

Capsule switching of *Neisseria meningitidis*

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ABSTRACT The different sialic acid (serogroups B, C, Y, and W-135) and nonsialic acid (serogroup A) capsular polysaccharides expressed by *Neisseria meningitidis* are major virulence factors and are used as epidemiologic markers and vaccine targets. However, the identification of meningococcal isolates with similar genetic markers but expressing different capsular polysaccharides suggests that meningococcal clones can switch the type of capsule they express. We identified, except for capsule, isogenic serogroups B [(α 2→8)-linked polysialic acid] and C [(α 2→9)-linked polysialic acid] meningococcal isolates from an outbreak of meningococcal disease in the U. S. Pacific Northwest. We used these isolates and prototype serogroup A, B, C, Y, and W-135 strains to define the capsular biosynthetic and transport operons of the major meningococcal serogroups and to show that switching from the B to C capsule in the outbreak strain was the result of allelic exchange of the polysialyltransferase. Capsule switching was probably the result of transformation and horizontal DNA exchange *in vivo* of a serogroup C capsule biosynthetic operon. These findings indicate that closely related virulent meningococcal clones may not be recognized by traditional serogroup-based surveillance and can escape vaccine-induced or natural protective immunity by capsule switching. Capsule switching may be an important virulence mechanism of meningococci and other encapsulated bacterial pathogens. As vaccine development progresses and broader immunization with capsular polysaccharide conjugate vaccines becomes a reality, the ability to switch capsular types may have important implications for the impact of these vaccines.

Neisseria meningitidis is a leading worldwide cause of meningitis and rapidly fatal sepsis in otherwise healthy individuals (1); over 350,000 cases of meningococcal disease were estimated to have occurred in 1995 (2). The problem of meningococcal disease is emphasized by the recurrence of major epidemics due to serogroups A, B, and C *N. meningitidis* over the last 20 years (3–9) [such as the devastating serogroup A outbreak in sub-Saharan Africa this year (W.H.O., 1996, unpublished bulletin); the recent dramatic increases in the incidence of serogroups B and C meningococcal disease in parts of North America (6–8); and the emergence in Europe and elsewhere of meningococci with decreased susceptibility to antibiotics (10)].

Differences in capsular polysaccharide chemical structure determine the meningococcal serogroups (11, 12). Meningococci of serogroups B, C, Y, and W-135 express capsules composed entirely of polysialic acid or sialic acid linked to glucose or galactose (12, 13), while the capsule of group A *N. meningitidis* is composed of *N*-acetyl mannosamine-1-phosphate (11). The currently available capsular polysaccharide vaccines for serogroups A, C, Y, or W-135 *N. meningitidis* are effective for control of meningococcal out-

breaks in older children and adults. However, because of poor immunogenicity in young children and short-lived immunity (14), these vaccines are not routinely used for long-term prevention of meningococcal disease. In the case of group B *N. meningitidis*, whose (α 2→8)-linked polysialic capsule is an immunotolerized self-antigen, a reliable polysaccharide vaccine is not yet available. However, rapid progress is being made in development of polysaccharide–protein conjugate vaccines, and it is hoped that, following the example of newly licensed *Haemophilus influenzae* type b vaccines, widespread introduction of the polysaccharide conjugates will lead to elimination of disease.

In some epidemic settings, simultaneous or closely linked meningococcal outbreaks have occurred in the same population due to different serogroups (5, 6, 15). Further, Caugant *et al.* (3, 16) have noted that meningococcal isolates of different serogroups may be members of the same enzyme type (ET)-5, ET-37, or ET-4 clonal complexes.

Since 1993 the number of cases of serogroup B meningococcal disease in Oregon and adjacent counties in Washington has doubled, and the overall incidence has been fivefold higher than rates observed in other parts of the United States (6). This increase was due to the first appearance in the United States of serogroup B meningococcal strains belonging to the ET-5 complex. ET-5 complex strains have been responsible for major epidemics in Norway, Iceland, Cuba, and South America over the last 20 years (3, 17, 18). Since 1994, cases of serogroup C meningococcal disease due to ET-5 complex strains were also noted in Oregon and Washington. Recent advances (19–27) in understanding the genetic basis for meningococcal capsule expression allowed us to carefully analyze the serogroup B and C ET-5 meningococcal strains responsible for the outbreak in the Pacific Northwest.

MATERIALS AND METHODS

Bacterial Strains. Forty serogroup B and C ET-5 complex meningococcal isolates recovered from Oregon, Washington, and California in 1994 and 1995 were used in these studies. In addition, meningococcal strains GA0078 (serogroup C), GA0290 (C), NMB (B), C114 (B), M986 (B), 2996 (B), KB (B), 269B (B), FAM18 (C), 6083 (W-135), GA0929 (Y), F8229 (A), F8239 (A), NM-44/76 (B), GA1002 (W-135), *N. gonorrhoeae* strain FA19, and *Neisseria lactamica* and other commensal *Neisseria* spp. (refs. 21 and 28; this study) were also used.

Molecular Epidemiology. Multiple enzyme electrophoretic (ET) typing and pulsed-field gel electrophoresis (PFGE) were performed as described (29, 30). Specific enzyme types (e.g., ET-301) were designated by the Centers for Disease Control Meningococcal Reference Laboratory (Atlanta). Serotyping of meningococcal strains was done as described (31), with the fol-

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Abbreviations: ET, enzyme type; PFGE, pulsed-field gel electrophoresis. Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U75650).

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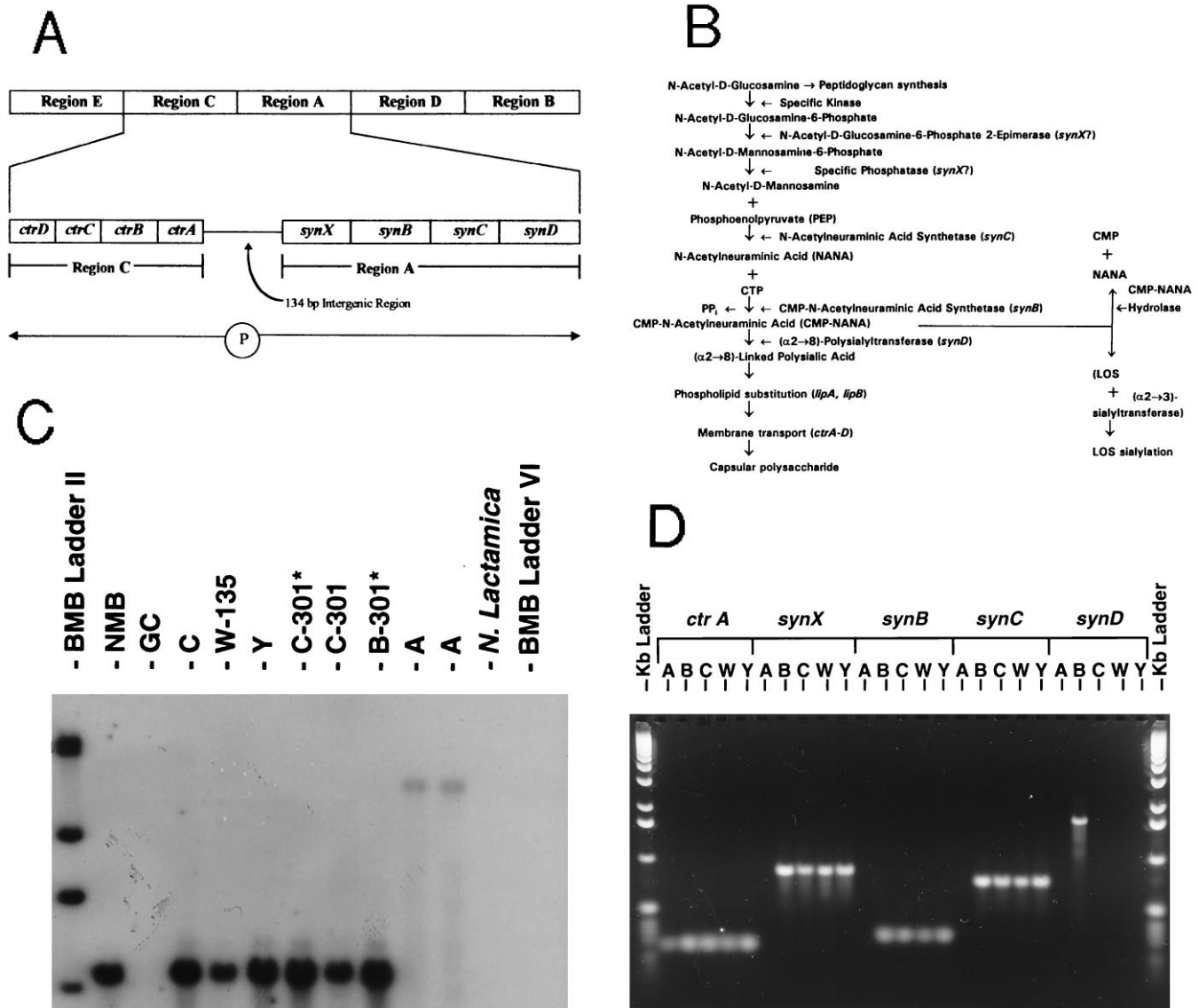


FIG. 1. Molecular analysis of capsule biosynthesis and membrane transport genes in prototype isolates of serogroup A, B, C, Y, and W-135 *N. meningitidis*. (A) Genetic basis for serogroup B meningococcal capsular polysaccharide. Meningococcal capsules are produced by genes encoded by the 24-kb *cps* gene complex (19), which is composed of five regions: E, C, A, D, and B. In serogroup B, four capsular biosynthetic genes (*synX-D*) are found in region A and are transcribed as an operon. Region C, adjacent to region A, contains four polycistronic genes, *ctrA-D*, encoding proteins that transport the phospholipid-substituted polysialic acid across the inner and outer membranes. The *ctr* genes are transcribed in the opposite orientation from the *syn* biosynthetic genes of region A, but use the same 134 bp promoter region (26). (B) The biosynthetic pathway for production of serogroup B capsule; SynX is either the *N*-acetyl-D-glucosamine-6-phosphate 2-epimerase that produces *N*-acetyl-D-mannosamine 6-phosphate or a specific phosphatase that converts *N*-acetyl-D-mannosamine 6-phosphate into *N*-acetyl-D-mannosamine (21); SynB is the CMP-*N*-acetylneuraminic acid (NANA) synthetase (23); SynC is the NANA synthetase (22); and SynD is the polysialyltransferase responsible for (α 2 \rightarrow 8)-linked polysialic acid chain polymerization and elongation (24). (C) Southern DNA hybridization showing *ctrA* homology in serogroups A (strains F8229, F8239), B (strains NMB, 1070 [B-301*]), C (strains FAM18, 1205 [C-301*], 1843 [C-301]), Y (strain GA0929), and W-135 (strain 6083) of *N. meningitidis*. Chromosomal DNA from each of the strains was prepared, digested with *Cla*I, electrophoresed through a 1.2% agarose gel, and transferred to a nylon membrane. The membrane was then probed with a 150 bp digoxigenin-labeled PCR product derived from the 5'-end of the serogroup B *ctrA* gene. *N. lactamica* and *N. gonorrhoeae* (GC) showed no hybridization. Molecular weight size standards (Boehringer Mannheim) flank the chromosomal digests. (D) PCR amplification of *ctrA* and *synX-synD* from serogroups A (strain F8239), B (strain NMB), C (strain FAM18), W-135 (strain 6083), and Y (strain GA0929) *N. meningitidis* using oligonucleotide primers derived from the individual gene sequences of serogroup B prototype strain NMB. Kb DNA ladders (BRL) flank the gel. [Fig. 1A and B are modified with permission from ref. 26 (Copyright 1996, American Society for Microbiology).]

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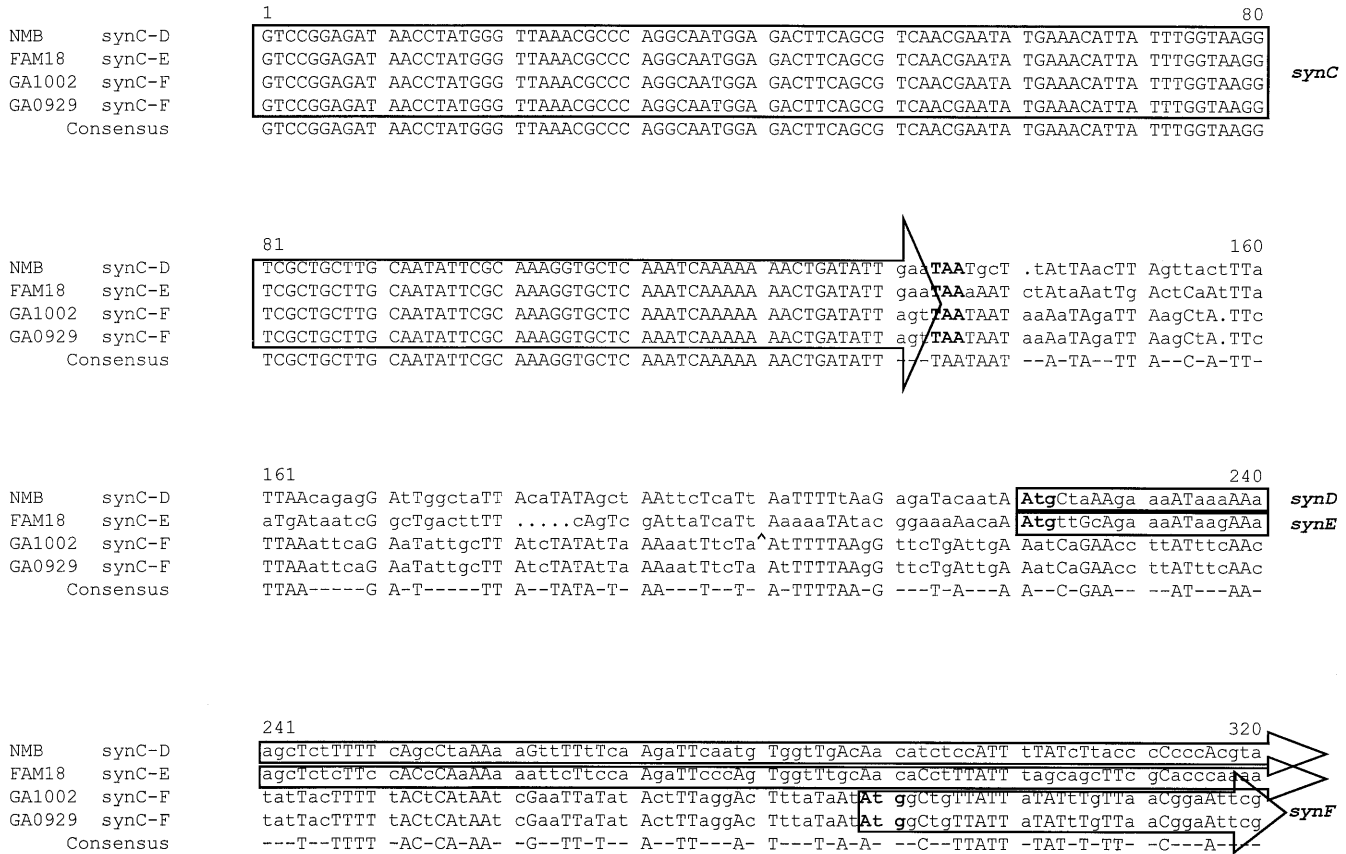


FIG. 2. Multiple nucleotide sequence alignment of the 3' end of *synC* and downstream sequence in serogroups B (strain NMB), C (strain FAM18), W-135 (strain GA1002), and Y (strain GA0929) *N. meningitidis* [Pretty multiple sequence comparison program of the Genetics Computer Group sequence analysis package version 7.3.1 UNIX (33)]. Consensus nucleotide matches (three or more identical) at each position are indicated in uppercase type, while differences from consensus are indicated by lowercase type. Dots indicate gaps introduced by the analysis program to facilitate alignment. The *synC* termination codon (TAA) and the *synD/E/F* start codons (ATG) are shown in boldface type. The location of an *IS1301* element located downstream of the *synC* gene in the otherwise identical sequence of a second serogroup W-135 strain, 6083, is shown in the GA1002 sequence by a [^]. The complete sequence of *synE* derived from serogroup C strain FAM18 is available through the GenBank data base under accession no. U75650.

lowing modifications. The serotyping procedure was modified to grow meningococci on brain heart infusion agar (Difco) supplemented with 1% horse serum (GIBCO), and to use a higher concentration of cells (cell density, OD₆₀₀ 1.0), different blocking buffer (PBS + 0.1% Tween 20), and shorter primary antibody incubation (2.5 h).

Molecular Biology. PCR, Southern DNA hybridization, and DNA sequencing techniques were performed as described (32). Automated sequencing using an Applied Biosystems model 377 automated DNA sequencing system was performed on some PCR templates. Oligonucleotide primers used for PCR, sequencing, and construction of Southern probes were as follows: 5' *ctrA*, 5'-GTGTGGAAGTTAATTGTAGGATG-3'; 3' *ctrA*, 5'-CCAC-CACCAACAATACTGCGG-3'; 5' *synX*, 5'-GCAATACCAT-TACGTTTATCTCTC-3'; 3' *synX*, 5'-GTTTCAGGATTGTT-GATTACTTCAGC-3'; 5' *synB*, 5'-GTCCTACGCCCTGCA-GAGCTGG-3'; 3' *synB*, 5'-CATTAGGCCTAAATGCCT-AGG-3'; 5' *synC*, 5'-GCTGAAGTTGTTAAACATCAAACAC-3'; 3' *synC*, 5'-GCTACGACAGATGCAAAGGCG-3'; 5' *synD*, 5'-AGAGGATTGGCTATTACATATAGC-3'; 3' *synD*, 5'-AGCTCTGTTGTCGATTACTCTCC-3'; 5' *FKBP*, 5'-CATTACACAGGTTGGCTGGAAGACGG-3'; 3' *FKBP*, 5'-GCAGCTCGACTTCAAATATCAAAGTGGC-3'; 5' *recA*, 5'-GCCAGCAGGAAGAAAACCTCG-3'; 3' *recA*, 5'-GCCGTT-GTAGCTGTACCACGC-3'; 5' *ctrA-synX*, 5'-CACCACCA-AACAATACTGCC-3'; 3' *ctrA-synX*, 5'-GCTTGTTCAT-TTTGCTACCAAGTGG-3'; 5' *galE*, 5'-CCAGCATCAATAT-CCTGCCACG-3'; 3' *galE*, 5'-CCATCATTGTGCAAGGCT-

CGC-3'. Nucleotide sequences were analyzed using either the DNAsar (Madison, WI) sequence analysis software or the Genetics Computer Group (Madison, WI) Sequence Analysis Software Package, Version 7.3.1 UNIX (33). Plate transformations of meningococcal strains were performed as described (32).

RESULTS

Molecular Epidemiology. Serogroup B and C meningococcal strains from the Pacific Northwest outbreak were studied by ET typing (29), serotyping (31), and PFGE (30). These included 35 ET-5 complex strains (29 were serogroup B and 6 were serogroup C) consecutively isolated during 1994 in Oregon and five serogroup B ET-5 complex strains recovered in 1994–1995 from Washington and California. In Oregon in 1994, ≈88% of serogroup B isolates studied were ET-5 complex strains and 17% of C isolates were ET-5 complex strains. None of the strains were from case-clusters or epidemiologically linked patients. All were ET-301 (a member of the ET-5 complex). All, except one, were serotype 4 or 15, all were subtype 1.7,16 and all except one expressed the L3,7,9 LOS immunotype. One predominant PFGE pattern (A) was seen in these isolates. None of the isolates differed from the predominant PFGE A pattern by more than three bands, indicating the isolates were closely related (34). These data, as well as similar data on other strains of this outbreak isolated in 1993–1996 showed identity or close-relatedness of the ET-5 serogroup B strains causing epidemic disease in the Pacific Northwest. In addition, the serogroup C strains isolated were identical to the dominant serogroup B strains by these molecular epidemiologic

markers. These data suggested that the epidemic meningococcal clone causing the outbreak in the Pacific Northwest may express either serogroup B [($\alpha 2 \rightarrow 8$)-linked polysialic acid] or serogroup C [($\alpha 2 \rightarrow 9$)-linked polysialic acid] capsular polysaccharide. The outbreak strains were distinct by ET typing, serotyping, subtyping, and PFGE from serogroup B and C meningococcal disease isolates recovered from other parts of the country during this time.

Genetic Structure of Capsule Biosynthesis and Transport Regions in Serogroups A, B, C, Y, and W-135 *N. meningitidis*. The genetic basis for serogroup B meningococcal capsule biosynthesis and membrane translocation has been under active study (19–27) and is summarized in Fig. 1A. The role of these genes in the capsule synthesis pathway is shown in Fig. 1B. Using Southern blot analysis, PCR, and DNA sequencing (21, 26), we assessed the genetic structure of the capsule transport and biosynthetic regions in strains from each of the other major meningococcal serogroups (Fig. 1C and D). The strains of the sialic acid capsule-expressing serogroups (B, C, Y, and W-135) were found to have a similar genetic organization consisting of the *ctrA* capsule transport gene linked by a short intergenic region to the oppositely transcribed biosynthetic genes *synX-synC*. Based on identical Southern hybridization patterns for *ctrA* (Fig. 1C), *synX*, *synB*, and *synC* (data not shown); identical PCR amplification products (Fig. 1D) of *ctrA*, *synX*, *synB*, and *synC*; and similar nucleotide sequences of the *ctrA-synX* intergenic region (26), we concluded that *ctrA* and *synX-synC* in serogroups C, Y, and W-135 *N. meningitidis* were homologues of the same genes in serogroup B meningococci. In contrast, *synD* [which encodes the serogroup B ($\alpha 2 \rightarrow 8$)-linked capsule polysialyltransferase (24)] was not detected in the serogroup C, Y, and W-135 strains by Southern hybridization or PCR amplification using probes specific for *synD* (Fig. 1D).

To investigate these observations further, the nucleotide sequence of the 3' end of *synC* and the sequence downstream of *synC* were determined in serogroups C, Y, and W-135. The sequences of the 3' end of *synC* from serogroups B, C, Y, and W-135 were identical up to the last codon where the sequences then diverged (Fig. 2). The 5' ends of the downstream ORFs, which encode the putative sialic acid capsule polymerases (designated in serogroup B as *synD*, in serogroup C as *synE*, and in serogroups Y and W-135 as *synF*), were distinct (Fig. 2). In the serogroup Y and W-135 strains the codon for the last amino acid in *synC* had been replaced by a different codon (creating a change from glutamine to serine) (Fig. 2). The nucleotide sequences downstream of *synC* were almost identical in serogroups Y and W-135 both in the intergenic region and in the first 800 bases of the 5' end of the predicted capsule polymerase, but were distinct from serogroups B and C.

Thus, meningococci expressing serogroup B, C, Y, or W-135 sialic acid capsules have similar *synX-synC* biosynthetic genes that are linked to *ctrA* of the capsule membrane transport operon. However, the genes encoding the sialic acid capsule polymerases in serogroups B, C, and Y/W-135 are different. Interestingly, meningococci of serogroups Y and W-135 are almost identical in the 5' end of this gene. These are known to be closely related serogroups, and simultaneous elaboration of both serogroup W-135 and Y capsular polysaccharides by a single strain of *N. meningitidis* has been reported (35).

In contrast to the sialic acid producing serogroups, serogroup A meningococci contain a *ctrA* homologue but did not have a *ctrA-synX* intergenic region or the sialic acid biosynthetic homo-

logues *synX-synD* homologues (Fig. 1D). The serogroup A *ctrA* differed in nucleotide sequence and Southern *ClaI* fragment size from the sequence and location of *ctrA* in the sialic acid capsule-expressing serogroups (Fig. 1C). Southern blot and PCR analyses revealed that for a specific serogroup, the genes (e.g., *synD*, *synE*, *synF*) involved in alternative capsule polymerization were not present elsewhere in the chromosome [e.g., serogroup B strains contained *synD* but not *synE* or *synF* homologues (data not shown)].

Transformation of Meningococcal Capsule Biosynthesis Operon *In Vitro*. Because meningococci are naturally competent for transformation, we postulated that conversion from one sialic acid-expressing capsule serogroup to another could be accomplished by homologous recombination of the sequences encoding the serogroup-specific capsule polymerase. To test this hypothesis, chromosomal DNA containing a class I *Tn916* insertion interrupting *synD* of the serogroup B strain NMB (26) was prepared and used to transform (32) the prototype serogroup C, Y, and W-135 meningococcal strains. Tetracycline-resistant transformants were obtained at a frequency of between 1×10^{-5} and 1×10^{-7} per recipient. Acquisition of the *Tn916* mutation and the adjacent *synD* sequence was confirmed by PCR and nucleotide sequence analysis of selected tetracycline-resistant transformants of these strains.

Analysis of Capsule Biosynthesis and Other Genes in Serogroup B and Serogroup C ET-5 Complex Strains from the Pacific Northwest Outbreak. We hypothesized that a transformation event involving the capsule biosynthesis genes might have produced the closely related serogroup B and C meningococcal strains recovered in the Oregon and Washington outbreak. Thus, we analyzed the capsule biosynthetic and transport operons as well as unlinked genes in two serogroup B and three serogroup C ET-5 complex strains (Table 1) recovered from this outbreak. These strains by ET-type (301), serotype (15), subtype (1.7,16), immunotype (L3,7,9), and PFGE type were identical, except for the type of capsule they produced.

(i) *Capsule biosynthesis operon.* By PCR and Southern hybridization profile, the strains had similar *ctrA* and *synX-synC* homologues, but the serogroup B ET-301 strains contained a *synD* homologue and the serogroup C ET-301 strains contained a *synE* homologue. This observation was confirmed by determination of the nucleotide sequences of the intergenic region following *synC* and the sequences of the 5' end of the downstream gene encoding the predicted polysialyltransferase. As shown in Fig. 3A, these regions were distinct in strain 1070 (serogroup B, ET-301) and 1205 (serogroup C, ET-301) isolates (only 63% nucleotide identity). The nucleotide sequence of *synD* in the B301 strain was 99% identical to *synD* of the prototype serogroup B strain NMB; and in the C301 strain, *synE* was 99% identical to *synE* of the prototype serogroup C strain FAM18. Nucleotide sequences of *synX* and *synC* from strains 1070 and 1205 demonstrated 1% (*synX*) and 5% (*synC*) diversity (Fig. 3A and B1), suggesting that in addition to the polysialyltransferase, the entire *synX-synD* biosynthetic operon had exchanged.

(ii) *Recombinational event.* In contrast to the biosynthesis operon, the 5' nucleotide sequence of *ctrA* and the *ctrA-synX* intergenic region were identical in B-301 strains 1070 and 1069 and C-301 strains 1205, 1198, and 1204, but differed from other B, C, Y, and W-135 strains (Fig. 3B1). For example, the two B-301 and

Table 1. *N. meningitidis* isolates of the ET-5 complex recovered from patients with invasive meningococcal disease in Oregon in 1994

ID no.	Date of onset of illness	Serogroup	Serotype	Subtype	Immunotype	ET type	PFGE type
B301#1 1070	06/21/94	B	15	1.7,16	L3,7,9	301	A
B301#2 1069	06/13/94	B	15	1.7,16	L3,7,9	301	A
C301#1 1205	11/19/94	C	15	1.7,16	L3,7,9	301	A
C301#2 1198	08/08/94	C	15	1.7,16	L3,7,9	301	A
C301#3 1204	10/29/94	C	15	1.7,16	L3,7,9	301	A

region separating *ctrA-synX*, and did not involve unlinked genes. The downstream recombinational exchange is not clearly established but may have occurred upstream or within *galE*. PCR studies using primers specific for the 3' end of *synC* and the 5' end of *galE* (37) indicated that *synD/synE* were downstream from *galE* by ≈ 2 kb in the prototype serogroup B strain, NMB, in the prototype serogroup C strain, FAM18, and in each of the B-301 and C-301 strains. PCR amplification of chromosomal DNA from B-301 and C-301 strains using internal *galE*-specific primers derived from the NMB *galE* sequence (37) yielded a 900-bp product. However, this product was not obtained with the serogroup C prototype strains FAM18 and two other non-ET-301 serogroup C strains (GA0078-ET-17 and GA0290-ET-27).

DISCUSSION

The data indicate that capsule switching in *N. meningitidis* can occur by gene conversion of the capsule polymerase and that this event occurs *in vivo*. Presumably, co-colonization of serogroup B and C strains in the human nasopharynx and genetic exchange of capsule biosynthesis genes by transformation and allelic-exchange are the events responsible for capsule switching. The high frequency (5–10%) of meningococcal carriage in the human nasopharynx of adults (38), which appears to increase in epidemic settings, may facilitate the chances of capsule switching. These data are supported by molecular analysis of meningococcal strain collections that contain isolates with otherwise identical genetic markers (e.g., ET-type) that express different capsular polysaccharides (3, 18).

In addition to the meningococcal epidemic in the Pacific Northwest, recent cases of meningococcal disease caused by B and C strains with identical serotypes and ET types in the Czech Republic and Canada (39, 40) suggest capsule switching may be common. Indeed, the ability to switch capsules provides a selective advantage to meningococci, inasmuch as they are thereby able to evade killing, opsonization, or neutralization by preexisting anticapsular antibody. Other experimental studies suggest capsule switching may not be an isolated event in meningococci, but appears to occur in encapsulated *Streptococcus pneumoniae* and *Haemophilus influenzae* (41, 42).

These data have important implications for understanding meningococcal pathogenesis, for developing vaccine prevention strategies based on capsular polysaccharide, and for epidemiologic investigations of disease due to encapsulated bacteria. For example, meningococci of different serogroups recovered during epidemic outbreaks or from cases of endemic disease can be identical in their expression of other virulence factors (e.g., outer membrane proteins). Because they do not express ($\alpha 2 \rightarrow 8$)-linked polysialic acid, the serogroup C ET-5 strains noted in this study may have significance in the current development of ET-5 outer membrane protein vaccines. Meningococcal capsule switching appears to occur among sialic acid-expressing strains by allelic replacement of the sialic acid capsule polymerase. Since the genetic structure responsible for capsule expression in group A meningococci is different (see above), homologous recombination with conversion of serogroup A strains to sialic acid capsule-expressing strains (by introduction of a sialic acid capsule biosynthetic operon) may be less likely.

These data suggest a general strategy by which meningococci and other encapsulated bacteria capable of causing epidemic outbreaks or endemic disease escape vaccine-induced or natural protective immunity. Thus, multivalent vaccines effective against all major capsular serogroups may be needed to control epidemics and possibly endemic disease. Evidence of capsule switching in encapsulated pathogens isolated in future outbreaks should be sought.

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