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Prevalence and Detailed Mapping of the Gonococcal Genetic Island in *Neisseria meningitidis*

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The 57-kb gonococcal genetic island (GGI) encodes a type IV secretion system (T4SS) that is found in most strains of *N. gonorrhoeae*. This T4SS functions to secrete single-stranded DNA that is active in natural transformation. The GGI has also been found in some strains of *N. meningitidis*. We screened 126 isolates of *N. meningitidis* and found the GGI in 17.5% of strains, with the prevalence varying widely among serogroups. The GGI is found in a significant number of serogroup C, W-135, and X strains but was not found in strains of serogroup A, B, or Y. Through detailed PCR mapping and DNA sequencing, we identified five distinct GGI types in meningococci. DNA sequencing and a genetic assay revealed that the GGI was likely integrated into the meningococcal chromosome by the site-specific recombinase XerCD and that the GGI can be excised and lost from the genome. Functional studies showed that in contrast with the gonococcal T4SS, the meningococcal T4SS does not secrete DNA, nor does it confer Ton-independent intracellular survival. Deletion of T4SS genes did not affect association with or invasion of host cells. These results demonstrate that the GGI is found in a significant proportion of meningococcal strains and that while some strains carry multiple insertions and deletions in the GGI, other strains carry intact T4SS genes and may produce functional secretion systems.

Neisseria meningitidis (meningococcus [Mc]) is a symbiont of the human nasopharynx and can also cause meningitis and septicemia (30). Up to 10% of the healthy adult population is colonized transiently with Mc, although this percentage can be significantly higher in college residence halls and military barracks (5). The closely related species *Neisseria gonorrhoeae* (gonococcus [Gc]) is the etiological agent of the sexually transmitted disease gonorrhea. Untreated gonococcal infections can progress to pelvic inflammatory disease or disseminate further, causing arthritis, endocarditis, and meningitis (12, 38). One significant difference between Mc and Gc is that Mc are able to produce a polysaccharide capsule, while Gc do not produce a capsule due to the absence of some of the genes that are required for capsule biosynthesis (26). Approximately 80% of gonococcal strains possess the gonococcal genetic island (GGI), while a limited number of meningococci have been found to have the GGI (10, 37). In gonococci, the GGI is 57 kb and contains genes for a type IV secretion system (T4SS) (10, 14).

Several genomic islands have been identified in *N. meningitidis*. In 2000, Klee et al. (19) found eight genomic islands in strains of Mc, five of which were conserved across a panel of representative strains of meningococci or were homologous with known virulence factors. Another screen using strains of Mc with various pathogenic potentials found a prophage prevalent in the chromosomes of invasive disease-causing meningococci (1). By screening a pan-*Neisseria* microarray with meningococcal genomic DNA, Snyder et al. (37) found the GGI in six Mc strains. However, these were mostly from serogroups that are not frequently associated with disease. It was unclear from these results whether the GGI is common in disease isolates and whether GGI-carrying strains can produce a functional T4SS. In Gc, the GGI encodes a T4SS that is responsible for secretion of single-stranded DNA, which is active in natural transformation (14, 28, 32). Other T4SSs have been shown to secrete proteins necessary for intracellular infection, to be required for cell adhesion, and to secrete DNA and proteins directly into host cells (4, 6, 23, 34, 40).

Here we found that the GGI is present in approximately 17% of Mc strains and that the percentage varies by serogroup. Mapping of the GGIs in *N. meningitidis* strains using PCR identified five different GGI types that had regions where insertions or deletions occurred relative to the gonococcal GGIs. Insertions or deletions were found to disrupt genes for type IV secretion in several strains; however, two strains have GGI types that are nearly complete in their T4SS-encoding regions. Measurement of GGI loss frequencies suggested that changes in the *difB* sequences diminished GGI loss to a rate lower than that seen in *N. gonorrhoeae*. We also report that meningococci with a GGI do not exhibit a T4SS-dependent increase in DNA donation for natural transformation or differences in association with or invasion of ME180 host cells. Despite the occurrence of GGI types that have T4SS that are highly similar to the GGI in gonococci, the meningococcal T4SS does not function in the same manner and may secrete other effectors we have not detected.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains and plasmids used in this study are listed in Table 1 and in Table S1 in the supplemental material. Recent clinical isolates of meningococci were grown on GCB agar (Difco) plates supplemented with IsoVitaleX (Becton Dickinson Co.) at 37°C with 5% CO_2 or grown with aeration in GC base liquid (GCBL) medium (1.5% Proteose peptone no. 3, 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.1% NaCl [pH 7.2]) containing IsoVitaleX and 0.042% $NaHCO_3$. All other meningococci and all gonococci were grown on GCB agar plates

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TABLE 1 Strains and plasmids

Strain or plasmid	Properties	Source or reference
N. meningitidis strains		
98/250521	Clinical isolate, serogroup H	PHLS
98/250521 Str	98/250521 transformed with pNMD42 [*rpsL*(*K43R*)]	This study
97/252675	Clinical isolate, serogroup H	PHLS
00/240868	Clinical isolate, serogroup Z	PHLS
01/241422	Clinical isolate, serogroup Z	PHLS
01/241471	Clinical isolate, serogroup Z	PHLS
01/241471 Str	01/241471 transformed with pNMD42 [*rpsL*(*K43R*)]	This study
ATCC 13102	Clinical isolate, serogroup C	ATCC
13102 Str	ATCC 13102 transformed with pNMD42 [*rpsL*(*K43R*)]	This study
JD1628	ATCC 13102 transformed with THH51 (*pacA*::*ermC*)	8
KL1011	01/241471 transformed with pHH35; *traH* deletion mutant	This study
KL1015	01/241471 transformed with ND515 (*exp1*::mTn*CmPhoA*)	This study
KL1017	01/241471 transformed with JD1628 (*pacA*::*ermC*)	This study
KL1018	KL1017 transformed with pKL14 (*pacA*::*ermC pilT*::*kan*)	This study
KL1019	KL1011 transformed with JD1628 (Δ*traH pacA*::*ermC*)	This study
KL1020	KL1019 transformed with pKL14 (Δ*traH pacA*::*ermC pilT*::*kan*)	This study
KL1021	01/241471 transformed with KL1015 (*exp1*::mTn*CmPhoA*)	This study
KL1022	97/252675 transformed with KL1015 (*exp1*::mTn*CmPhoA*)	This study
KL1023	01/241471 transformed with KL1021 (*exp1*::mTn*CmPhoA* backcross)	This study
KL1024	97/252675 transformed with KL1022 (*exp1*::mTn*CmPhoA* backcross)	This study
KL1025	KL1023 transformed with MS11 [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA*]	This study
KL1028	98/250521 Str transformed with (98/250521XND515) (*exp1*::mTn*CmPhoA* backcross)	This study
KL1029	KL1023 transformed with pNMD42 [*rpsL*(K43R)]	This study
KL1030	KL1024 transformed with pNMD42 [*rpsL*(K43R)]	This study
KL1031	KL1028 transformed with pKL15 [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA rpsL*]	This study
KL1032	KL1029 transformed with pKL15 [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA rpsL*]	This study
KL1033	KL1030 transfomed with pKL15 [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA rpsL*]	This study
KL1034	KL1031 transformed with pVD300*recA4* [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA rpsL recA*::*tetM*]	This study
KL1035	KL1032 transformed with pVD300*recA4* [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA rpsL recA*::*tetM*]	This study
KL1036	KL1033 transformed with pVD300*recA4* [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA rpsL recA*::*tetM*]	This study
KL1037	01/241471 Str transformed with pKL19 [*rpsL*(*K43R*) Δ*tonB*::*ermC*]	This study
KL1038	13102 Str transformed with pKL19 [*rpsL*(*K43R*) Δ*tonB*::*ermC*]	This study
KL1040	13102 Str transformed with KL1025 [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA*]	This study
KL1041	13102 Str transformed with KL1040 [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA* backcross]	This study
KL1044	KL1041 transformed with pKL15 [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA rpsL*]	This study
KL1045	KL1044 transformed with pVD300*recA4* [*rpsL*(*K43R*) *exp1*::mTn*CmPhoA rpsL recA*::*tetM*]	This study
KL1046	KL1032 from loss assay [*rpsL*(*K43R*) ΔGGI]	This study
KL1047	KL1046 transformed with pKL19 [*rpsL*(*K43R*) ΔGGI Δ*tonB*::*ermC*]	This study
KL1048	JD1628 transformed with pKL14 (*pacA*::*ermC pilT*::*kan*)	This study
KL1049	JD 1628 transformed with pHH35 (*pacA*::*ermC* Δ*traH*)	This study
KL1050	KL1049 transformed with pKL14 (*pacA*::*ermC* Δ*traH pilT*::*kan*)	This study
KL1051	KL1044 transformed with ND518 [*rpsL*(*K43R*) ΔT4SS]	This study
KL1052	KL1051 transformed with pKL19 [*rpsL*(*K43R*) ΔT4SS Δ*tonB*::*ermC*]	This study
KL1053	13102 Str transformed with pSB2 (pSB2 insertion into 13102)	This study
KL1054	13102 Str transformed with pKL22 (pKL22 insertion into 13102)	This study
N. gonorrhoeae strains		
MS11	Wild-type *N. gonorrhoeae*	39
FA19	Wild-type *N. gonorrhoeae*	H. S. Seifert
ND515	MS11 transformed with HH522 (*exp1*::mTn*CmPhoA*)	11
ND518	ND500 transformed with pJS3D (ΔGGI Erm island)	This study
ND569	ND519 transformed with pNMD38 (*exp1*::mTn*CmPhoA difA*$^+$ *difA*$^+$ *rpsL*)	11
Plasmids		
pKL6	*traD* complement in pKH37	31
pKL13	01/24147171 77F-83R in pNMD37 at SpeI/SacI	This study
pKL14	Kan^r from pKH99 digested with EcoRV/Ecl136II in pHH42 digested with AgeI and blunted	This study
pKL15	*rpsL ermC* from pKL13 into pKL6 at HindIII	This study
pKL16	3′ end of *NGO1380*-5′ end of *exbB* from 13102 into pIDN1 at BamHI/PstI	This study

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid	Properties	Source or reference
pKL17	*tonB* deletion from pKL16, religated at XmaI	This study
pKL18	*ermC* from pIDN1 inserted in *tonB* deletion from pKL17 at XmaI	This study
pKL19	Δ*tonB*::*ermC* insert from pKL18 in pKH6 at SacI	This study
pKL22	13102 183F-144R in pIDN1 at PspOMI/KpnI	This study
pSB2	13102 195F-64R inserted in pIDN1 at SacI/SacII	This study
pSB3	Chromosme walking product containing 3.5 kb of GGI from 13102 adjacent to *yfeA*	This study
pSB4	Chromosme walking product containing 4.2 kb of GGI from 13102 adjacent to *yecA*	This study
pVD300*recA4*	*tetM* inserted in *recA*	35
pHH35	*traH* deletion, 990-bp in-frame deletion in pJD1181	11
pHH42	*pilT* mutant (K136Q)	42
pKH6	*cat* from pGCC6del inserted at HindIII/ClaI of pIDN1, *ermC* cut out with SalI and XbaI	This study
pKH37	Complementation vector	20
pKH99	Kan^r from pHSS6 into BamHI site of pIDN2	This study
pNMD37	PCR product of *difB* and flanking DNA outside GGI from IN522 into pNMD36 at SpeI/SacI	This study
pNMD42	MS11 *rpsL*(*K43R*) into pIDN2 at KpnI/XhoI	This study
pHSS6	Cloning vector	36
pIDN1	Cloning vector	15
pIDN2	Cloning vector	15

with Kellogg's supplements at 37°C with 5% CO_2 or in GCBL with Kellogg's supplements and 0.042% $NaHCO_3$ with aeration (18, 24).

GGI screening. Recent clinical isolates were obtained from the Wisconsin State Laboratory of Hygiene (WSLH), Active Bacterial Core Surveillance Team and the Emerging Infection Programs Network of the U.S. Centers for Disease Control and Prevention (CDC), and L. H. Harrison (University of Pittsburgh). Colonies were resuspended in colony lysis solution (1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl [pH 8.0]) and lysed at 95°C for 15 min (41). DNA from lysates was used as the template for PCR to screen for the GGI. We used primers 77F and hlhggiR (see Table S2 in the supplemental material for primer sequences) to amplify *dif* and flanking DNA to test for the absence of the GGI, and we used 137F and 118R to amplify a conserved fragment within the GGI. Due to the heterogeneous nature of sequences between *dif* and *ung* in meningococci, Southern blotting was used to confirm the absence of the GGI in several isolates. Chromosomal DNA isolation and purification was performed as described by Dillard (7). Probes were generated by PCR amplification of fragments from *traI* (53F-235R), *oriT* (247F-254R), *ydhAB* (193F-218R), and *yea* (197F-72R). Southern blotting was performed according to the method described by Sambrook et al. (33).

PCR walking and GGI typing. Chromosomal DNA was isolated and purified from gonococcal strain MS11 and meningococcal strains 01/241471, 98/250521, 01/241422, 97/252675, 00/240868, A22, and ATCC 13102 using the DNeasy kit (Qiagen) according to manufacturer's instructions. Meningococcal strains were obtained from the Manchester Public Health Laboratory Service (PHLS), D. A. Caugant (Norwegian Institute of Public Health), and the American Type Culture Collection (ATCC). We performed successive PCRs beginning with DNA flanking the GGI. Each target was approximately 2 kb in *N. gonorrhoeae* strain MS11, and each product overlapped the next by about 500 bp (see Table S3 in the supplemental material for primers). The amplified DNA was run on a 0.8% agarose-TBE gel. Any fragments from meningococci that were a different size than the MS11 fragment were sequenced at the University of Wisconsin—Madison Biotechnology Center using the BigDye fluorescent sequencing method according to the manufacturer.

Recent clinical isolates with the GGI were analyzed for GGI type by colony lysis PCR (see Table S1 in the supplemental material). GGI$^+$ *N. meningitidis* isolates were judged to carry GGI type 1 if there was a 3.5-kb fragment from a PCR using primers 155F and 106R, representing a deletion from *yecB-parB* (see Table S3 in the supplemental material for primer sequences and targets). To test for GGI type 2, insertions in *yaa* and *traD* were observed using primers 83F-82R and 137F-118R, respectively. GGI type 3 was identified by the presence of a 1.4-kb product from a PCR with 77F-76R (truncation of *traD*) and a 1.4-kb product from 45F-46R (IS*1655* in *traK*). To distinguish GGI type 3 from GGI type 4, we looked for a 1.1-kb product (type 3) or a 1.2-kb product (type 4) from a reaction with 63F-184R. GGI type 5 was identified through amplification from 155F-106R and a 3.0-kb product from 113F-1105-6R, indicating a deletion from *traE-ydbA*.

Loss assay. We constructed strains to select for GGI loss in four strains: 01/241471, 97/252675, 98/250521, and ATCC 13102. Each strain was transformed by spot transformation with pNMD42 to introduce an *rpsL* allele conferring streptomycin (Str) resistance at the chromosomal locus. This strain was transformed with chromosomal DNA from ND515, carrying *exp1*::mTn*CmPhoA*, in order to introduce a chloramphenicol (Cm) resistance marker and *phoA* into the GGI, allowing blue/white screening. The resulting strain was transformed with pKL15 to introduce an erythromycin (Erm) marker and an *rpsL* allele in the GGI. The *rpsL* allele in the GGI encodes for streptomycin sensitivity, which is dominant over streptomycin resistance (22). In order to prevent homologous recombination between the two *rpsL* genes, the strain was transformed with pVD300*recA4*, which has a tetracycline (Tet) resistance marker inserted into *recA*. Each of the final strains was resistant to Tet, Erm, and Cm, PhoA positive on XP indicator plates, and sensitive to Str.

Loss frequency was determined essentially as described by Domínguez et al. (11). Individual colonies were streaked onto GCB Cm plates (10-μg/ml final concentration) and grown overnight to ensure the presence of the GGI at the beginning of the assay. To allow for GGI loss, Cm-resistant colonies were grown overnight on GCB agar plates without selection. After 16 to 18 h of growth, bacteria were swabbed from plates into GCBL and plated in duplicate on GCB plates without selection to enumerate total CFU/ml and on GCB-Tris-XP (40 μg/ml) plates with Str (100 μg/ml) to select and screen for GGI loss. Loss frequency is the ratio of Str-resistant, PhoA$^-$ CFU to total CFU. Reported frequencies are the geometric means from three independent experiments.

Coculture transformation. Donor strains for coculture transformation were constructed in ATCC 13102 and 01/241471 backgrounds, as these strains had the most complete T4SS-encoding regions. 01/241471 was transformed with chromosomal DNA from JD1628 (ATCC 13102 *pacA*::*ermC*) to introduce an Erm resistance marker. Test strains were then transformed with pKL14 to insert a kanamycin (Kan) resistance marker in *pilT* to ensure that the strain could act only as a DNA donor in natural trans-

formation. Negative-control strains were constructed by transforming strains with *pacA*::*ermC* with pHH35 to generate an in-frame deletion of *traH* (11). *traH* is required for DNA secretion via the T4SS (15). The *traH* deletion strains were then transformed with pKL14 to introduce the *pilT* mutation. Recipients were constructed by transformation of ATCC 13102 or 01/241471 with pNMD42, introducing Str resistance.

We grew strains overnight on GCB plates without selection. Individual colonies were then restreaked onto appropriate selection, GCB with Erm (10 μg/ml) for donors and GCB with Str (100 μg/ml) for recipients. After 16 to 18 h growth, strains were inoculated in GCBL plus supplements at an optical density at 540 nm (OD_{540}) of 0.19 to 0.21. After 2 h growth, strains were diluted 1:32 in fresh GCBL. At this point, donors and recipients were mixed together and grown in coculture for 3 h. At 0 h and 3 h, strains were plated in duplicate onto GCB without selection, GCB Erm, GCB Str, and GCB Erm (5 μg/ml) Str (100 μg/ml). Transformation frequency was calculated as the number of transformants (CFU/ml on GCB Erm Str) per donor (CFU/ml on GCB Erm). Reported frequencies are the geometric means from three independent experiments.

Intracellular survival assay. We deleted *tonB* in ATCC 13102 and 01/241471 by spot transformation with pKL19 and selection on GCB plates containing Erm (10 μg/ml). We also constructed *tonB* deletions in strains lacking the GGI obtained by selection in the loss assay (01/241471 background) or by transformation with chromosomal DNA (ATCC 13102 background). Strains were grown overnight on GCB plates. Piliated colonies were selected, restreaked, and grown for 16 h on GCB plates.

Intracellular growth in the absence of inorganic iron was assayed using a modification of the method described by Zola et al. (42). ME180 cervical epithelial cells were seeded at a density of 2.5×10^5 cells/ml to a 12 well plate and grown to 70 to 80% confluence at 37°C with 5% CO_2 in McCoy's 5A medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) (Gibco). Meningococci were resuspended in McCoy's 5A supplemented with 10% HI-FBS and 24 μM $Fe(NO_3)_3$ and allowed to infect monolayers of ME180 cells at a multiplicity of infection (MOI) of 10 for 4 h at 37°C with 5% CO_2. After initial infection, wells were washed twice with Chelex-treated PBS and then treated with McCoy's 5A with 10% HI-FBS and 25 μg/ml gentamicin (Sigma) for 1 h to kill any extracellular meningococci. After gentamicin treatment, cells from time zero were lysed by treatment with 0.5% saponin in Chelex-treated PBS for 1 min, harvested, and plated for meningococcal CFU counts onto GCB plates. The remaining cells had media replaced with McCoy's 5A plus 10% HI-FBS and were incubated at 37°C with 5% CO_2 for 4 or 24 h. One hour before each time point, cells were treated with gentamicin, followed by lysis and harvest. Each experiment was performed in triplicate, and final CFU/ml values are the geometric means from three independent experiments.

Association and invasion assay. ME180 cells were seeded into 24-well plates at a density of 4.0×10^5 cells/ml in a total volume of 0.5 ml of McCoy's 5A with 10% HI-FBS and grown to 80% confluence. The cells were infected with meningococcal strains ATCC 13102 and 01/241471 and isogenic $T4SS^-$ strains at an MOI of 100 for 3.5 h at 37°C with 5% CO_2. Total CFU/ml was enumerated by plating supernatants and lysates to GCB plates. Lysis occurred by incubation with 0.5% saponin in PBS for 5 min. Wells to enumerate associated and invaded CFU/ml were washed six times with PBS. Contents of association wells were lysed and plated on GCB agar plates. Invaded wells were treated with 25 μg/ml gentamicin for 1 h and incubated at 37°C with 5% CO_2. Wells were washed an additional six times with PBS, and lysates were plated onto GCB. Each experiment was carried out in triplicate. Association and invasion frequencies shown are the geometric means from three individual experiments.

RESULTS

Survey of *N. meningitidis* strains for the GGI. When the GGI was initially discovered in *N. gonorrhoeae*, attempts were made to identify it in other *Neisseria* species using low-stringency Southern hybridization (10). It was not found in any symbiotic *Neisseria* species or any of 9 *N. meningitidis* strains. However, the two GGI genes used for the hybridization studies turned out to be variable in the GGI, and Snyder et al. (37) identified versions of the GGI in six Mc strains. Given the small number of Mc strains tested, it was not clear how common the GGI is in the meningococcal population. Therefore, we screened clinical isolates of *N. meningitidis* for the presence of the GGI using three panels of isolates. One panel contained *N. meningitidis* isolates from the Wisconsin State Laboratory of Hygiene (WSLH), which were collected from patients in Wisconsin with invasive meningococcal disease. The second was a panel from the University of Pittsburgh that also contained invasive disease isolates. The third panel was obtained from the U.S. Centers for Disease Control and Prevention (CDC) and contained invasive and noninvasive strains (see Table S1 in the supplemental material for a complete list of strains). To screen isolates by PCR, we used two sets of primers, one set internal to the GGI and a second set to amplify across the *dif* region that is the GGI insertion site in the chromosome (14). Results were considered conclusive if a product of the correct size was seen for only one reaction. Isolates that did not give conclusive results were screened by Southern blotting. Of the 126 isolates screened, we found that 22 isolates (17.5%) had the GGI. The percentage of GGI-positive strains varied by serogroup (Table 2).

TABLE 2 Summary of results of GGI screening in *N. meningitidis*

Serogroup	No. of GGI^+ isolates/total	No. of isolates with island type				
		1	2	3	4	5
All Mc	22/126	2	1	6	1	12
A	0/21					
B	0/23					
C	6/22		1			5
D	0/1					
H	2/2			1		1
W135	6/15			5	1[a]	
X	2/11	1				1
Y	0/23					
Z	6/8	1				5

[a] GGI identified in draft genome sequence of strain α275 (GenBank accession number AM889138).

PCR walking, DNA sequencing, and GGI typing. It was apparent from the results of Snyder et al. that some versions of the GGI in *N. meningitidis* carried deletions relative to the 57-kb Gc GGI (37). Therefore, we examined the GGIs of seven Mc strains for gene content using PCR, paying particular attention to the presence of genes required for type IV secretion. Six of the seven strains had been previously shown to have the GGI (37). The seventh strain we identified as possessing a distinct GGI type during the course of this study. The GGI-encoded T4SS in *N. gonorrhoeae* has been shown to secrete DNA active in natural transformation and to facilitate TonB-independent iron uptake during intracellular growth of gonococci (42). Thus, the presence of the T4SS genes in Mc might affect horizontal gene transfer or intracellular growth of *N. meningitidis* strains possessing the T4SS genes. In order to assess the content of the GGI in Mc, we performed overlapping PCR across the GGI and sequenced regions that gave PCR products different in size from that of *N. gonorrhoeae* strain MS11.

We found four different island types among seven strains by PCR walking and an additional GGI type in the draft genome of strain α275 (Fig. 1 and 2). The first island type, present in strain

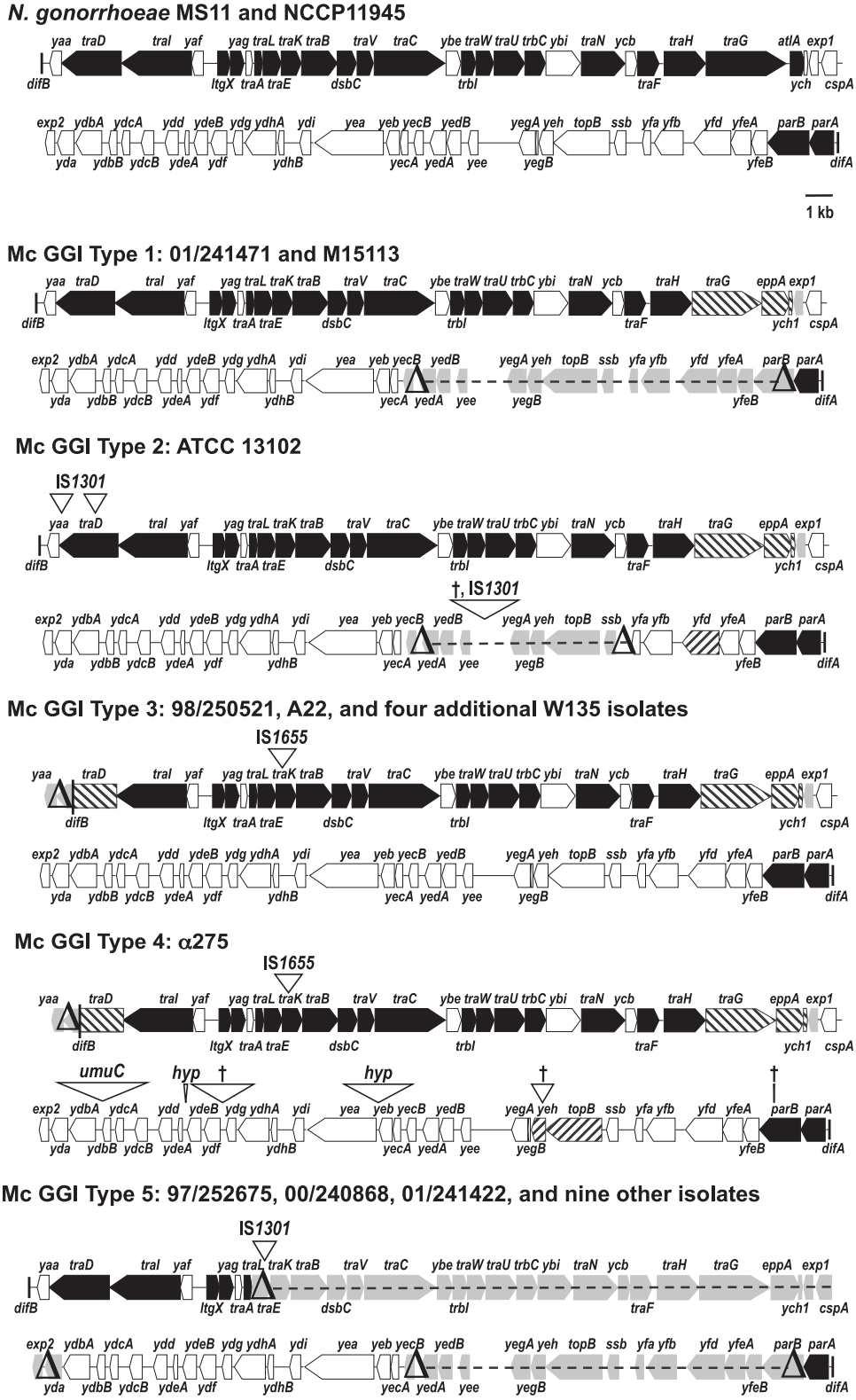

FIG 1 Maps of GGIs in *N. gonorrhoeae* and *N. meningitidis*. Five GGI types have been identified in meningococci by overlapping PCR and bioinformatic analysis. Genes in black have been shown to be necessary for T4SS-dependent DNA secretion in gonococci. Genes in white are not necessary for DNA secretion. In meningococcal maps, genes filled with diagonal bars are similar to, yet distinct from, genes found in gonococcal strain MS11. Genes in light gray with deltas or dashes through them are deleted in meningococci. Inverted triangles represent insertions in meningococcal sequences, with the insertion sequence or homologous sequence indicated above (bases drawn to scale); a dagger indicates an insertion with no homologous sequence found.

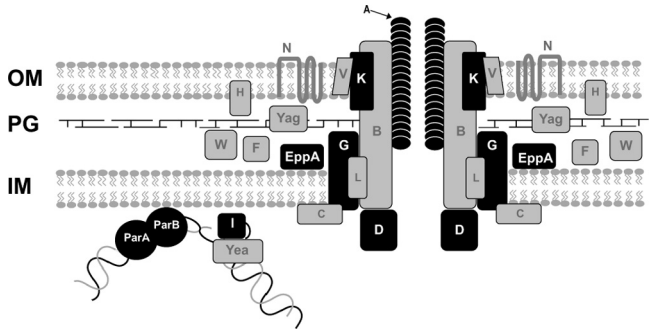

FIG 2 Schematic of the type IV secretion system in *N. meningitidis*. In gonococci, the partitioning protein homologs ParA and ParB may bind DNA and bring it to the T4SS apparatus. The relaxase, TraI, nicks DNA and it is unwound by a helicase, possibly Yea. The DNA-TraI complex may then interact with the putative coupling protein, TraD, and the DNA is secreted through the T4SS into the extracellular environment. TraK, the predicted secretin, may contribute to pore formation in the outer membrane. TraA is a predicted pilin. EppA is an endopeptidase that could potentially degrade peptidoglycan to allow for apparatus formation in the periplasm. Proteins depicted in black show differences between meningococci and gonococcal strain MS11, while proteins in gray are similar. OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

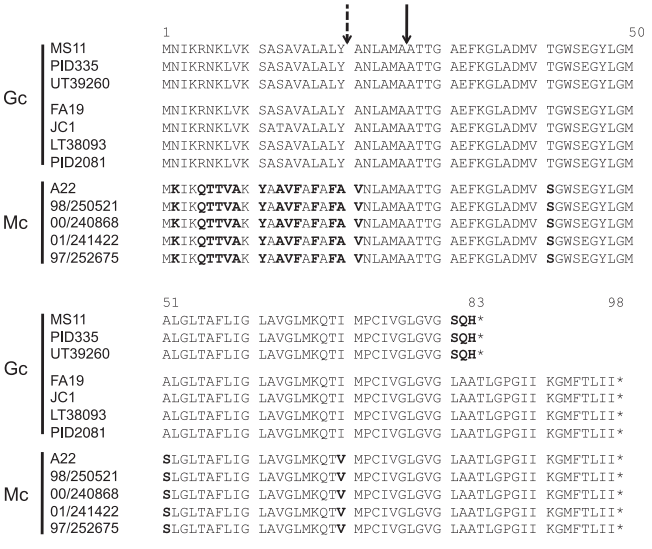

FIG 3 Alignment of TraA from *N. gonorrhoeae* and *N. meningitidis*. There are three alleles of *traA*. Gonococci have a short or long allele, and meningococci carry a long allele with a different signal sequence. Amino acid differences are in bold. The signal sequence cleavage site is indicated by a solid arrow. An alternate potential cleavage site in meningococci is indicated by a dashed arrow.

01/241471, lacks the lytic transglycosylase gene, *atlA*, and instead encodes a peptidoglycan endopeptidase, EppA. This arrangement of genes (lacking *atlA* and carrying *eppA*) is also found in one-sixth of gonococcal strains (10, 20, 28). There is also a deletion of *exp1* and an 11-kb deletion encompassing genes from *yecB* through *parB*. In *N. gonorrhoeae*, *atlA* and *parB* are both necessary for DNA secretion (10, 20, 25). However, all of the genes for structural components of the T4SS appear intact. This strain has the long allele of *traA*, and the signal sequence encoded by *traA* is different from that of gonococci (Fig. 3). Previous studies have shown that *traA* is not required for DNA secretion in Gc (16). In contrast with Snyder et al. (37), we were able to detect and sequence *traE* from 01/241471 and *parA* in all GGI$^+$ meningococcal strains analyzed in the present study. These results, particularly the lack of *atlA* and *parB*, raise the question of whether this strain carries a functional T4SS and whether it could secrete DNA or possibly some other substrate. This GGI type is also found in a serogroup X strain, M15113 (Table 2). A model of the T4SS highlighting the components that differ between the meningococcal and gonococcal systems is shown in Fig. 2.

The second island type is found in strain ATCC 13102. As with all the Mc strains that carry this region, *atlA* and *exp1* are absent and *eppA* is present in this island type. IS*1301* is inserted into *traD* downstream of the region encoding the first Walker A box. A second copy of IS*1301* is inserted into *yaa*, a gene of unknown function. This strain also has the long allele of *traA*. There is an 8.2-kb deletion from *yeb* to *ssb*. It is replaced by 2.5 kb of sequence that contains an insertion of IS*1301* preceded by two insertions of a 50-bp segment of GGI sequence found in the intergenic region between *ydf* and *ydg*. Between the two insertions of the *ydf-ydg* intergenic region is 1.3 kb of sequence that is not homologous to any known sequence. ATCC 13102 is the only strain we have found to carry this version of the GGI (Table 2).

The third island type is present in strains 98/250521 and A22. Four W135 recent clinical isolates we screened also have this island type. Many characteristics of this island type are also found in sequenced strain α275, carrying the fourth island type. Like the island types in 01/241471 and ATCC 13102, these strains have *eppA* and lack *atlA* and *exp1*. They also have a deletion in *traD*, resulting in the loss of 300 bp of *traD* coding sequence and the deletion of the *yaa* open reading frame. TraD is the predicted T4SS coupling protein, which would bind substrates for secretion by the T4SS apparatus, and it was recently shown that *traD* is necessary for DNA secretion (31), although a *traD* truncation mutant gives an intermediate level of DNA secretion (14). These GGIs also carry an insertion of IS*1665* in *traK* immediately following the *traK* start. There was no consensus ribosome binding site or other in-frame start codon in the insertion, so it is unlikely that TraK is produced in these strains. TraK is predicted to be a secretin, an outer membrane protein that forms part of the channel for type IV secretion (14). Pachulec (25) recently found that *traK* is necessary for DNA secretion from *N. gonorrhoeae*. We found the genes *traA* and *ybe* to be present in strain A22 and *yecB* in both A22 and 98/250521, which differs from the results of Snyder et al. (37).

Recently a draft genome of strain α275 was made available (GenBank accession number AM889138). This strain contains a GGI that is 64 kb, 7 kb longer than the gonococcal strain MS11 GGI (28). This GGI represents a fourth island type in meningococci. It is highly similar to the GGI type 3 from *difB* through *ydbA*. The sequence from *ydbB* through *parA* contains numerous insertions (Fig. 1). The insertions range in size from 30 bp to 3.3 kb. The largest insertion contains sequence homologous to the *umuC* domain of DNA polymerase V. Two of the insertions have homology to hypothetical proteins, and three insertions have no known homology. There is a 56-bp deletion in *yeh*, an 80-bp deletion in *topB*, and a 30-bp deletion in *parB*. This island type is unique to α275.

The fifth island type is present in strains 01/241422, 00/240868, and 97/252675, as well as five serogroup C strains, three serogroup Z strains, and one serogroup X strain (Table 2). These strains have

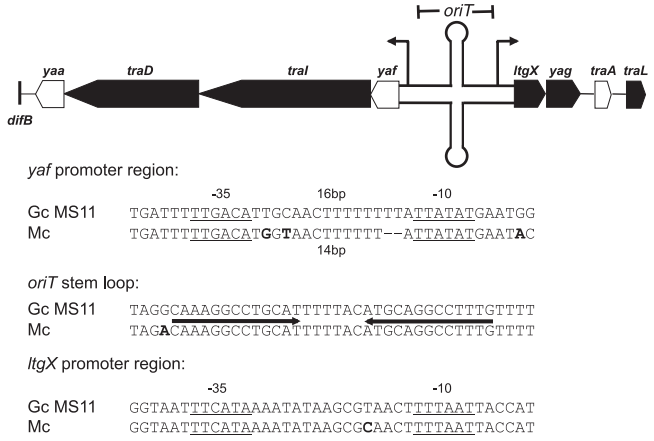

FIG 4 Analysis of the *yaf-ltgX* intergenic region. The intergenic region between *yaf* and *ltgX* contains the *oriT* and likely promoters for the transcript starting with *yaf* and the transcript starting with *ltgX*. The *oriT* is drawn as a stem-loop, and promoters are drawn as elevated arrows on the map. The *yaf* promoter is likely defective in meningococci due to a 2-bp deletion in the spacer region between the putative −10 and −35 sites. The *oriT* and the *ltgX* promoter are conserved between gonococci and meningococci. The meningococcal sequence shown is 100% identical in seven strains we sequenced.

two large deletions. The 22-kb region from *traE* through *yda* is deleted and replaced by one or two copies of IS*1301*. The deletion removes a majority of the T4SS genes. The 11-kb deletion from *yecB* through *parB* seen in GGI type 1 is also present in these strains. Most genes in this region have no homologues or are homologous to other genes of unknown function. There is a single-stranded binding protein, SsbB, and a homolog of topoisomerase IV, TopB, encoded in this region as well. Neither is required for DNA secretion (25).

We used PCR to assign a GGI type to the 22 recent clinical isolates that carry the GGI (Table 2; also, see Table S1 in the supplemental material). All isolates gave results consistent with one of the five island types we established above. All GGI$^+$ serogroup C isolates, except for ATCC 13102, carry island type 5. GGI type 3 was found only in serogroup W135 strains, and α275 was the only W135 strain with GGI type 4. Types 3 and 4 are identical from *difB* to *ydbA*; after *ydbA*, GGI type 4 has numerous insertions and deletions. Nearly all serogroup Z strains carry island type 5; 01/241471 is the only group Z strain with island type 1. We did not observe a correlation between invasive disease and presence or absence of the GGI in meningococci.

Sequences of the *oriT* and *traI* promoter regions. Since several of the Mc GGIs contain deletions in T4SS genes, we hypothesized that these strains might carry mutations in the origin of transfer, *oriT*, or promoters for the T4SS operons. We sequenced the region between *yaf* and *ltgX*, which contains the *oriT* and promoters for the putative *yaf traI traD yaa* operon and the putative operon starting with *ltgX*, which contains most of the genes required for type IV secretion in *N. gonorrhoeae*, from seven meningococcal strains (14, 31, 32). The *oriT* is found in the 156-bp fragment within the PsiI and NgoMIV sites in the *yaf-ltgX* noncoding region (our unpublished results). When aligned with the gonococcal sequence, all meningococcal sequences have an identical *oriT* stem-loop sequence (Fig. 4). There are six separate base pair differences between gonococci and meningococci within the entire *oriT*. Meningococci also have a 2-bp deletion and a 2-bp insertion within 20 bp of each other. The proposed −10 and −35 sequences for the *ltgX* operon promoter are also intact, and spacing is conserved. However, the spacing between the presumed −10 and −35 sites for the promoter of the apparent *yaf* operon is altered in meningococci. In gonococci, there is 16 bp between the −10 and −35 hexamers; however, in meningococci, a 2-bp deletion results in a 14-bp spacer between the sites (Fig. 4).

***traA* sequencing.** The gonococcal genetic island in *N. gonorrhoeae* contains *traA*, encoding the putative T4SS pilin. Sequencing in gonococci revealed two alleles of *traA*, a long form and a short form (Fig. 3). A single nucleotide insertion results in a frameshift, and early termination of the short allele. Despite *traA* being required for F-plasmid conjugation, *traA* is not required for DNA secretion via the T4SS in gonococci (16). As type IV secretion of DNA occurs in a contact-independent manner in Gc, it is not unreasonable that a pilus would not be required for DNA secretion but would be necessary for contact-dependent conjugation in other systems. Jain et al. showed that the long allele of *traA* encodes a protein that when coexpressed in *E. coli* with *trbC* is processed and circularized (17). That study found that the signal sequence was cleaved after A26, which was the predicted cleavage site.

Meningococci contain a third allele of *traA*. To date, all *traA*$^+$ meningococci have been found to have a 98-amino-acid TraA, but the signal sequence is different from that in gonococci (Fig. 3). There are also 3 amino acid changes in the predicted mature form of the protein, T41S, A51S, and I70V, from gonococci to meningococci (Fig. 3). Signal sequence prediction showed that the most likely cleavage site was after A26, as in gonococci. Another possible cleavage site, after A20, was also identified; however, the percent likelihood of cleavage at that site as predicted by SignalP 4.0 (27) was much lower than the value for a site after A26.

GGI loss. The GGI is flanked by a direct repeat. One copy of this repeat constitutes the *dif* site, a sequence recognized by the site-specific recombinases XerCD to separate chromosome dimers during replication (2, 3, 14). In Gc, XerCD action at the *dif* site *difA* and a second, partial *dif* site, *difB*, leads to excision of the GGI and its loss from the population at low frequency (11). We sequenced the *dif* site closest to *traD*, *difB*, in several Mc strains and found three different sequences (Fig. 5). The first sequence, *difB2* (found in strains A22, 98/250521, and α275), has multiple changes in the XerD-binding site compared to the consensus sequence. The second sequence, *difB3* (found in strains 01/241471 and 97/252675), has three base pair changes within the XerD-binding site and one base pair change within the spacer region (11). These changes result in a palindromic *dif*. The third sequence, *difB4* (found in strain ATCC 13102), is similar to the palindromic *difB* but does not have the G-to-A change in the spacer region. Previous work has shown that the sequence of *dif* influences excision of the GGI from the gonococcal chromosome, with strains that have a sequence more similar to consensus having a higher loss frequency (11).

To quantify GGI loss, we utilized an assay that selects for absence of the GGI, thus allowing for detection of low-frequency events (Fig. 6) (11). The assay strain has a streptomycin sensitivity marker inserted within the GGI and a streptomycin resistance marker elsewhere on the chromosome. As streptomycin sensitivity is dominant over streptomycin resistance, a strain that has the GGI will be streptomycin sensitive, and a strain that has lost the GGI will be streptomycin resistant (11, 22). Bacteria were grown

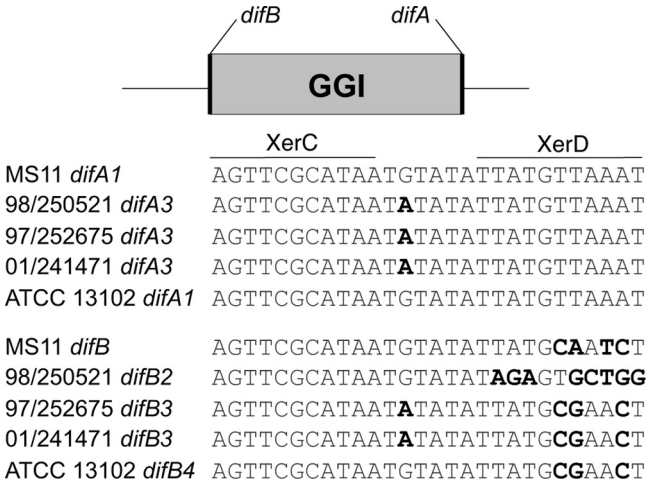

FIG 5 Alignment of *difA* and *difB* from *N. gonorrhoeae* and *N. meningitidis*. The 28-bp *dif* sequence is the binding site of the site-specific recombinases XerCD. *difA* is conserved at the XerC- and XerD-binding sites in gonococci and meningococci with and without a GGI. *difB* is found only in strains with a GGI. XerC- and XerD-binding sites are indicated by a line above the first sequence in the alignment. Bases that are in bold differ from the consensus *dif* sequence. The changes in *difB* of 01/241471 and 97/252675 result in a palindrome. Numbers assigned to *difA* and *difB* sequences are based on those proposed by Domínguez et al. (11).

for 18 h on nonselective GCB plates, swabbed into liquid medium, and plated on GCB agar plates containing streptomycin to select for bacteria that had excised and lost the GGI.

Domínguez et al. (11) showed that the loss frequency for the gonococcal strain MS11 is 1.15×10^{-6}. If the MS11 *difB* sequence is replaced with the consensus *dif* sequence (*difA*), the frequency of loss is 1.84×10^{-3}. We found the loss frequency for meningococcal strain 01/241471 to be $3.45 \times 10^{-8} \pm 2.76 \times 10^{-8}$. We could not detect loss for strains 97/252675, 98/250521, or ATCC 13102, indicating that the loss frequency is 6.25×10^{-9}, the limit of detection, or less (Table 3).

GGI acquisition by meningococci. The sequence of the DNA flanking the GGI also provides insight into the mechanism of GGI acquisition by meningococci. The genomes of all 15 sequenced Mc strains currently available in the National Center for Biotechnology Information (NCBI) database show the presence of a gene, *NMA1385*, between *difA* and *ung*. This gene is not present in gonococcal genome sequences, and no gene is present between *difA* and *ung* in Gc (14). If GGI acquisition occurred by site-specific recombination mediated by XerCD, *NMA1385* would still be present in meningococci with a GGI (Fig. 7A). However, if GGI transfer occurred by homologous recombination between gonococci and meningococci, recombination would be predicted to occur at *yheS* and *ung*, resulting in the loss of *NMA1385* (Fig. 7B). We used PCR to amplify the region between *parA* and *ung* and found a fragment of increased size relative to that in gonococci.

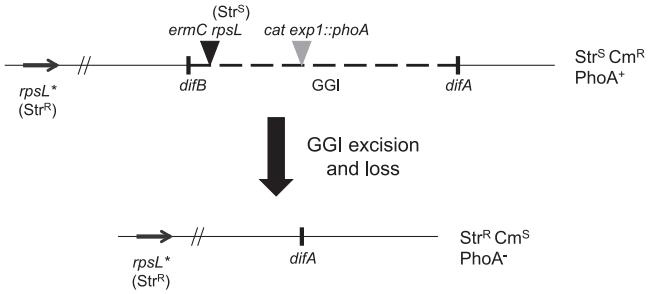

FIG 6 Schematic of the GGI loss assay. A strain that has both streptomycin-sensitive (Str^s) and -resistant (Str^r) alleles of *rpsL* is also marked with erythromycin resistance (Erm^r) and chloramphenicol resistance (Cm^r) and is positive for alkaline phosphatase activity (PhoA$^+$). Any cell that has the GGI is Str^s, Cm^r, and PhoA$^+$. If the GGI is excised from the chromosome and the cell undergoes division, the GGI will be lost from that cell, since the GGI appears to be incapable of replicating on its own. A cell that has lost the GGI is then Str^r, Cm^s, and PhoA$^-$.

TABLE 3 Frequency of GGI loss is lower in *N. meningitidis* than in *N. gonorrhoeae*

Parent[a]	Strain	*dif* type	Loss frequency
MS11 (Gc)	MS11	AB	1.15×10^{-6}[b]
MS11 (Gc)	ND569	AA	1.84×10^{-3}[b]
01/241471 (Mc)	KL1035	AB	$3.45 \times 10^{-8} \pm 2.76 \times 10^{-8}$
97/252675 (Mc)	KL1036	AB	$<6.25 \times 10^{-9} \pm 1.16 \times 10^{-8}$
98/250521 (Mc)	KL1034	AB	$<3.14 \times 10^{-9} \pm 1.22 \times 10^{-9}$
13102 (Mc)	KL1045	AB	$<5.28 \times 10^{-10} \pm 1.57 \times 10^{-9}$

[a] Each parent strain was modified by transforming with a *rpsL*(*K43R*) marker and then inserting an *ermC-rpsL* cassette and an mTn*CmPhoA* cassette in the GGI and a tetracycline resistance marker in *recA*.
[b] Frequency as reported by Domínguez et al. (11).

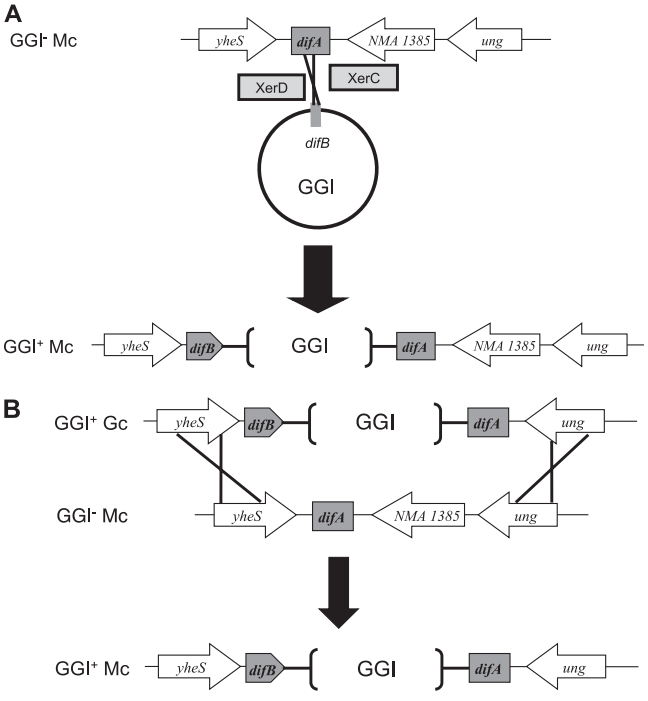

FIG 7 Possible mechanisms of GGI integration into the meningococcal chromosome. (A) Integration by XerCD site-specific recombination. The GGI as a mobile element contained a sequence homologous to the *dif* sequence. The site-specific recombinases XerC and XerD acted on the *dif* sequence in the GGI and *dif* sequence in the meningococcal chromosome, resulting in integration of the GGI. The gene *NMA1385* is still present in this strain with the GGI. (B) Integration by homologous recombination. After uptake of the GGI from gonococci by a meningococcal cell, the GGI is integrated into the chromosome by homologous recombination at *yheS* and *ung*. The gene *NMA1385* is no longer present.

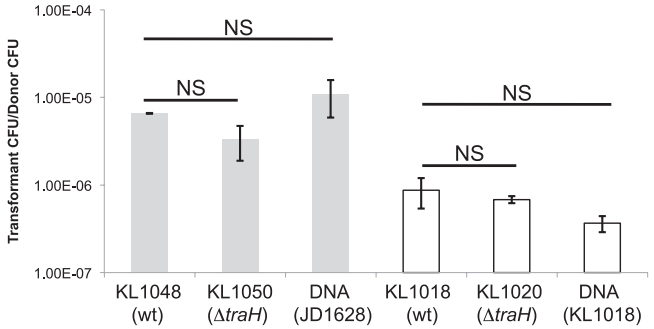

FIG 8 *N. meningitidis* does not secrete DNA in a type IV secretion system-dependent manner. Differently marked strains of meningococci were grown together for 3 h and then plated with selection to identify doubly resistant transformants. Gray bars indicate that the recipient was Mc strain ATCC 13102; white bars indicate that the recipient was strain 01/241471. The frequencies shown are the geometric means from at least three independent experiments; error bars represent the standard error of the mean. NS, no significant difference by analysis of variance (ANOVA) or the Tukey-Kramer method.

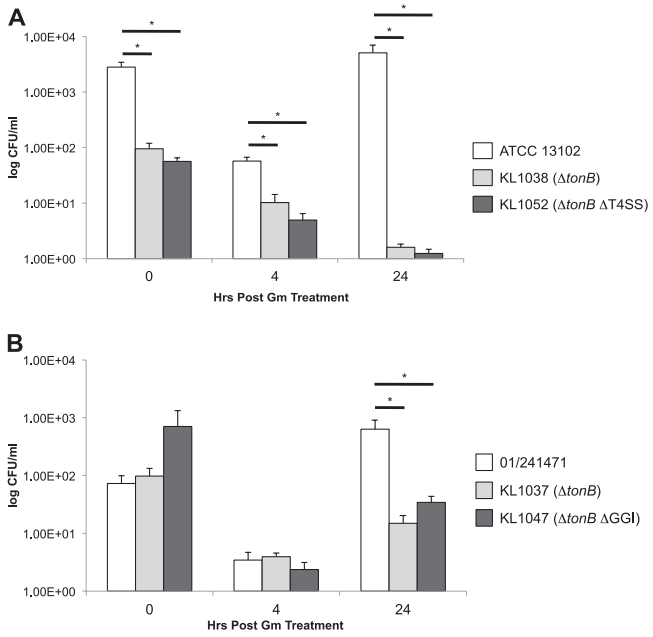

FIG 9 T4SS$^+$ Mc do not show Ton-independent intracellular survival. ME180 epithelial cells were infected with Mc at an MOI of 10 for 3 h followed by a 1-h treatment with 25 μg/ml gentamicin (Gm). Measurements were taken at 0, 4, and 24 h after Gm treatment. Before each additional time point, wells were treated a second time with Gm. Each experiment was performed in triplicate, with each value shown being the geometric mean from at least three independent experiments. Error bars represent the standard errors of the means. *, $P < 0.05$ by ANOVA and the Tukey-Kramer method.

Sequencing revealed that the fragment contained *NMA1385*, indicating that GGI integration in Mc likely occurred via site-specific recombination.

Coculture transformation. Gonococci with a functional T4SS show significantly higher DNA donation frequencies in coculture transformation than strains without a functional T4SS (10, 15, 32). We utilized coculture transformation to detect T4SS-dependent DNA secretion in meningococci. The two strains of meningococci that were used as parental strains, ATCC 13102 and 01/241471, were selected because they contained the most complete T4SS-encoding region of the GGI. The donor strains, KL1048 and KL1018, were marked with erythromycin resistance by inserting *ermC* into the *pacA* gene. The donors also had a kanamycin marker inserted into the *pilT* gene so that DNA uptake could not occur, ensuring that the donor could not act as a recipient in natural transformation. A second pair of donors, KL1050 and KL1020, had a deletion in *traH*, in addition to having *pacA*::*ermC* and *pilT*::*kan*. In gonococci, mutation of *traH* abolishes T4SS-dependent DNA secretion (14, 15). The recipients were marked with a streptomycin resistance marker.

Strains were grown for 2 h in liquid culture separately and diluted, and then a donor was mixed and grown with the recipient. The recipient strains were also grown with 1 μg of purified chromosomal DNA carrying *pacA*::*ermC* to quantify the transformation frequency of the recipient with this chromosomal marker. After 2 h of coculture, bacteria were plated onto GCB agar with erythromycin (Erm), Str, or Erm and Str in order to count the number of donor, recipient, and transformant CFU (Fig. 8). Although the transformation frequency appeared to be slightly lower for the T4SS mutants, there was no significant difference in DNA donation frequency between wild-type (KL1048 or KL1018) and Δ*traH* (KL1050 or KL1020) strains. Wild-type gonococcal strains show 50- to 500-fold-higher levels of DNA donation than mutants defective in type IV secretion (10, 14, 25). No such effect was seen in meningococci, indicating that these strains do not exhibit T4SS-dependent DNA secretion.

Ton-independent intracellular survival. Zola et al. (42) demonstrated that gonococci that have the type IV secretion system encoded in the GGI are able to survive intracellularly under iron starvation conditions without a functional Ton complex. The Ton complex is responsible for energy transduction for a variety of outer membrane transporters, including those responsible for iron internalization (28, 42). We constructed mutations in 01/241471 and ATCC 13102 backgrounds to determine if meningococci with the GGI also exhibit Ton-independent intracellular survival. We infected ME180 cervical epithelial cells with wild-type, Δ*tonB* (KL1037, KL1038), Δ*tonB* ΔGGI (KL1047), and Δ*tonB* ΔT4SS (KL1052) meningococci at a multiplicity of infection (MOI) of 10 for 3 h. After the initial infection, wells were washed and treated with medium containing 25 μg/ml gentamicin (Gm) for an hour to kill any bacteria that were not inside the epithelial cells. Measurements were taken at 0, 4, and 24 h after initial Gm treatment. An hour before each of these time points, wells were treated with 25 μg/ml Gm as before. The wild-type strains 01/241471 and ATCC 13102 exhibited an initial decrease in CFU at 4 h after Gm treatment but had rebounded by 24 h posttreatment. Thus, the wild type meningococcal strains showed growth characteristics similar to those of wild-type gonococcal strains in this assay (42). Neither the Δ*tonB*, the Δ*tonB* ΔGGI, nor the Δ*tonB* ΔT4SS strains rebounded at 24 h (Fig. 9). There was a significant difference between the wild-type and mutant strains, but there was no significant difference between the mutant strains that carry the T4SS genes and those that do not at 24 h posttreatment. These results indicate that the T4SS in meningococci does not contribute to Ton-independent intracellular survival.

Association with and invasion of ME180 cervical epithelial cells. To determine if the GGI affects the ability of meningococci

to associate with or invade epithelial cells, we infected ME180 cervical epithelial cells with the wild type and an isogenic T4SS$^-$ mutant in two different backgrounds. ME180 cells were infected at an MOI of 100 for 3.5 h. There was no significant difference between either 01/241471 or ATCC 13102 and its respective T4SS$^-$ mutant for both association index and invasion index, indicating that the GGI and T4SS do not affect meningococcal adhesion or invasion of host cells under these conditions (see Fig. S1 in the supplemental material).

DISCUSSION

The presence of the gonococcal genetic island in some strains of *N. meningitidis* leads us to ask several intriguing questions. How did meningococci acquire the GGI? Do meningococci secrete T4SS substrates? Can GGI$^+$ meningococci obtain iron via the T4SS? Does the presence of the GGI affect interactions between meningococci and host cells? In the process of answering these questions, we carefully mapped the GGI in seven *N. meningitidis* strains and found five distinct GGI types.

One way that *N. meningitidis* could have acquired the GGI is through natural transformation and homologous recombination with chromosomal DNA from *N. gonorrhoeae*. However, sequencing of the regions flanking the GGI in *N. meningitidis* strains suggests that this was not the method of GGI acquisition. *N. meningitidis* strains carry a gene, *NMA1385*, between *difA* and *ung*, but this gene is not found in *N. gonorrhoeae*. If the GGI were introduced into *N. meningitidis* strains by homologous recombination, then this gene would have been lost in GGI$^+$ strains (Fig. 7). DNA sequencing revealed that this gene is present in all GGI$^+$ *N. meningitidis* strains examined, suggesting that the GGI was not acquired by homologous recombination. Furthermore, sequencing of the *dif* sites suggests the GGI was acquired by site-specific recombination. As in gonococci, the GGI is flanked by a direct repeat, *difA* and *difB*, with *difB* carrying a few sequence changes relative to *difA*, but these sequence changes differ between *N. gonorrhoeae* and *N. meningitidis*, suggesting that the GGI was separately acquired by these two species or that the GGI was present in an ancestor of the pathogenic *Neisseria* species and has undergone different mutational events as these species evolved. The sequence changes in *difB* are found in the XerD-binding region (11, 14). Despite these mismatches to the consensus *dif* sequence, we were able to measure GGI loss from *N. meningitidis*. This result suggests that, as in gonococci, the *dif* sites can be acted on by XerCD to excise the GGI from the chromosome by site-specific recombination.

The GGI in meningococci varies significantly from strain to strain (Fig. 1). Five island types were identified, and these show great differences in size and gene content. Four of the five island types have insertion sequences present, and all of the island types have at least one deletion compared to the *N. gonorrhoeae* GGI sequences. Mc GGI type 1 is approximately 46 kb, compared with 57 kb in the gonococcal strain MS11. We did not find any insertion sequences in this island; however, there is an 11-kb deletion. The type 2 Mc GGI in ATCC 13102 has three separate insertions of IS*1301* and a deletion of 8 kb. This island is approximately 51 kb. The type 3 island is about 56 kb. It has IS*30* inserted in *traK* and has a deletion of the first 1.2 kb of the GGI, including part of *traD*. The island in α275, type 4, is 64 kb and contains numerous insertions, including an insertion of IS*30* and *umuC*. UmuC is a subunit of DNA polymerase V which acts in SOS-activated DNA replication and mutagenesis (29). The type 4 GGI also has a deletion in the first 1.2 kb of the island, including *yaa* and the 3′ end of *traD*. The smallest island, type 5, is approximately 24 kb. There are two deletions, one spanning most of the type IV secretion genes that is 22 kb and another extending from *yecB* through *parB* that is 11 kb. IS*1301* replaces the deleted type IV secretion genes in those strains. These data show that some GGIs are very similar to those of *N. gonorrhoeae* and may produce a type IV secretion system (Fig. 1 and 2). However, many of them have acquired multiple insertions and deletions and do not have the necessary components for a type IV secretion system. Through recombination and deletion, the genes are being mutated and lost. However, those that have all the type IV secretion genes appear not to have any mutations, suggesting that the type IV secretion system may function in these strains.

We measured DNA transfer in coculture for the GGI$^+$ *N. meningitidis* strains that appeared to have intact type IV secretion systems. We detected transfer in coculture, but DNA donation occurred at the same frequency from *traH* strains as from the wild-type parental strains. These data suggest that the meningococcal strains do not donate DNA via type IV secretion. These data led us to wonder whether the *oriT* was present in these strains. DNA sequencing of the *yaf-ltgX* intergenic region revealed that the *oriT* is conserved. However, the sequence also identified a 2-bp deletion between the putative −35 and −10 hexamers in the promoter for the operon containing the genes encoding the relaxase TraI and putative coupling protein TraD (Fig. 4) (31). This deletion may significantly diminish transcription of these genes. The promoter for the other transcript from this region had no sequence changes, suggesting that meningococci may make the structural subunits of the type IV apparatus.

Previous studies showed that meningococci and gonococci require a functional Ton complex to survive intracellularly under iron-limiting conditions (13, 21). Recently, it was demonstrated that gonococci producing the type IV secretion system exhibit Ton-independent intracellular survival (42). The mechanism of survival is unknown, but these data suggest that the T4SS is active during intracellular growth. We examined meningococci for Ton-independent intracellular survival and found that T4SS presence did not confer Ton-independent survival, indicating that the T4SS likely functions differently in meningococci than in gonococci. We also tested those same strains and isogenic T4SS$^-$ mutants for differences in association with and invasion of host cells. We found that there were no significant differences between the parental and T4SS$^-$ strains. Thus, under the conditions tested, no differences in meningococcal infection were conferred by the T4SS.

The GGI is more common in meningococci than previously thought. We identified multiple island types, and it is likely that more types exist. Some GGIs had insertions of genes, such as *umuC*, and undiscovered islands probably have insertions of other interesting genes, some of which may contribute to meningococcal fitness. Approximately one-sixth of Gc strains lack the *atlA* gene, which is required for type IV secretion of DNA, and carry in its place *eppA* and a variant allele of *traG*, just as we have found here for Mc strains (10, 20, 28). Those *eppA*$^+$ Gc strains do not secrete DNA (9), and we have found that the GGI$^+$ Mc strains similarly do not show increased DNA donation for transformation (Fig. 8). The existence of these multiple Gc and Mc strains with this different T4SS gene arrangement suggests that this puta-

tive T4SS functions differently from the MS11 T4SS and has a function in the pathogenic *Neisseria* that is still to be determined.

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