GenBank accession no. AE004969), *N. meningitidis* strain MC58 (Tettelin *et al.*, 2000), *N. meningitidis* strain Z2491 (Parkhill *et al.*, 2000), and *N. meningitidis* strain FAM18 (<ftp://ftp.sanger.ac.uk/pub/pathogens/nm>). Probes were also included for additional sequences from the *N. gonorrhoeae* strain MS11 GGI (Dillard & Seifert, 2001; Dillard & Hamilton, 2002; Hamilton *et al.*, 2005), neisserial genes from GenBank/EMBL that are not in the genome sequences currently available, genes identified through our ongoing investigations of MMEs (Saunders & Snyder, 2002; Snyder *et al.*, 2004), and antibiotic resistance cassette markers used in mutagenesis. Each probe of 150–450 bp targets the most conserved (>90% similar) non-repetitive region within each coding sequence, achieving maximal hybridization to orthologous genes in different strains and species. Where possible, significant cross-hybridization was eliminated, or noted, to minimize the potential for cross-hybridization with other genes, which would generate false positive results. Because only one sequence of a GGI is currently available, it was not possible to specifically select probes from within the most conserved regions of these coding sequences; therefore, a greater number of weak or false negative results may occur if these genes are divergent. The PCR product probes generated were suspended in Genetix Spotting Solution for Amine slides. The microarray slides were printed with a Genetix QArray Mini microarray printer using 150 µm solid aQu pins onto Genetix Amine slides.

Bacterial strains used. Representative strains from serogroups C (strains SB25 and 2120), W-135 (strain A22), X (strain 860060), Y (strain 860800), and Z (strain E32) were obtained from the MLST (multilocus sequence typing) meningococcal strain collection (<http://pubmlst.org/neisseria/>). To supplement these, additional strains of atypical serogroups were obtained from the Meningococcal Reference Laboratory [Manchester Public Health Laboratory Service (PHLS), UK]. In all, the CGH data analysed were from *N. meningitidis* strains MC58 (serogroup B, sequence strain), Z2491 (serogroup A, sequence strain), FAM18 (serogroup C, sequence strain), SB25 (serogroup C, MLST strain), 2120 (serogroup C, MLST strain), A22 (serogroup W-135, MLST strain), 860060 (serogroup X, MLST strain), 860800 (serogroup Y, MLST strain), E32 (serogroup Z, MLST strain), 00/240794 (serogroup D, reference strain NCTC 009714), 01/241825 (serogroup 29E, PHLS strain), 98/250521 (serogroup H, PHLS strain), 97/252572 (serogroup H, PHLS strain), 97/252675 (serogroup H, PHLS strain), 01/241809 (serogroup X, PHLS strain), 01/241693 (serogroup X, PHLS strain), 00/240868 (serogroup Z, PHLS strain), 01/241422 (serogroup Z, PHLS strain), and 01/241471 (serogroup Z, PHLS strain). *N. gonorrhoeae* strains MS11 and FA19, as strains known to contain the GGI (Snyder *et al.*, 2004), were hybridized to the pan-*Neisseria* microarray-v2 as controls.

Comparative genome microarray hybridization. Chromosomal DNA was extracted (McAllister & Stephens, 1993) from the neisserial strains following growth on GC medium (BD) with Kellogg and ferric nitrate supplements (Kellogg *et al.*, 1963) at 37 °C, in 5% (v/v) CO₂ overnight. DNA (20 µg) was fluorescently labelled through direct incorporation of FluoroLink Cy3-dCTP or FluoroLink Cy5-dCTP (Amersham Biosciences) using 5 units DNA polymerase I Klenow fragment (Bioline) and 3 µg random hexamer primers (Invitrogen). Unincorporated nucleotides and random primers were removed using QIAquick Nucleotide Removal columns

(Qiagen) according to the manufacturer's instructions. Labelled DNA-DNA probe microarray hybridizations were conducted in 4 × SSC, 0.29% SDS under LifterSlips (Erie Scientific) at 65 °C overnight. The slides were washed at 65 °C in 1 × SSC, 0.05% SDS for 2 min, at room temperature in 0.06 × SSC for 2 min, and in fresh 0.06 × SSC for 2 min before drying by airbrush (Paasche, with SimAir compressor). Microarray slides were scanned using a ScanArray Express HT Microarray Scanner (Perkin Elmer) and analysed using ScanArray Express software v2.1 (Perkin Elmer).

Each strain was compared against two other strains in a three-way experimental design, such that strains A, B, and C were compared as A vs B, B vs C, and C vs A. The presence or absence of any gene in one pairwise analysis was validated by comparison to the results of the unrelated comparative hybridization. If necessary, hybridizations were repeated to resolve ambiguities.

Dinucleotide signature analysis and G+C content determination. Gene-by-gene dinucleotide signature analysis has previously been applied to the complete genome sequence of *N. meningitidis* strain MC58 (Tettelin *et al.*, 2000) and to the genes of the neisserial *dcw* cluster in *N. gonorrhoeae* (Snyder *et al.*, 2001). A revised version of this methodology was used for this study, which compensates for the sampling effects of addressing variable gene lengths that tend to artefactually increase the divergence of shorter sequences (Saunders *et al.*, 2005). The variation in the signature of each individual coding region, in comparison to the signature of the genome as a whole, can indicate genes that have been acquired from other species with different compositional biases. Dinucleotide signatures were determined for the complete genome of *N. gonorrhoeae* strain FA1090 for reference, for each of the coding regions of this genome, and for the *N. gonorrhoeae* strain MS11 GGI (GenBank accession no. AY803022). The deviation from the mean genome signature of each coding sequence was determined and compared against the mean divergence of all annotated coding sequences in the genome.

The percentage of G and C bases within each gene was determined and compared with the G + C content of the *N. gonorrhoeae* strain FA1090 genome sequence.

GGI location determination using microarrays. The determination of the location of the GGI was attempted using a modification of the CGH protocol described above. Specific primers that pointed out from the GGI (Table 1) were used instead of random primers. Primers at the GGI01 end of the GGI were used with FluoroLink Cy3-dCTP, and primers at the GGI56/GGI57 end of the GGI were used with FluoroLink Cy5-dCTP (Amersham Biosciences). All other conditions and methods were identical to those used for CGH.

Table 1. Oligonucleotide primers used in this study

Primer	Sequence
GGI01out1	5'-CTACAGCAATTCGATACTACG-3'
GGI01out2	5'-GAAGTTTACAGACTAAGCCGG-3'
GGI56out1	5'-GTGCAGGCTCAAGGTTGC-3'
GGI56out2	5'-ATTGGCAACGACATTAACATGC-3'
GGI57out3	5'-AACTGCACCCTGAGTATCC-3'
GGIdelF	5'-ATGCGGCCAAAAATCTATACC-3'
GGIdelR	5'-ACAGCAGCCGTATTTTCAGG-3'
GGIRjoinFA	5'-GCGGTGTCAGGTTCAATTTGGC-3'
GGIRjoinMS	5'-AACATCCGCAATCGAACAGG-3'
WGGIdelR	5'-ATGCCCACCTTGAAAAATATGG-3'

GGI mutant construction. To attempt to construct whole island knock-out mutants in the GGI-containing *N. meningitidis* strains, the *N. gonorrhoeae* strain FA1090 region was PCR amplified using *Pfu* Turbo polymerase (Stratagene) with primers GGIdelF and GGIdelR (Table 1) and cloned into pCR-Script-Cam (Stratagene). This region of 2003 bp contains a portion of the genes that flank the GGI in gonococcal strain MS11 (*ung* and XNG0740, see Fig. 1). Through blunt-end digestion of the pCR-Script-Cam vector, with *EcoRV* and *SmaI*, and religation, the *EcoRI* site in the multiple cloning site of the vector was removed. The cloned product contains a hypothetical gene (XNG0741) with naturally occurring *EcoRI* sites just inside the coding region at each end, which were used to insert one of two antibiotic resistance cassettes. A construct was generated containing a kanamycin resistance cassette from pCR2.1 (Invitrogen) fused to the neisserial *recA* promoter and a second construct was made using the kanamycin resistance determinant *aphA-3*, which lacks a promoter and reduces the likelihood of polar effects (Ménard *et al.*, 1994). These plasmids were named pGGIdelP_{recA}kan and pGGIdelaphA-3. PCR amplicons generated using a 1:1000 dilution of the plasmids were used to transform *N. meningitidis* strain 98/250521, as well as *N. gonorrhoeae* strains FA1090 and FA19 as controls. Transformants were selected on GC agar containing 50 mg kanamycin l⁻¹.

Fig. 1. The MME_{ung} and the integration of the GGI at the *dif* site. The MME region adjacent to *ung* has been described previously (Snyder *et al.*, 2004). It is now apparent, however, from CGH and sequencing data, that the GGI present in *N. gonorrhoeae* strain MS11 has inserted at the *dif* site between two hypothetical genes. This event is not in accordance with the MME model; it is rather an insertion into an existing MME region. In *N. meningitidis* strain MC58, the MME_{ung} contains a methylase and NMB1224, rather than the hypothetical gene XNG0741 present in gonococcal strains FA1090 and MS11. The annotated coding region NMB1224 contains a *dif* site, which is the point of integration of the GGI in *N. meningitidis* strain A22 (GenBank accession no. DQ171942).

Sequencing of GGI ends. PCR amplifications from strain MS11 used HotStarTaq polymerase (Qiagen) and primer pairs GGIout1 with GGIdelF, GGIout1 with GGI57out3, and GGI57out3 with GGIdelR (Table 1). The product from *N. gonorrhoeae* strain MS11 generated using primer pair GGI01out1 with GGIdelF was sequenced directly, whereas the other two strain MS11 products were TOPO TA cloned (Invitrogen) for subsequent sequencing using primers M13 Forward and M13 Reverse (Invitrogen). Primer pair GGIRjoinMS with GGIjoinFA was used to generate a new PCR amplicon that was internal to the strain MS11 GGI57out3/GGIdelR product, which was then sequenced using these primers. A PCR product spanning the meningococcal strain A22 *dif* site was generated using GGI56out1 and WGGIdelR, which was designed using the NMB1224 sequence from *N. meningitidis* strain MC58. This spanned the junction between the chromosomal sequence and the GGI sequence. The product was TOPO TA cloned (Invitrogen) and sequenced using M13 Forward and M13 Reverse (Invitrogen). Sequencing used ABI Prism BigDye Terminator Cycle Sequencing version 3.0 (Applied Biosystems), and was resolved on an ABI Prism 3100 DNA Sequencer (Applied Biosystems). Sequences were analysed using Trev from the Staden package (Bonfield *et al.*, 2002) and the Wisconsin Package from GCG (Accelrys). Sequence data were deposited into GenBank under accession numbers DQ171942, DQ171943, DQ171944, and DQ171945.

RESULTS

Comparative genome microarray hybridizations

Controls. Each microarray was assessed for the presence of the genes of the GGI, as determined by hybridization to the printed probes. Hybridizations with the sequenced meningococcal strains (MC58, Z2491, and FAM18) were conducted as negative controls; these did not indicate the presence of the GGI-associated genes. Positive control hybridizations were performed using *N. gonorrhoeae* strains MS11 and FA19, which are known to contain the GGI (Dillard & Seifert, 2001; Snyder *et al.*, 2004). Four of the GGI probes do not consistently generate a signal, although the cause for this is unclear. These probes (for GGI09, GGI43, GGI45, and GGI48) generated clear and bright PCR product bands during microarray production. Additionally, our recent reannotation of the GGI includes a coding sequence (CDS) that was previously unannotated for which no probe has been designed (GGI46). Probes were not made to the repeated sequences flanking GGI26 (sequences annotated as GGI25 and GGI27).

To verify that each of the tested strains was correctly identified as *N. meningitidis*, the microarray results were assessed for the presence of genes such as the *ctr* (NMB0071, NMB0072, NMB0073, and NMB0074) and *lip* (NMB0082 and NMB0083) capsule biosynthesis genes and *opc* (NMB1053). In all cases, these characteristic meningococcal genes were present in the tested meningococcal strains.

Serogroup H. A near complete version of the GGI, with 47 of the 50 probed GGI annotated coding regions, is present in *N. meningitidis* strain 98/250521 (Table 2). Two other serogroup H strains were assessed: *N. meningitidis* strains 97/252572 and 97/252675 (Table 2). Strain

Table 2. Comparative genome hybridization to GGI probes

Columns represent the hybridization of GGI probes in comparative genome microarray hybridization using the pan-*Neisseria* microarray-v2 on *N. meningitidis* strains 98/250521, A22, 97/252675, 00/240868, 01/241422, and 01/241471, and on *N. gonorrhoeae* strains MS11 and FA19. + and – indicate hybridization and lack of hybridization to the probe for the indicated GGI sequence. Gene numbers are from the annotation of GenBank accession no. AY803022 (GGI gene) and from our own annotation of this sequence (CDS).

GGI gene	CDS	Annotation	98/250521 serogroup H	A22 serogroup W135	97/252675 serogroup H	00/240868 serogroup Z	01/241422 serogroup Z	01/241471 serogroup Z	MS11 & FA19
NG5001	GGI01	<i>traD</i> , channelling/conjugative DNA transfer protein	+	+	+	+	+	+	+
NG5002	GGI02	<i>traI</i> , helicase/nickase/conjugative relaxase	+	+	+	+	+	+	+
NG5003	GGI03	Hypothetical	+	+	+	+	+	+	+
NG5004	GGI04	<i>iagB</i> , transglycosylase/similar to coliform <i>pilT</i>	+	+	+	+	+	+	+
NG5005	GGI05	Hypothetical	+	+	+	+	+	+	+
NG5006	GGI06	Hypothetical	+	–	+	+	+	+	+
NG5007	GGI07	<i>traL</i> , pilus assembly protein	+	+	+	+	+	+	+
NG5008	GGI08	<i>traE</i> , pilus assembly protein	+	+	–	+	+	–	+
NG5010	GGI10	<i>traB</i> , pilus assembly protein	+	+	–	–	–	+	+
NG5011	GGI11	<i>dsbC</i> , thiol–disulphide interchange protein	+	+	–	–	–	+	+
NG5012	GGI12	<i>htdD/traV</i> , pilus assembly protein	+	+	–	–	–	+	+
NG5013	GGI13	<i>traC</i> , pilus assembly protein	+	+	–	–	–	+	+
NG5014	GGI14	Hypothetical	+	–	–	–	–	+	+
NG5015	GGI15	<i>traF1</i> , pilus assembly protein	+	+	–	–	–	+	+
NG5016	GGI16	<i>traW</i> , pilus assembly protein	+	+	–	–	–	+	+
NG5017	GGI17	<i>traU</i> , pilus assembly protein	+	+	–	–	–	+	+
NG5018	GGI18	<i>trbC</i> , pilus transfer protein	+	+	–	–	–	+	+
NG5019	GGI19	<i>traN</i> , frameshifted mating stabilization protein	+	+	–	–	–	+	+
NG5021	GGI20	Hypothetical	+	+	–	–	–	+	+
NG5022	GGI21	<i>traF2</i> , pilus assembly protein	+	+	–	–	–	+	+
NG5023	GGI22	<i>traH</i> , pilus assembly protein	+	+	–	–	–	+	+
NG5024	GGI23	<i>traG</i> , self-annotation from Q93NF1	+	+	–	–	–	+	+
NG5025	GGI24	<i>atlA</i> , bacteriophage lysozyme/lysin	–	–	–	–	–	–	+
NG5028	GGI26	<i>cspA</i> , cold shock protein	+	+	–	–	–	+	+
NG5030	GGI28	Hypothetical	+	+	–	–	–	+	+
NG5031	GGI29	Hypothetical	+	+	+	+	+	+	+
NG5032	GGI30	Conserved hypothetical	+	+	+	+	+	+	+
NG5033	GGI31	<i>prlF</i> , <i>htrA</i> suppressor protein	+	+	+	+	+	+	+
NG5034	GGI32	Hypothetical	+	+	+	+	+	+	+
NG5035	GGI33	Frameshifted conserved hypothetical protein	+	+	+	+	+	+	+
NG5036	GGI34	Hypothetical	+	+	+	+	+	+	+
NG5037	GGI35	Hypothetical	+	+	+	+	+	+	+
NG5038	GGI36	Hypothetical	+	+	+	+	+	+	+
NG5039/NG5040	GGI37	Frameshifted restriction methylase	+	+	+	+	+	+	+

Table 2. cont.

GGI gene	CDS	Annotation	98/250521 serogroup H	A22 serogroup W135	97/252675 serogroup H	00/240868 serogroup Z	01/241422 serogroup Z	01/241471 serogroup Z	MS11 & FA19
NG5041	GGI38	Hypothetical	+	+	+	+	+	+	+
NG5043	GGI39	GGI helicase	+	+	+	+	+	+	+
NG5044	GGI40	GGI acetyltransferase	+	+	+	+	+	+	+
NG5045	GGI41	Conserved hypothetical	+	+	+	+	+	+	+
NG5046	GGI42	Conserved hypothetical	-	-	-	-	-	-	-
NG5048	GGI44	Conserved hypothetical	+	+	+	+	+	+	+
NG5050	GGI47	Putative exported/outer-membrane protein	+	+	+	+	+	+	+
NG5053	GGI49	GGI topoisomerase	+	+	+	+	+	+	+
NG5054	GGI50	ssb, GGI single-strand binding protein	+	+	+	+	+	+	+
NG5055	GGI51	Hypothetical	+	+	+	+	+	+	+
NG5056	GGI52	Hypothetical	+	+	+	+	+	+	+
NG5057	GGI53	Bacteriophage DNA replication protein	+	+	+	+	+	+	+
NG5058	GGI54	Hypothetical	+	+	+	+	+	+	+
NG5059	GGI55	Hypothetical	+	+	+	+	+	+	+
NG5060	GGI56	GGI <i>parB/spo0J</i> , putative partitioning protein	+	+	+	+	+	+	+
NG5061	GGI57	GGI <i>parA/soj/minD</i> , putative partitioning protein	-	-	-	-	-	+	+

97/252572 did not hybridize to the GGI probes. Strain 97/252675 hybridized to some of the probes, indicating that portions of the GGI (GGI01–GGI07 and GGI29–GGI41) are present, but large contiguous sections appear to be absent.

Serogroup W-135. One serogroup W-135 strain was assessed, *N. meningitidis* strain A22 (Table 2). The GGI probe hybridization pattern was very similar to that of the serogroup H strain 98/250521, which contains a near complete GGI. Forty-five of the 50 GGI genes interrogated were present in strain A22.

Serogroup Z. Four strains of *N. meningitidis* serogroup Z were assessed: E32, 01/241471, 00/240868, and 01/241422 (Table 2). Strain E32 did not hybridize to the GGI probes. Strains 00/240868 and 01/241422 have identical GGI probe hybridization profiles, which are similar to that of serogroup H strain 97/282675, containing GGI01–GGI08 and GGI29–GGI41. The most complete GGI of those identified in the serogroup Z strains is in strain 01/241471, although GGI42–GGI56 are apparently absent. Other GGI genes are also absent or divergent in this strain, while one of the hybridized probes is not hybridized by any of the other meningococci assessed in this study (GGI57).

Other meningococcal strains. Of the combined MLST/PHLS strain set, strains of serogroups C (strains SB25 and 2120), D (strain 00/240794), 29E (strain 01/241825), X (strains 860060, 01/241809, and 01/241693), and Y (strains O929 and 860800) did not hybridize with GGI-associated probes.

Dinucleotide signature analysis and G+C content

The dinucleotide signatures and G+C content of the predicted coding regions of the GGI are supportive of the horizontal acquisition origin of some genes and segments of the GGI. The weighted deviation of the dinucleotide signature and the G+C content were calculated for each of the 57 coding regions (Fig. 3). The mean divergence of the gonococcal genes in the FA1090 genome is 0.062 %, with an SD of 0.021 and 95 % confidence intervals of 0.0009. Compared with the FA1090 genes, GGI23 (*traG*) is the second most divergent gene not encoded by a repeat (a different source of signature divergence) assessed. These results show that several components of the GGI have sequence characteristics suggesting that they are not neisserial in origin. However, some regions may not have a foreign source, and/or have been in the neisseriae sufficiently long to have ameliorated to a typical neisserial composition.

Determination of GGI chromosomal location by microarray hybridization

In an attempt to determine the chromosomal location of the meningococcal GGI, primers were designed at the ends of

the GGI sequence, such that they would extend outward. We hypothesized that if a specific primer extension product could be generated with incorporated fluorescent nucleotides (as is done using random primers for CGH), then the location of the GGI could be determined based on the gene probes that hybridize to the specific labelled product. Unfortunately, the use of specific primers in the CGH protocol produced non-specific hybridizations. This is probably due to the temperature necessary for Klenow extension (37°C), leading to non-specific annealing of the primers. It was noted, however, that the genes of the GGI were the brightest of the features on the microarray following this labelling procedure – which was the opposite of the predicted result. This led us to speculate whether the GGI was present as an extrachromosomal circle, which could account for outward pointing primers producing signal for internal genes.

Knock-out mutants of the GGI

N. gonorrhoeae strains FA1090 and FA19 and *N. meningitidis* strain 98/250521 were transformed with PCR products generated using specific primers (GGIdelF and GGIdelR, Table 1) and pGGIdelP_{recA}kan or pGGIdelaphA-3 as the templates. Kanamycin-resistant colonies were obtained for all three strains following transformation, and colony PCR using kanamycin cassette directed primers produced amplicons of the expected sizes. Transformants of strains FA19 and 98/250521 were grown overnight without selection for

DNA preparation. Hybridization to the pan-*Neisseria* microarray-v2 indicated that the GGI genes are still present in these strains, further suggesting that the GGI genes were present in more than one location.

Sequencing demonstrates the chromosomal and extrachromosomal nature of the GGI

Given the observations suggesting both chromosomal and additional extrachromosomal locations for the GGI, primers were designed to generate products that would be specific and confirm the presence of this element in both states. Sequencing from *N. gonorrhoeae* strain MS11 (GenBank accession nos DQ171943 and DQ171944) joined both ends of the established GGI sequence (AY803022) to the *N. gonorrhoeae* strain FA1090 genome sequence (AE004969) at the *dif* site (Figs 1 and 2) identified previously (Hamilton *et al.*, 2005). In contrast to our previous observations, these sequence data (GenBank accession no. DQ171943) revealed that the strain FA1090 hypothetical gene XNG0741 is present in strain MS11 and that the GGI has inserted within the MMEung at the *dif* site (Fig. 1), rather than according to the MME model (Snyder *et al.*, 2004). However, the variation in gene presence within this MME between strains FA1090, MC58, and FAM18 would still classify this location as an MME.

Hybridization of these GGI-containing *N. meningitidis* strains to the genes within the *N. meningitidis* strain MC58

Fig. 2. The GGI is present both in the chromosome and as an extrachromosomal plasmid. Sequencing of *N. gonorrhoeae* strain MS11 using primers within *ung* (GenBank accession no. DQ171943) and XNG0740 (GenBank accession no. DQ171944) confirmed its location in the chromosome (see Fig. 1), having integrated at the *dif* site between XNG0741 and XNG0740. Sequencing of a PCR product generated with primers pointing out from the ends of the GGI revealed that this element is also present as an extrachromosomal circle (GenBank accession no. DQ171945). The *dif* site serves as the junction between the beginning and end of the GGI sequence (GenBank accession no. AY803022).

MMEung (Snyder *et al.*, 2004) suggests that this region is similar to that of the meningococci, rather than the gonococci (Fig. 1). In *N. meningitidis*, the *dif* site is within the annotated hypothetical protein coding region NMB1224. Sequencing of *N. meningitidis* strain A22 using primers WGGIdifR and GGI56out1 revealed that this region is similar to the MMEung in *N. meningitidis* strain MC58 and that the GGI has inserted at the *dif* site (Fig. 1) at this location (GenBank accession no. DQ171942).

Sequencing of the strain MS11 amplicon generated using primers GGI01out1 and GGI57out3 (GenBank accession no. DQ171945) confirmed the existence of a circularized form of the GGI in which the two 'outward' ends are joined at the *dif* site (Fig. 2).

DISCUSSION

N. meningitidis serogroup H strain 98/250521 and *N. meningitidis* serogroup W-135 strain A22 hybridize to nearly all of the probes to the GGI. These are the first meningococcal strains to be identified that contain the GGI, albeit one that may be incomplete in comparison to the model GGI from *N. gonorrhoeae* strain MS11. *atlA*, the GGI *parA*, and a conserved hypothetical gene (GGI42) are apparently absent or divergent from both of these meningococcal strains. The probes for *atlA* and the conserved hypothetical gene did not hybridize to any of the meningococcal strains, while the GGI *parA* probe only hybridized to one of the strains (01/241471).

Differential presence of genes within the GGI has been previously noted and is the basis for the defined classes of GGI (Dillard & Seifert, 2001). Class I GGIs contain the genes that have been sequenced from *N. gonorrhoeae* strain MS11 (GenBank accession no. AY803022) (Dillard & Seifert, 2001; Dillard & Hamilton, 2002; Hamilton *et al.*, 2005). Class II GGIs contain the *sac-4* allele of *traG*. The absence of *atlA* from the GGI of some gonococcal strains has been reported previously as class III GGIs and is associated with an alternative sequence present in the equivalent location, although its nature has not been determined (Dillard & Seifert, 2001). The absence of *atlA* in these meningococcal versions of the GGI suggests that they belong to class III. It would be interesting, therefore, to compare a class III gonococcal GGI with those from *N. meningitidis* strains 98/250521 and A22. In addition, all but one of the strains lacked hybridization to the GGI *parA* probe; therefore, this may indicate either a meningococcal or a GGI class III-specific variation in this sequence, particularly since the associated GGI *parB* gene is present in these strains and sequencing from strain A22 indicated that at least a portion of *parA* is present (GenBank accession no. DQ171942).

The previous classification of the GGI as specific to *N. gonorrhoeae* was based on Southern blots conducted using just two hybridization probes: to *atlA* and *traG*, both of which were known to be variably present in the GGIs (Dillard & Seifert, 2001). The absence of the *atlA* gene in

class III GGIs of *N. gonorrhoeae* is also seen in the meningococcal strains; therefore, the use of this gene as a probe would have prevented identification of any class III GGIs in the strains tested. Further, class II GGIs contain a divergent allele of *traG* (*sac-4*), therefore a *traG* probe would not necessarily have identified strains with class II GGIs. The *N. meningitidis* strains tested previously were of serogroups A, B, C, and D (Dillard & Seifert, 2001). Our microarray hybridizations with DNA from these serogroups also did not reveal GGI sequences.

Although probes on the pan-*Neisseria* microarray are designed to the most conserved regions of the genes where multiple sequences of individual genes are available, there was only one GGI sequence available for design, from *N. gonorrhoeae* strain MS11. If there are differences (probably greater than at least 20 % divergence) between different allelic versions of genes, then this would lead to the lack of hybridization to the probe, and detection in this system. Specific primer pairs designed using the *N. gonorrhoeae* strain MS11 island sequence frequently failed to generate specific PCR products from meningococcal templates, further indicating divergence of these sequences.

If the GGI is contiguous and present in the same location in the *N. meningitidis* strains as it is in *N. gonorrhoeae* strain MS11, then generation of knock-out mutants of the entire GGI would make this clear when the parent and mutant DNA are hybridized to the microarray. However, after preparation of DNA from the transformants and hybridization to the microarray, it was revealed that the genes of the GGI are still present. The same was true for the *N. gonorrhoeae* strain FA19 transformants; therefore, sequencing was conducted to confirm the chromosomal location of the GGI. It was noted previously that an artificial construct containing the *dif* site readily excised from the chromosome (Hamilton *et al.*, 2005). If the reverse is true, it may be that the GGI reintegrated at the *dif* site that is still present in the mutant constructs.

Sequence data revealed that, in strain MS11, the GGI is present both within the chromosome and as an extra-chromosomal circular DNA element (Fig. 2). The genes of the GGI share significant similarity with the genes of the F plasmid (Hamilton *et al.*, 2005), therefore its presence as a free and as a chromosomally integrated plasmid is, on this basis, to be expected. The type IV secretion system encoded by the GGI has been shown to secrete chromosomal DNA, much the same as other integrated conjugative plasmids. Unlike most conjugation systems, cell-cell contact is not required, although this may be because it is not necessary in the naturally competent *Neisseria* species. It has additionally been shown that this secreted DNA is capable of neisserial transformation (Hamilton *et al.*, 2005). Indeed, a neisserial conjugative plasmid was identified previously, but while the GGI is 57 kb, the conjugative plasmid was reported as being 37 kb (Elwell & Falkow, 1977). These may represent different unrelated plasmids, or it is possible that these are variants of the GGI plasmid.

Fig. 3. Dinucleotide signature analysis and G+C content of the *N. gonorrhoeae* strain MS11 GGI. Each annotated feature is plotted along the *x* axis, with the dinucleotide signature weighted deviation from the genome signature (bars) on the left *y* axis and the G+C content of each gene (solid line) on the right *y* axis. The dotted line delineates 52%, the mean neisserial G+C content.

In its circularized form, the adjacent coding regions of the GGI are now orientated in the same direction. Strand switching for the coding regions occurs only between GGI03 and *iagB* (GGI04) and between *atla* (GGI24) and *cspA* (GGI26) (Fig. 2). While as an integrated island, the organization is less structured, in the circularized form, the global organization of the genes and potential transcriptional units is more coherent.

The absence of some of the GGI genes in different strains suggests either that the complete GGI has developed from the combination of distinct segments, or that portions of it have been deleted or added. Compared with the genome sequence, there are distinct differences in the G+C content and dinucleotide signatures of the genes of the GGI of *N. gonorrhoeae* strain MS11, which suggests that these genes may have had a foreign origin (Fig. 3). However, it also shows features of considerable amelioration, or having origins not greatly divergent in composition, that suggest that it has been present within the *Neisseria* species for a relatively long time. A 'foreign' origin is also consistent with the absence of the common neisserial intergenic Correia repeat (Correia *et al.*, 1986, 1988), but it may also be that these elements are not maintained on neisserial plasmids.

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