

Genetic Basis for Nongroupable *Neisseria meningitidis*

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Nongroupable *Neisseria meningitidis* may constitute one-third or more of meningococcal isolates recovered from the nasopharynx of human carriers. The genetic basis for nongroupability was determined in isolates obtained from a population-based study in which 60 (30.9%) of 194 meningococcal isolates from asymptomatic carriers were not groupable. Forty-two percent of nongroupable isolates were related to serogroup Y ET-508/ST-23 clonal complex strains, the most common groupable carrier isolate from the study population. Nongroupable isolates were all rapidly killed by 10% normal human serum. The capsule loci of 6 of the ET-508/ST-23 complex strains and of 25 other genetically diverse nongroupable meningococci were studied in detail. Serogroup A or novel capsule biosynthesis genes were not found. Nongroupable isolates were genetically serogroup Y, B, or C isolates that did not express capsule but were related to groupable isolates found in the population (class I); capsule deficient because of insertion element-associated deletions of capsule biosynthesis genes (class II); or isolates that lacked all capsule genes and formed a distinct genetic cluster not associated with meningococcal disease (class III).

Neisseria meningitidis (meningococcus) is the etiologic agent of meningitis and rapidly fatal sepsis throughout the world. The polysaccharide capsules of serogroups A, B, C, Y, X, and W-135, which are expressed on the surface of *N. meningitidis*, form the basis for serogrouping and are essential for invasive meningococcal disease. Capsule inhibits the complement-mediated bactericidal activity of human serum, retards opsonization and engulfment by phagocytic cells, may promote meningo-

coccal shedding from mucosal surfaces, may enhance transmission from one individual to another, and increases the survival of meningococci in the environment [1–7]. However, in the natural reservoir, the human nasopharynx, nongroupable, presumably unencapsulated meningococci are common [1, 8–14] and may have a selective advantage. An absence of capsule has been shown to facilitate the adherence of meningococci to the human nasopharyngeal epithelium [1, 15]. Meningococcal surface structures (e.g., Por, Opa, and Opc proteins and lipooligosaccharide [LOS]), which are unmasked by capsular polysaccharide, appear to enhance attachment, microcolony formation, and human epithelial cell entry.

N. meningitidis capsule expression is determined by a genetic island in the meningococcal chromosome [16] that is composed of 2 divergently transcribed operons [17] (figure 1). The sialic acid capsule biosynthesis operons (*syn* or *sia*) and the serogroup A biosynthesis operons (*myn* or *sac*) consist of 4 genes. In serogroups B, C, Y, and W-135, *N. meningitidis synA–C* encode the

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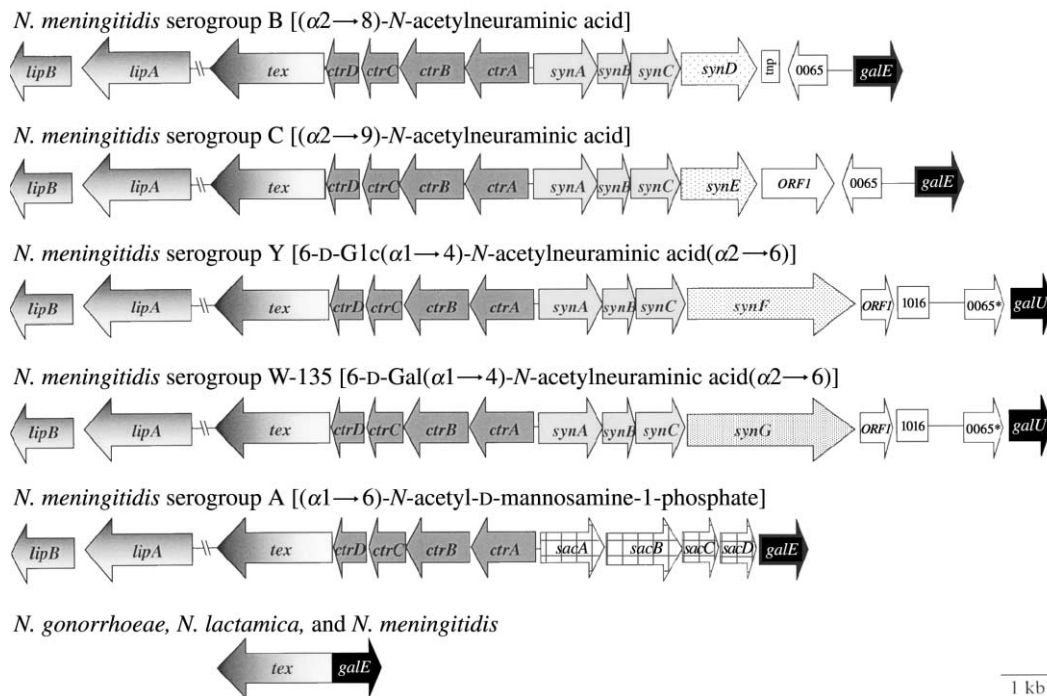


Figure 1. Genetic organization of the capsule biosynthesis and transport loci of *Neisseria meningitidis* serogroups B, C, Y, W-135, and A. The capsule transport operon, *ctr* (dark-gray arrows), is highly conserved among all 5 serogroups. The enzymes encoded by *synA–C* (light-gray arrows) synthesize CMP-*N*-acetylneuraminic acid or sialic acid (CMP-NANA) and are conserved among the sialic acid-producing serogroups B, C, Y, and W-135. Sialyltransferase, which catalyzes the serogroup-specific linkage of sialic acid homopolymers or sialic acid with glucose or galactose, is encoded by the fourth gene of the *syn* operon and varies from serogroup to serogroup (dots). The serogroup A capsule is composed of (α 1 \rightarrow 6)-linked *N*-acetyl-D-mannosamine-1-phosphate, and the genes comprising this capsule synthesis operon, *sacA–D* (windowpane-patterned arrows) are unique to this serogroup. Flanking the *ctr* and *syn* operons are *tex* (vertically shaded arrows) and either *galE* or *galU* (black arrows). The nucleotide sequences between the polymerase and *galE/galU* vary as shown for the representatives of each serogroup sequenced (strains NMB [B], FAM18 [C], GA0929 [Y], and GA1002 [W-135]). In *Neisseria gonorrhoeae* and *Neisseria lactamica*, *tex* and *galE* are adjacent. Two other genes involved in the expression of capsule polymers, *lipA* and *lipB* (horizontally shaded arrows), are located 4.8 kb 5' of *tex* and are present in encapsulated *N. meningitidis* but are not found in *N. gonorrhoeae* or *N. lactamica*. Squares, IS1016 homologues; 0065, homologue of NMB0065 from MC58 genome database. ORF1, putative capsule O-acetylation genes [69]; *, Homologue of NMB0065, which is not identical to the genes denoted "0065."

enzymes that synthesize CMP-*N*-acetylneuraminic acid or sialic acid (CMP-NANA), and the fourth gene encodes the polymerase that catalyzes the serogroup-specific linkage of CMP-NANA (serogroups B and C) or the linkage of sialic acid with other sugars (e.g., serogroups Y and W-135) into nascent capsular polysaccharide chains [18–22]. In serogroup A meningococci, *sacA–D* (*mynA–D*) forms a distinct biosynthesis operon that is divergently transcribed from the *ctr* operon and encodes genes required for serogroup A *N*-acetylmannosamine-1-phosphate capsule biosynthesis [23]. Novel capsule biosynthesis genes are also present in serogroup X (Y. Tzeng, C. A. Noble, and D.S.S., unpublished data) and in serogroups Z, K, L, and 29E. The capsule transport operon (*ctr*) also consists of 4 genes: *ctrA*, *ctrB*, *ctrC*, and *ctrD*. These genes are generally conserved among encapsulated *N. meningitidis*, regardless of serogroup, and form a predicted ABC transporter that translocates capsular polysaccharide chains from the cytoplasm to the surface of the meningococcus [24, 25]. The products of

lipA and *lipB* are proposed to be involved in the cytoplasmic phospholipid substitution of the capsular polysaccharide chains [26].

Phase variation between the capsule-on and -off phenotypes is well described in *N. meningitidis* in vitro [15, 17, 27, 28]. However, the precise genetic mechanisms of nongroupability in the meningococcal population carried in vivo have not been determined. Recently, meningococcal carriage was assessed in a large population of high school students in 2 demographically similar Georgia counties [9]. To better understand the biology of *N. meningitidis* in vivo, the genetic basis of nongroupable carriage isolates from that study was defined.

MATERIALS AND METHODS

Collection and characterization of human nasopharyngeal carrier isolates of *N. meningitidis* and other strains. Methods for the collection of nasopharyngeal meningococcal carrier

Table 1. Nucleotide primers to genes of the capsule region and used in the study.

Primer	Sequence	Location
JS51	5'-GCAATACCATTACGTTTATCTCTC-3'	<i>synA</i>
JS40	5'-GTTTCAGGATTGTTGATTACTTCAGC-3'	<i>synA</i>
JS33	5'-ACTTGGTCATACAATTAATGCTGC-3'	<i>synB</i>
JS54	5'-GCATGCATATACAACGGATCC-3'	<i>synB</i>
JS52	5'-GCTGAAGTTGTTAAACATCAAACAC-3'	<i>synC</i>
JS68	5'-GCTACGACAGATGCAAAGGCG-3'	<i>synC</i>
JD17	5'-CCTGCTCATCTCTATATGCTG-3'	<i>synD</i>
JD74	5'-GATGAAGGGACCTGGAACATATGC-3'	<i>synD</i>
SE9	5'-TCCAAGATTCCCAGTGGTTTGC-3'	<i>synE</i>
SE29	5'-ACTTCATCTGAAAACAGGATAGCG-3'	<i>synE</i>
SE4	5'-CTTCACTCATCCTCTTCAACTGG-3'	<i>synF</i>
SE51	5'-CCTGCTCGGAAGATTCACCTTTG-3'	<i>synF</i>
SE22	5'-AGATCAGGTTTTGAGCCTATTCG-3'	<i>sacA</i>
SE36	5'-AGCGGAATGCACAAAAGAGAATG-3'	<i>sacA</i>
JS75	5'-GTGTGGAAGTTTAATTGTAGGATG-3'	<i>ctrA</i>
LJ4	5'-CCACCACAAACAATACTGCCG-3'	<i>ctrA</i>
JD1	5'-TCTTGCCGGTGCCTGAGTTT-3'	<i>ctrB</i>
JD2	5'-CTCGGTTGCGAGATCACTTC-3'	<i>ctrB</i>
JD3	5'-GCGGACGGTGTAGACGATATC-3'	<i>ctrD</i>
JD4	5'-CAGCACCATCGCATTATCGCAA-3'	<i>ctrD</i>
JD5	5'-GTTGAGCCGTTGCTGATGACA-3'	<i>ctrC</i>
JD6	5'-TGCATACTCTGCACTTTCGG-3'	<i>ctrC</i>
Tex1	5'-CAATATCTGCGGAGTTGGAAGAGC-3'	<i>tex</i>
Tex3	5'-CTTCGACACGACGATTTGTGC-3'	<i>tex</i>
SS3CK1	5'-CCAGCATCAATATCCTGCCACG-3'	<i>galE</i>
CK29A	5'-CTTCCATCATTGTGCAAGGCTGC-3'	<i>galE</i>
Tex4	5'-CGGCAAGCAACACGTCCGCC-3'	<i>GCtex</i>
GalEGC1	5'-GGACGGCGGCAGACGAGTTGC-3'	<i>GCtex</i>
SS3CK1-GC	5'-GCAAATTACCGGCAGAAACATACC-3'	<i>GCgalE</i>
CK29A-GC	5'-CGAATCTTCCATCATTGCTGC-3'	<i>GCgalE</i>
JD39	5'-AAATACAACCACGCGCCGAACCT-3'	<i>lipA</i>
JD42	5'-GCGCGGGATGGGGAGCAGAT-3'	<i>lipA</i>
JD43	5'-GCGTATGCTTAAAGTTCAACC-3'	<i>lipB</i>
JD46	5'-CGCTGGTGTGTTGATGGTGA-3'	<i>lipB</i>

NOTE. GC, *Neisseria gonorrhoeae*. These primers were designed for the *N. gonorrhoeae* *tex* or *galE* sequences.

isolates used in the present study have been reported elsewhere [9]. In brief, a bent sterile swab was used to access the nasopharyngeal tonsillar region superior to the uvula of high school students in 2 Georgia counties. The swab was immediately plated on Martin-Lewis medium (Remel) that contained vancomycin-colistin-nalidixic acid, placed in individual isolation bags with CO₂ tablets, and transported at room temperature, within 4 h after inoculation, to the Georgia Public Health Laboratory of the Georgia Department of Human Resources (Atlanta). To ensure consistency in specimen collection, a limited number of personnel were trained specifically in culture col-

lection techniques. To ensure that *N. meningitidis* would survive under the study conditions, a reference isolate of *N. meningitidis* was streaked onto a Martin-Lewis plate, placed in an isolation container with CO₂, and transported to the high school at room temperature on each culture day. The control plate was returned to the laboratory and handled in the same manner as the study cultures under investigation. All of the control plates grew viable *N. meningitidis*.

Martin-Lewis plates were incubated at 35°C in a 5% CO₂-enriched atmosphere and were examined for growth typical of *N. meningitidis*. Colonies with typical morphology were further characterized as *N. meningitidis* by a positive oxidase test (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride 1%; Becton Dickinson Microbiology Systems), the presence of gram-negative diplococci, the ability to ferment dextrose and maltose but not lactose and sucrose by the rapid fermentation method [29], and a lack of polysaccharide production on 5% sucrose agar. Isolates defined as *N. meningitidis* were subcultured to heart infusion agar with 5% defibrinated sheep blood and serogrouped using A, B, C, D, W-135, X, Y, and Z grouping antisera (Difco Laboratories). All *N. meningitidis* isolates were stored at -70°C in tryptose phosphate broth with 20% glycerol. To independently confirm species and serogroup, all isolates were subsequently reconfirmed at the Meningitis and Special Pathogens Laboratories of the Centers for Disease Control and Prevention (CDC, Atlanta). The well-characterized serogroups B, C, Y, and W-135 meningococcal isolates NMB, FAM18, GA0929, and GA1002 were also used in these studies, as was the unencapsulated Tn916 insertion mutant (*synA*⁻) of isolate NMB, designated M7 [19, 30]. In addition, *Neisseria lactamica* strain 66015 and *Neisseria gonorrhoeae* strain FA19 were used as controls.

Multilocus enzyme electrophoresis typing (MLEE) and multilocus sequence typing (MLST). MLEE was performed at CDC using 24 constitutive enzymes, as described by Reeves et al. [31] and as reported elsewhere [9], for these isolates. Numbers were assigned to enzyme alleles on the basis of enzyme mobilities, and each unique set of alleles was defined as an electrophoretic type (ET). An index of genetic relatedness was determined by weighing the degree of diversity at each of the 24 enzyme loci, and similarities among the ETs were assessed by phylogenetic analysis. Phylogeny was generated, as described elsewhere [9], by the unweighted pair-group method with arithmetic means. MLST was performed at the University of Oxford, as described elsewhere [32], on the basis of the nucleotide sequences of 7 meningococcal genes (*abcZ*, *akd*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm*).

Polymerase chain reaction (PCR), single specific primer (SSP)-PCR, and restriction site (RS)-PCR. Chromosomal DNA was prepared using the DNeasy Tissue Kit (Qiagen). A chromosomal DNA preparation from each nongroupable iso-

late was used as the template for each PCR. The meningococcal and gonococcal primers used in this study are listed in table 1. For each PCR, 0.625 U of AmpliTaq DNA Polymerase (Perkin Elmer) and 0.625 U of Taq Extender (Stratagene) were used. Serogroup Y isolates were distinguished from serogroup W-135 isolates according to the method of Borrow et al. [33], using amplification of the polymerase gene and subsequent digestion of the PCR products with *Xba*I.

In brief, for SSP-PCR, overnight chromosomal DNA digests were performed using various blunt-cutting enzymes. Chromosomal fragments were ligated overnight into linearized and shrimp alkaline phosphatase-treated pUC18. The ligation mix was subsequently used as the template in a PCR with one primer to the pUC18 sequence (5'-TCA CAC AGG AAA CAG CTA TGA CC-3' or 5'-CCA GTC ACG ACG TTG TAA AAC G-3') and the second primer to a known meningococcal sequence (see table 1). PCR products >500 bp were gel purified (Qiagen) and sequenced with each primer.

The RS-PCR method of Weber et al. [34] was also used. The restriction site primers used for RS-PCR in the study were as follows: 5'-TCA CAC AGG AAA CAG CTA TGA CCN NNN NNN NNN GGA TCC-3', 5'-TCA CAC AGG AAA CAG CTA TGA CCN NNN NNN NNN AAG CTT-3', 5'-TCA CAC AGG AAA CAG CTA TGA CCN NNN NNN NNN GAA TTC-3', and 5'-TCA CAC AGG AAA CAG CTA TGA CCN NNN NNN NNN GAT C-3'.

Southern DNA hybridization. The Genius nonradioactive DNA labeling and detection system (Roche Molecular Biochemicals) was used for Southern DNA hybridization. Specific DNA probes to each *ctr* and *syn* gene were PCR amplified (table 1), random-prime labeled with digoxigenin, and used to probe Southern DNA blots [35] under high-stringency conditions, according to the manufacturer's protocols.

Nucleotide sequencing. Automated dye-terminator nucleotide sequencing was done at the Emory University DNA Core Sequencing Facility. The template was prepared by PCR and then purified using the QIAquick PCR Purification Kit (Qiagen). Eight to 11 μ L of template and 7.5 pmol of primer were used in each sequencing reaction.

Whole-cell ELISA. Whole-cell ELISA was done according to the published protocols [23], with minor modifications: 100 μ L of a 1:3 dilution of the cell suspension (OD at 650 nm, 0.1) was added to the microtiter plates for serogroup Y ELISA or 100 μ L of a 1:27 dilution for serogroup C ELISA; the serogroup Y capsule-specific primary monoclonal antibody (mAb) 5-2-Y was used at a 1:50 dilution, and the serogroup C-specific mAb 4-2-C was used at a 1:2000 dilution; secondary antibody was used at a 1:2500 dilution for serogroup Y or 1:10,000 for serogroup C; and incubation was at 37°C instead of 33°C.

Normal human serum (NHS) bactericidal assays. Bac-

tericidal assays were done as described elsewhere [36, 37]. In brief, meningococci were grown to the midlog phase in gonococcal broth, and then diluted to 10⁵ cells/mL in HEPES/MEM buffer. Five microliters of diluted cells were added to 40 μ L of HEPES/MEM and 5 μ L of NHS in a microtiter-plate well (Falcon tissue culture plate 3072). Assays were done in duplicate for each isolate, and 3 separate assays were done for isolates that were not 100% killed. The NHS was collected and pooled from 5 adult human donors without a history of gonococcal or meningococcal disease. NHS was either heated for 30 min at 55°C before addition to cells, to inactivate complement, or not heated. NHS was used in these experiments at a final concentration of 10% (vol/vol). The cells-NHS-buffer mixture was incubated at 37°C with 3.5% CO₂ for 30 min, after which time 10 μ L of sample from each well was plated onto 2 gonococcal agar plates. The unencapsulated control isolate M7, which contains a Tn916 insertion in *synA*, the first biosynthesis gene [19, 20], was plated onto gonococcal agar that contained 5 μ g/mL tetracycline. Plates were incubated overnight at 37°C with 3.5% CO₂, and survival was assessed by colony count. Data were expressed as percentage survival or as log₁₀ survival. The significance of differences between the means of 2 variables was determined using Student's *t* test with unpaired values and a 2-tailed hypothesis (GraphPad).

Tricine SDS-PAGE analysis of LOS preparations. A mini Protean apparatus (Bio-Rad) was used for Tricine-SDS-PAGE, which was done according to the description of Schagger and von Jagow [38]. Crude LOS preparations were prepared from whole-cell lysates of meningococcal growth suspended in distilled water. The protein concentrations of these preparations were approximated by the Bradford Assay (Bio-Rad). Proteinase K digests consisted of 1 μ g of protein in 2% SDS to which 2 μ L of 25 mg/mL proteinase K (Sigma) was added, and the mixture was incubated at 60°C overnight. The reaction was stopped with the addition of 38 μ L of loading buffer (1 M Tris [pH 8.45], 10% glycerol, 2% SDS, 5% β -mercaptoethanol, and 0.05% bromophenol blue). A 15- μ L aliquot of each sample was boiled for 4 min before being loaded onto a 16% Tricine-SDS-PAGE gel. After electrophoresis, the gels were fixed in 40% ethanol-5% acetic acid overnight. The gels were silver stained according to the method of Hitchcock and Brown [39]. For exogenous complementation of meningococcal isolates with CMP-NANA, gonococcal agar plates were made that contained 50 μ g/mL CMP-NANA (Sigma). Isolates from freezer stocks were streaked on these plates and were incubated at 37°C with 3.5% CO₂ overnight.

RESULTS

Identification of *N. meningitidis* nongroupable carrier isolates. In a population-based carriage study of 2730 high

school students located in 2 Georgia counties, 196 asymptomatic carriers of *N. meningitidis* were identified [9]. Of the 196 isolates, 91 were serogroup Y, 38 were serogroup B, 3 were serogroup Z, 2 were serogroup C, and 62 were not groupable. Of the 62 nongroupable isolates, 2 were subsequently determined to be *N. lactamica*. Of the remaining nongroupable isolates, 52 were autoagglutinating, and 8 were nonagglutinating. Of the 60 nongroupable isolates, 25 were identical, according to MLEE, to genetic types associated with serogroup Y (e.g., ET-508, ET-527, ET-1212, ET-1215, or other ET-508/ST-23 complex members) [9]. Seven of these 25 were confirmed by PCR to contain *synF* encoding the serogroup Y capsule polymerase, and 6 others were seen, as noted below, to contain *synF*. Three identical ET-1221 autoagglutinating isolates were confirmed by PCR to contain *synD*, encoding the serogroup B polysialyltransferase.

Thirty-one nongroupable isolates, 7 of 8 nonagglutinating isolates and 24 of 52 autoagglutinating isolates, were selected for further study. These 31 nongroupable isolates were of genetically diverse ET types (figure 2), spanning the population structure (genetic distance, >0.45) of *N. meningitidis*. All of these nongroupable isolates were repeatedly confirmed to be *N. meningitidis* (see Materials and Methods).

Sensitivity of nongroupable meningococcal isolates to human sera. The expression of capsule is the major requirement for serum resistance of *N. meningitidis* [37]. The 31 nongroupable isolates all exhibited rapid killing (>98.1%–100% killing in ≤30 min; $P \leq .0013$) in a low concentration (10%) of normal human serum (data not shown). This rapid killing was similar to that seen for the genetically defined unencapsulated meningococcal mutant M7 (*synA::Tn916*) [19]. In contrast, the serogroup B encapsulated parent of mutant M7, isolate NMB, which was obtained from a patient with meningitis, was serum resistant [37]. The sensitivity to human serum further indicated that the nongroupable carrier isolates were unencapsulated.

Unencapsulated *N. meningitidis* with an intact capsule genetic locus (class I). The 31 isolates were first screened by PCR for the presence and size of the genes of the meningococcal capsule locus (biosynthesis and transport operons) and *lipA* and *lipB*, using primers internal to each gene. None of the nongroupable isolates contained the genes (*sacA–D*) of the biosynthesis operon encoding the serogroup A mannosamine capsule, as tested by PCR [23].

Ten (32.3%) of 31 nongroupable isolates contained the sialic acid biosynthesis operon, including a specific sialyltransferase gene, and were designated class I. Four (M4957, M4952, M5074, and M4956) were found to have *synD* (serogroup B), 2 (M4954 and M4949) were found to have *synE* (serogroup C), and 4 (M4946, M5017, M4943, and M5016) were found to have *synF* (serogroup Y). Four (M4949, M5017, M4943, and M5016) of

6 *synE*- or *synF*-bearing class I isolates did not express capsular polysaccharide, as tested by whole-cell ELISA (data not shown). The fifth and sixth isolates (M4954 and M4946) expressed <10% of wild-type levels of capsular polysaccharide. The genetic relationships of these isolates are shown in figure 2.

Sialic acid, which is synthesized by proteins encoded by *synA–C*, is incorporated into the capsular polysaccharides of serogroups B, C, Y, and W-135 [20, 22, 40] and is also added to the terminal α -chain galactose of meningococcal LOS [41]. Four class I nongroupable isolates (M4946, M5017, M4943, and M5016) displayed a wild-type *syn* operon, as tested by PCR, including a wild-type serogroup Y polymerase. In these isolates, LOS was examined for sialylation (figure 3). None of these 4 isolates sialylated LOS, which suggested that they did not synthesize CMP-NANA. Because this result could also be due to alteration of the LOS sialyltransferase (Lst), the 4 isolates were exogenously supplemented with 50 $\mu\text{g}/\text{mL}$ CMP-NANA, and their LOS profiles were again examined (figure 3). Three of 4 isolates sialylated LOS, indicating that Lst was functional.

Three class I nongroupable carrier isolates (M4952, M5074, and M4956) had an intact *synA–C* locus according to PCR and contained *synD*, which encodes the serogroup B polysialyltransferase. Variation in a poly-C tract at the 5' end of *synD* has been described as a mechanism for capsule phase variation of serogroup B *N. meningitidis* in vitro [27, 28]. A tract of 7 dC residues allows for the expression of capsule, whereas an insertion or deletion of 1 dC results in a missense mutation within *synD* and capsule is not expressed. Nucleotide sequencing of *synD* of the 3 nongroupable isolates revealed that 2 contained a deletion of 1 dC within the poly-C tract, and 1 contained an insertion of 1 dC (figure 4). Thus, slipped-strand mispairing within *synD* was predicted to be the mechanism for the nongroupability of 3 class I isolates.

Three class I nongroupable *N. meningitidis* isolates demonstrated wild-type *syn* and *ctr* operons according to PCR and expressed an endogenously sialylated LOS (data not shown). One (M4957) had *synD* without evidence of slipped-strand mispairing, and 2 (M4954 and M4949) contained *synE*. Nucleotide sequencing of the *ctrA–synA* intergenic promoter regions of these 3 isolates revealed all to be wild type [17], with the exception of 1 isolate, which had a C \rightarrow T substitution 17 nt before the *synA* start codon. Nucleotide sequencing of the *synE* serogroup C polymerase revealed them to be wild type.

Overall, class I nongroupable *N. meningitidis* isolates contained intact serogroup Y, B, or C capsule biosynthesis and transport operons, and nongroupability was the result of the predicted slipped-strand mispairing of *synD* or presumed point mutations affecting capsule biosynthesis or transport.

Insertion (IS) elements and recombination events within the capsule biosynthesis operon (class II). Eleven of 31 non-

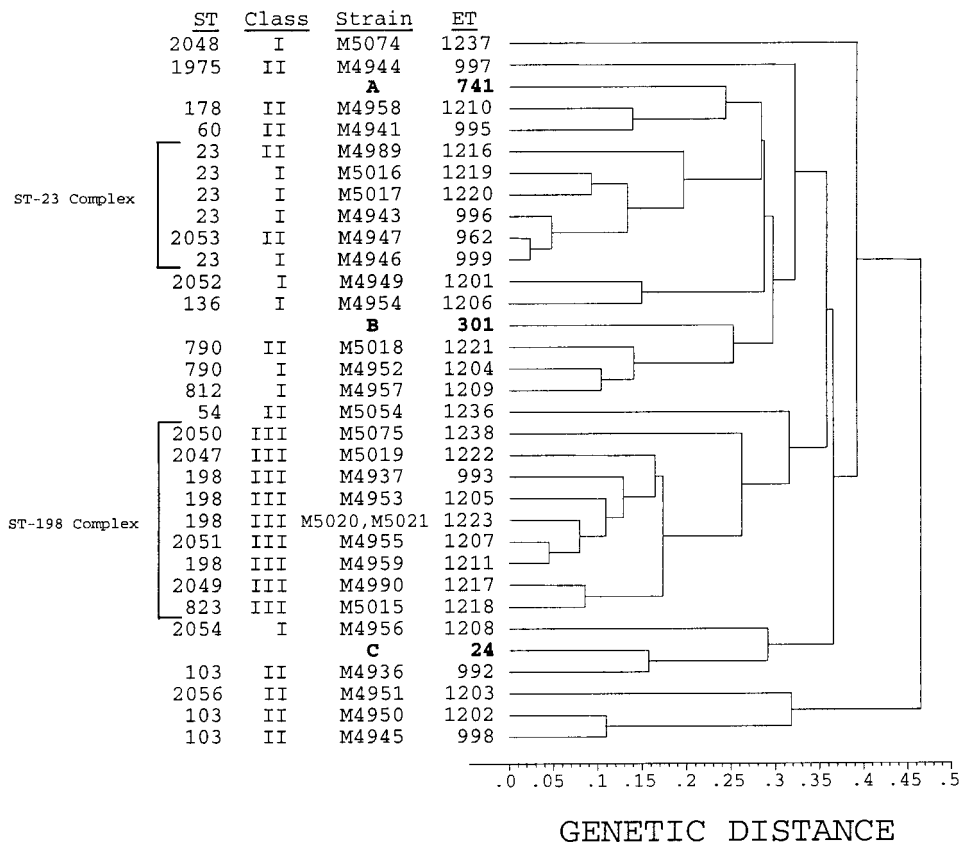


Figure 2. Phylogeny illustrating the genetic diversity (>0.45) of 31 nongroupable *Neisseria meningitidis* carriage isolates collected in a population-based study. Phylogeny is based on multilocus enzyme electrophoresis typing. The disease-associated serogroup B, C, and A isolates (*bold*) are included for reference: ET-24 is an ET-37 complex serogroup C representative, ET-301 is a member of the ET-5 serogroup B complex, and ET-741 is serogroup A subgroup III. Sequence types (ST), based on multilocus sequence typing, are also listed, and ST-23 and ST-198 clonal complexes are indicated by brackets. ET, electrophoretic type.

groupable isolates contained IS elements and extensive recombination events in the capsule biosynthesis operon. Seven isolates (M4936, M4944, M4945, M4950, M4958, M4941, and M5054) lacked all *syn* genes but contained the *ctr* operon according to PCR and Southern hybridizations (illustrated in figure 5A and 5B). SSP-PCR gene walking in the 6 isolates, followed by nucleotide sequencing, revealed an IS1301 element in the 5' end of *ctrA* (figure 6) and loss of the *syn* operon. Of these isolates, 3 contained nucleotide sequences adjacent to IS1301. The sequences were related (up to 68% identity over 127 of 129 aa) to the *Bacillus subtilis* gene encoding the teichoic acid biosynthesis protein TagD. A seventh isolate (M4945) contained the same foreign *tagD* insertion in *ctrA*, although the IS1301 element was not found (figure 6).

Two distinct IS1301-associated insertions in *ctrA* were noted. M4958, M4941, and M5054 had 44 bp of novel nucleotide sequence inserted after nucleotide 75 of the *ctrA* open-reading frame (ORF). M4936, M4944, M4950, and M4945 had 193–222 bp of nucleotide sequence that was common to all 7 isolates

inserted after nucleotide 89 in the *ctrA* ORF (figure 6). The 5' end of *ctrA* was absent in all 7 isolates. Thus, IS1301 was associated with the import of foreign nucleotide sequences and the deletion of the entire *syn* operon in 7 class II nongroupable isolates.

An eighth class II isolate (M5018) contained *synA–D* but with an insertion of IS1301 in *synA* (figure 6). The copy of IS1301 was intact but was inverted with respect to the sequence published elsewhere [15] and was inserted at nt 675 (of 1133) in *synA*, 88 bp downstream of the site reported to be involved in reversible IS1301-mediated capsule phase variation [15, 42]. The *synA* gene was otherwise intact in M5018, and *synB–D* were wild type according to PCR.

A ninth class II isolate (M4947) contained a novel insertion sequence element of 756 bp within *synC* (figure 6). The element was composed of a 5' region of 20 bp usually found downstream of *synF*, followed by 202 bp that were 63% identical to a degenerate IS1016 transposase-encoding gene family from the *N. meningitidis* MC58 genome database (available at: <http://>

www.tigr.org) [43]. The 3' end of the IS1016 element formed an inverted repeat of nucleotide sequence identical to the 5' end. The *synC* gene was otherwise intact in this isolate, as were *synA*, *synB*, and *synF*.

A tenth class II isolate (M4989) contained a similar IS1016-like insertion of 776 bp within *synF* (figure 6). The IS1016-like insertion was composed of the same 3' nucleotide sequence downstream of *synF* and the same predicted IS1016 family degenerate transposase homologue as that described above for M4947. However, the insertion in this isolate lacked the sequence found immediately 5' of the transposase sequence in M4947, and the entire insert was inverted, compared with M4947.

An eleventh class II isolate, M4951, lacked *syn* genes and *ctrA-C* but contained *ctrD* (figure 6). A nucleotide sequence normally found downstream of *synE* was adjacent to the extreme 3' end of *ctrC* in M4951, indicating, by sequence homology to the serogroup C genome sequence (available at: <http://www.sanger.ac.uk>), that this isolate was related to serogroup C. IS1301 and IS1016 were not detected in the capsule region of this isolate.

Nongroupable *N. meningitidis* with an *N. lactamica*/*N. gonorrhoeae*-like genetic organization (class III). Ten of 31 nongroupable meningococcal carrier isolates did not contain known capsule biosynthesis or transport genes, according to PCR or Southern hybridizations (figure 5C and 5D). To confirm

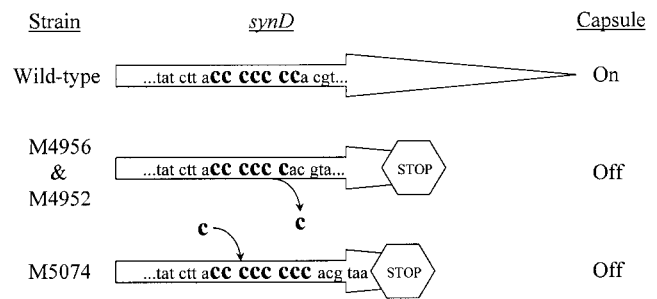


Figure 4. Class I *Neisseria meningitidis*, not groupable because of predicted slipped-strand mispairing within *synD*, which encodes the serogroup B sialyltransferase. In wild-type *N. meningitidis* serogroup B, the *synD* poly-C tract contains 7-dC residues, and capsule is expressed [27]. When a deletion (as in isolate M4956 and M4952) or an insertion (as in isolate M5074) of 1 dC residue occurs, a result of local denaturation and mispairing followed by replication or repair [27, 70], a premature stop codon is generated, and capsule is not expressed.

that these isolates lacked the entire capsule genetic island and did not contain a novel capsule locus, PCR was done using primers within genes known to flank the biosynthesis and transport operons (figure 7). PCR primers designed to the *N. meningitidis tex* and *galE* nucleotide sequences yielded no product (data not shown), but PCR primers designed to *N. gonorrhoeae tex* and *galE* showed that *tex* and *galE* were adjacent in each

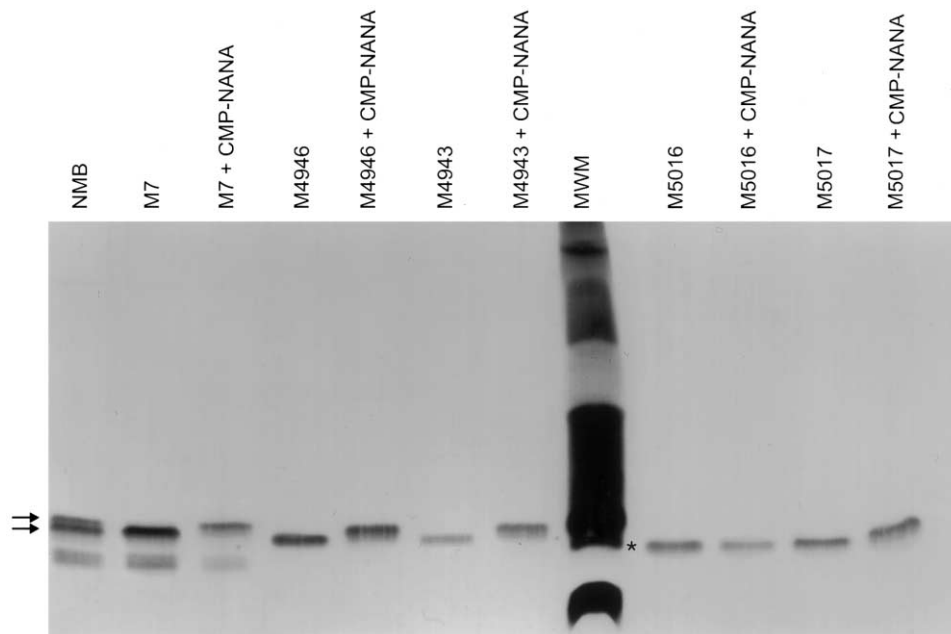


Figure 3. Silver-stained 16.5% tricine SDS-PAGE gel of lipooligosaccharide (LOS) of class I nongroupable meningococcal carrier isolates with and without complementation by 50 μ g/mL exogenous CMP-*N*-acetylneuraminic acid or sialic acid (CMP-NANA). The wild-type serogroup B isolate, NMB, synthesizes both sialylated and unsialylated LOS (arrows). M7, a *synA* mutant of isolate NMB, is able to express only unsialylated LOS (lower arrow). Three nongroupable isolates, M4946, M4943, and M5017, were complemented by CMP-NANA but not isolate M5016. MWM, molecular weight marker; *, Bovine trypsin inhibitor (6.2 kDa).

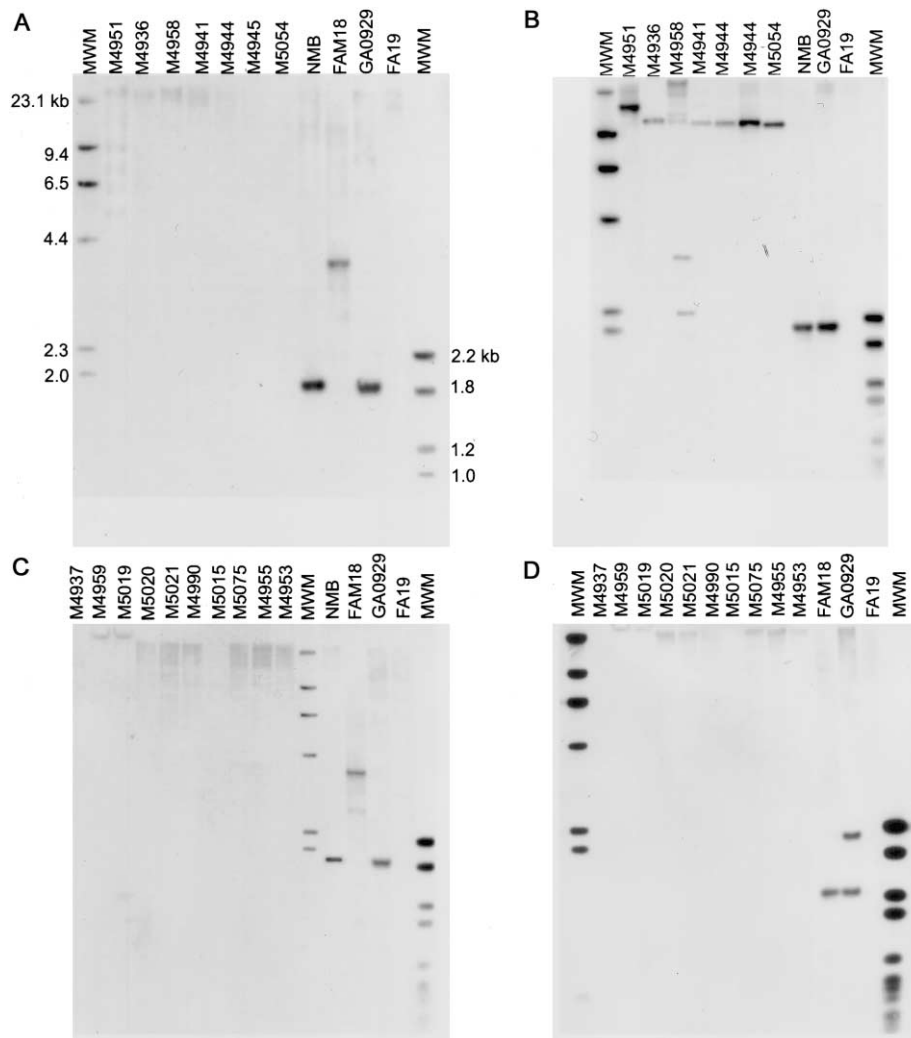


Figure 5. Southern hybridizations showing nongroupable *Neisseria meningitidis* that lack capsule transport and/or biosynthesis genes. *A*, Chromosomal DNA (*PvuII* digest) of class II nongroupable meningococcal isolates M4951, M4936, M4958, M4941, M4944, M4945, and M5054, like the *N. gonorrhoeae* control isolate FA19, did not hybridize with the *synB* probe in contrast to control isolates NMB (serogroup B), FAM18 (serogroup C), and GA0929 (serogroup Y). These same 7 isolates hybridized to the *ctrD* probe (*B*). *C* and *D*, Chromosomal DNA of the 10 class III nongroupable meningococcal isolates M4937, M4959, M5019, M5020, M5021, M4990, M5015, M5075, M4955, and M4953 that were without *ctr* and *syn*, as tested by polymerase chain reaction, and that did not hybridize to *ctrB* (*C*) or *synB* (*D*) gene probes. High- and low-molecular-weight markers (MWM) were used for each blot.

of these 10 class III nongroupable meningococcal isolates. The *tex-galE* arrangement was also found when gonococcal primers in *N. lactamica* (data not shown) and *N. gonorrhoeae* (figure 7) were used. The 10 isolates also did not contain *lipA* or *lipB*.

To further confirm that these class III isolates were *N. meningitidis*, all 10 isolates were found to have *sodC*, a gene that is found in *N. meningitidis* but not in any other neisserial species [44]. The *fbp* gene, which is found in *N. meningitidis* and commensal *Neisseria* but not in *N. gonorrhoeae* [45], was also present in the 10 isolates. All isolates in this group oxidized dextrose and maltose but not sucrose or lactose. None of the isolates expressed β -galactosidase. The class III isolates were

members of the ST-198 complex and were clustered by ET (figure 2).

DISCUSSION

The genetic basis for nongroupable *N. meningitidis* was assessed using a population-based collection of nasopharyngeal carrier isolates from North American high school students. The isolates collected and studied spanned the population diversity of *N. meningitidis* [9]. Nongroupable *N. meningitidis* has been hypothesized to be the result of the expression of novel capsular polysaccharide biosynthesis genes, alterations in the capsule

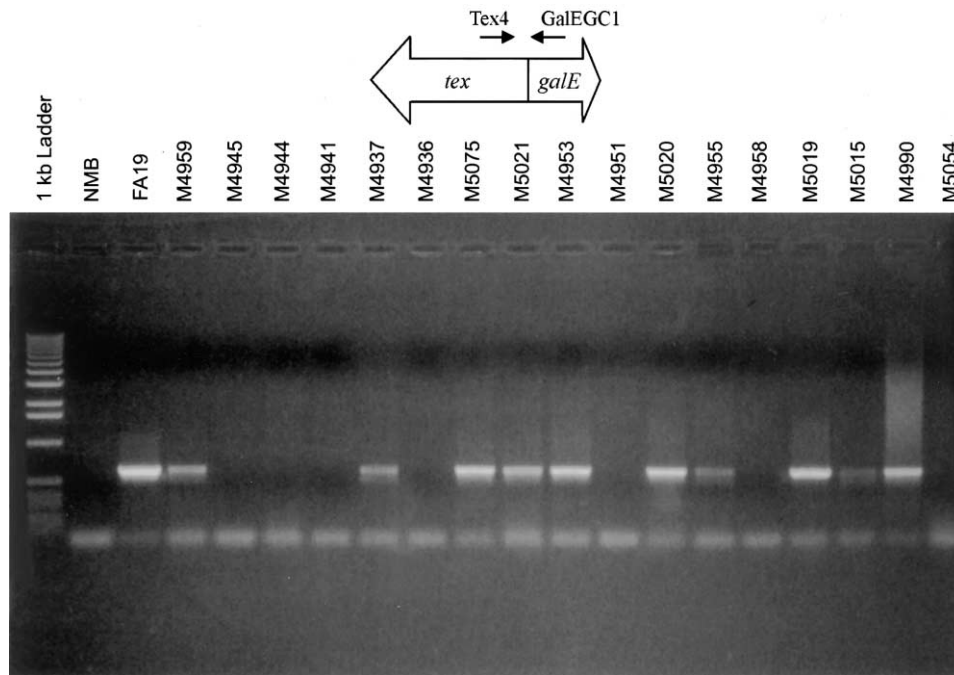


Figure 7. The capsule locus is absent in class III nongroupable *Neisseria meningitidis*. The *tex*-to-*galE* polymerase chain reaction (PCR) products in 17 nongroupable *N. meningitidis* carrier isolates that did not hybridize with *ctr* or *syn* probes and lacked PCR products for capsule transport and/or biosynthesis genes are shown. Primers Tex4 and GalEGC1 were used in this reaction. Note that only the 10 isolates that lacked *ctr* and *syn* genes yielded PCR products: M4959, M4937, M5075, M5021, M4953, M5020, M4955, M5019, M5015, and M4990. Isolates that yielded no product are class II isolates. FA19, *N. gonorrhoeae* wild-type control isolate; NMB, *N. meningitidis* serogroup B wild-type control isolate. Lane 1, 1-kb ladder.

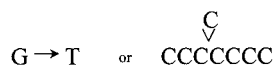
tionally active in *N. meningitidis* [15], and IS1301 insertion into and precise excision out of *synA* has been shown in vitro to be a mechanism for capsule phase variation [15, 42]. The present study demonstrated that IS1301 is found in meningococcal capsule operons in nongroupable isolates from carriers. New *synA*::IS1301 and *ctrA*::IS1301 insertion sites were noted in our study. However, IS1301 was always associated in our population with deletions and recombinations within the *syn* operon and with the import of foreign nucleotide sequences. Thus, recombination events within the capsule biosynthesis operon, including deletions and insertions of foreign DNA, are found in nongroupable *N. meningitidis* and are associated with IS1301. Although IS1301 is common in serogroup Y isolates [42], IS1301 was found within the capsule loci in genetically unrelated isolates throughout the population structure of *N. meningitidis*.

The translated *tagD*-like sequence imported into the capsule loci of many class II isolates appears to be foreign but most closely related to TagD of *B. subtilis*. TagD is the glycerol 3-phosphate cytidylyltransferase in *B. subtilis* and other gram-positive bacteria and has been proposed to have a central role in teichoic acid polymer formation [51, 52]. The *N. meningitidis* serogroups A, B, and C genome databases also contain a gene, *aut*, that has homology to the *tagD* found in class II isolates

and to *B. subtilis tagD*. The meningococcal *aut* is the homologue of domain II of *rfaE* in *Escherichia coli* K12 [53], which catalyzes adenosine triphosphate (ADP) transfer to form ADP-D-glycero-D-manno-heptose in lipopolysaccharide inner-core biosynthesis [54].

An IS1016-like element was also identified within the sialic acid biosynthesis operon of nongroupable isolates. The element was composed of inverted repeats that flanked DNA homologous to the IS1016 family transposase. Of interest, the flanking repeats of this element were composed of a nucleotide sequence found 3' of *synF* and 5' of *galE* in the meningococcal genomes. Complete IS element-mediated deletion of the capsule locus has been noted in nontypeable isolates of *Haemophilus influenzae* [55]. IS1016 flanks the *cap* locus of division I *H. influenzae* isolates in directly repeated copies, forming a compound transposon [56]. Other IS1016 family transposase nucleotide sequences are present downstream of the capsule transport operon in *N. meningitidis* [43, 57] (D.S.S., unpublished data). Nontypeable pharyngeal *H. influenzae* isolates studied by St. Geme et al. [55] contained IS1016 elements in place of the capsule locus. Additionally, the *H. influenzae* isolate Rd, which expressed serotype D capsule, lost the capsule locus because of IS1016-associated recombination between the 2 flanking IS1016 copies [55, 56]. The sialic acid (*syn*) operon of *N. meningitidis*

I. Slipped-strand Mispairing or Point Mutations in Serogroup B, C, or Y Capsule Locus



II. IS Element Insertions and Recombination in Capsule Biosynthesis Region



III. Absence of Capsule Locus: *N. lactamica*/*N. gonorrhoeae*-like

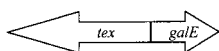


Figure 8. Major classes of nongroupable *Neisseria meningitidis*. Class I, presumed slipped-strand mispairing or point mutations within the serogroup B, C, or Y sialic acid biosynthesis or capsule transport operons. Class II, recombination events associated with insertion (IS) elements and loss of capsule biosynthesis operon genes. Class III, the complete absence of a meningococcal capsule locus. These isolates possessed a *Neisseria lactamica*/*Neisseria gonorrhoeae*-like organization and nucleotide sequence.

appears to be a hot spot for IS insertions and recombination [15, 30, 58]. Recently, Arreaza et al. [59] described insertion of another element, IS435IN1, in the meningococcal capsule locus that was accompanied by the duplication of target DNA flanking the element.

In the completed *N. meningitidis* serogroup B MC58 and serogroup A Z2491 genomes, Tettelin et al. [43] and Parkhill et al. [57] recognized the capsule locus as a genetic island located between *tex* and *galE*, the presumed result of horizontally transferred DNA. We found that 17% of nongroupable *N. meningitidis* did not contain a capsule locus or other capsule expression genes. In these isolates, the *tex* and *galE* adjacent genetic arrangement was identical to that found in *N. gonorrhoeae* and commensal *Neisseria* species. Furthermore, the nucleotide sequence of *tex* and *galE* in these meningococcal isolates had greater homology to these genes in *N. gonorrhoeae* and commensal *Neisseria* species.

ET and MLST genetic typing data in our study demonstrated close genetic relatedness among nongroupable isolates belonging to 2 groups (figure 2). All isolates that contained the serogroup Y polymerase were members of the ET-508/ST-23 complex. ET-508/ST-23 complex isolates were also the most common groupable isolates in this population [9] at a time when serogroup Y was the major cause of endemic disease in the state. Of interest, ST-23 complex isolates were not found in a Czech Republic carriage study [60]. The second genetic cluster of nongroupable isolates was formed by all of the class III isolates, which belonged to the ST-198 complex (figure 2).

Recently, Claus et al. [61] also noted that 16.4% of carriage isolates from Bavaria harbored the capsule-null genotype. In the Bavarian carriage collection, 32% of isolates with capsule-null loci were of the ST-198 complex, whereas the remaining capsule-null isolates represented 5 other ST complexes. Among these capsule-null Bavarian meningococci was an ST-44 complex (lineage 3 by MLEE) isolate, which in the Georgia carriage study was only found among class I and II nongroupable isolates. Of interest, ST-198 complex isolates were not found in the Czech carriage study [60]. The capsule-null locus may represent the ancestral state of *N. meningitidis*, with some genetically diverse meningococci having retained this ancestral genotype while others have not.

Class I nongroupable meningococci likely represent reversible capsule loss that is associated with genetically diverse meningococci and that one would imagine would be an ideal adaptation for both the long-term survival of *N. meningitidis* in the nasopharynx and the aerosol transmission. By contrast, the insertions identified among class II isolates are likely irreversible and probably occur frequently among nasopharyngeal meningococci, given the large number of isolates in this class that harbored distinct insertions. Some of these insertions must become fixed within the population, because class II isolates were genetically distinct, but the lack of clonal expansion of isolates harboring class II insertions within the population sampled suggests that they are short-term solutions for survival and represent evolutionary dead ends. It is interesting that certain clonal complexes, as represented by class III isolates, have evolved to survive without ever expressing a capsule, which might mean that they have transmission dynamics that differ from those traditionally attributed to meningococci.

The exchange of genetic cassettes via inter- and intraspecies transformation has been well described in *N. meningitidis* and *N. gonorrhoeae*. Examples are the *exl* [62] and *pilS/pilE* loci [63], the penicillin-binding protein 2 (*penA*) gene (which converts a sensitive meningococcus to penicillin resistance [64]), the adenylate kinase (*adk*) gene [65], at a locus involved in sulfonamide resistance (*dhps*) [66], and at the shikimate dehydrogenase (*aroE*) and glutamine synthetase (*glnA*) genes [67]. Interspecies recombination events occur in vivo: an *N. meningitidis* vaginal isolate was characterized that had acquired the *N. gonorrhoeae* *porB* gene [68]. Transformation and recombination-mediated switching of meningococcal capsular types has also been reported in vivo [30, 58] and results in the transformation of the *syn* operon. The transformation and recombination of the capsule genetic loci observed in our nongroupable isolates into encapsulated isolates would abrogate capsule expression. One-third of *N. meningitidis* nasopharyngeal isolates are consistently nongroupable, representing a large pool of DNA that will maintain this phenotype. Nongroupable meningococcal isolates represent a reservoir that will influence

diagnostic assays, decisions about prophylaxis, and, potentially, herd immunity to new conjugate meningococcal vaccines. Knowledge of the nongroupable meningococcal population is also important in understanding meningococcal biology and pathogenesis.

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