

## Characterization of the Gene Cassette Required for Biosynthesis of the ( $\alpha$ 1 $\rightarrow$ 6)-Linked *N*-Acetyl-D-Mannosamine-1-Phosphate Capsule of Serogroup A *Neisseria meningitidis*

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The ( $\alpha$ 1 $\rightarrow$ 6)-linked *N*-acetyl-D-mannosamine-1-phosphate meningococcal capsule of serogroup A *Neisseria meningitidis* is biochemically distinct from the sialic acid-containing capsules produced by other disease-associated meningococcal serogroups (e.g., B, C, Y, and W-135). We defined the genetic cassette responsible for expression of the serogroup A capsule. The cassette comprised a 4,701-bp nucleotide sequence located between the outer membrane capsule transporter gene, *ctrA*, and *galE*, encoding the UDP-glucose-4-epimerase. Four open reading frames (ORFs) not found in the genomes of the other meningococcal serogroups were identified. The first serogroup A ORF was separated from *ctrA* by a 218-bp intergenic region. Reverse transcriptase (RT) PCR and primer extension studies of serogroup A mRNA showed that all four ORFs were cotranscribed in the opposite orientation to *ctrA* and that transcription of the ORFs was initiated from the intergenic region by a  $\sigma$ -70-type promoter that overlapped the *ctrA* promoter. The first ORF exhibited 58% amino acid identity with the UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) 2-epimerase of *Escherichia coli*, which is responsible for the conversion of UDP-GlcNAc into UDP-*N*-acetyl-D-mannosamine. Polar or nonpolar mutagenesis of each of the ORFs resulted in an abrogation of serogroup A capsule production as determined by colony immunoblots and enzyme-linked immunosorbent assay. Replacement of the serogroup A biosynthetic gene cassette with a serogroup B cassette by transformation resulted in capsule switching from a serogroup A capsule to a serogroup B capsule. These data indicate that assembly of the serogroup A capsule likely begins with monomeric UDP-GlcNAc and requires proteins encoded by three other genes found in the serogroup A *N. meningitidis*-specific operon located between *ctrA* and *galE*.

Recurrent, devastating epidemics of meningitis and septicemia due to serogroup A *Neisseria meningitidis* continue to plague many parts of the developing world (2, 28, 29, 31, 32, 42, 43) and can produce attack rates that approach 1% of the population. The serogroup specificity of *N. meningitidis* is determined by the structure of the expressed capsular polysaccharide. The serogroup A capsule is composed of repeating units of ( $\alpha$ 1 $\rightarrow$ 6)-linked *N*-acetyl-D-mannosamine-1-phosphate (26). This structure is chemically distinct from those of the capsules of the other major disease-associated meningococcal serogroups, B, C, Y, and W-135, which are composed of or contain sialic acid. The genes required for biosynthesis of the sialic acid-containing capsules of *N. meningitidis* have recently been characterized (38), but the genetic basis for serogroup A capsular polysaccharide biosynthesis has not been defined.

We and others (15, 38) have previously shown that serogroup A *N. meningitidis* contains a homolog of *ctrA*, the first of four polycistronic genes, *ctrA* to *-D*, found in meningococcal serogroups B, C, Y, and W-135. The *ctrA* to *-D* genes are members of the ABC transporter family and encode proteins involved in the transport of the assembled capsule across the inner and outer membranes (14). The serogroup A *ctrA* homolog, however, is distinct in Southern *Clal* fragment size (38) and 5' nucleotide sequence (15) from *ctrA* in the sialic acid

capsule-expressing serogroups. Adjacent to the 5' end of *ctrA* in serogroups B, C, Y, and W-135 is a 134-bp intergenic region separating the *ctrA* to *-D* transport cluster from the sialic acid biosynthesis and capsule polymerase genes, *synA* to *-D* (*E*, *F*, *G*) (37, 38). In this report we characterize the genetic cassette immediately upstream of *ctrA* in serogroup A *N. meningitidis* and establish its role in ( $\alpha$ 1 $\rightarrow$ 6)-linked *N*-acetyl-D-mannosamine-1-phosphate capsule biosynthesis.

### MATERIALS AND METHODS

**Strains, plasmids, and growth conditions.** Serogroup A meningococcal strains F8229 and F8239 were originally isolated during an outbreak in Nairobi, Kenya in 1989 (32) and were provided by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia. Strain F8229 (CDC1750) is encapsulated and was clinically isolated from the cerebrospinal fluid of a patient with meningitis. Strain F8239 (CDC16N3) is an unencapsulated variant originally isolated as a serogroup A strain from the pharynx of an asymptomatic carrier. These strains belong to clonal group III-1 and are closely related to strains that have caused epidemics in Saudi Arabia, Chad, Ethiopia, and other parts of the world (2, 28, 31, 32). F8229-ORF1 $\Omega$ , F8229-ORF2 $\Omega$ , F8229-ORF2*aphA*-3, F8229-ORF3 $\Omega$ , and F8229-ORF4 $\Omega$  are serogroup A mutants created by insertional mutagenesis, as described below. Strains F8229-43 and F8229-43<sup>R</sup> were created by transformation of strain F8229 with chromosomal DNA obtained from serogroup B meningococcal mutant strain 43 (37).

*Escherichia coli*  $\alpha$ InvF' (Invitrogen, San Diego, Calif.) was used as the host strain for all cloned PCR products and recombinant plasmids created during these studies. Plasmid pHP45 (33) was the source of the spectinomycin-resistant  $\Omega$ -fragments used for polar gene mutagenesis (see below), and plasmid pUC18K (27) was the source of the *aphA*-3 kanamycin resistance cassette used for nonpolar mutagenesis (see below).

Meningococcal strains were grown on gonococcal (GC) base agar (Difco Laboratories, Detroit, Mich.) or in GC broth (30) at 37°C with 3.5% CO<sub>2</sub>, with the exception of meningococcal mutants grown under kanamycin selection, which was performed on brain heart infusion base agar (Becton Dickinson and

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TABLE 1. Oligonucleotide primers used in this study

Primer	Nucleotide sequence (5'→3')
LJ4	CCACCACCAACAATACTGCCG
SE26	GCGTTTAAACAAGCGTTCTAAGCC
SE33	GTAAGTCTCAGAAGATAAAGATTGG
SE40	GAATAGCACTACATGCACTTCCC
SE41	CAGGCGAGTGCCAAAGACG
SE46	GAAGCTGTAGCTGCAGGAATCG
SE56	AATCAATTTCAATATCTTACAGCC
SE57	TTACCTGAATTTGAGTTGAATGGC
SE61	CAAAGGAAGTTACTGTTGTCTGC
SE63	TTCATATAACTTGCAGAAAAGATG
JS102	GAGCCTATTCGAAATCAAAGCTG
JS103	AGATACCATTAGTGCATCTATGAC
JS104	CATGAAACTCAGCACAGATAGAAC
JS105	GTTATTTAAATCTAGCCATGCTGG
GalE1	CGTGGCAGGATATTGATGCTGG

Co., Cockeysville, Md.) containing 2.5% fetal bovine serum (GIBCO BRL, Gaithersburg, Md.) at 37°C with 3.5% CO<sub>2</sub>. *E. coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates (GIBCO BRL) at 37°C. Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were used at the following final concentrations: kanamycin, 60 µg/ml; spectinomycin, 100 µg/ml; ampicillin, 100 µg/ml; and tetracycline, 5 µg/ml.

**Nucleic acid purification.** Chromosomal DNA was isolated from *N. meningitidis* by the procedure described by DiLella and Woo (12). RNA was prepared by a previously described modification of the method published by Baker and Yanofsky (3, 37). DNA was digested with restriction endonucleases obtained from New England Biolabs, Beverly, Mass.

**Standard PCR and SSP-PCR.** Standard PCRs were performed as previously described (39). Amplitaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) was used for these reactions, and in some reactions, Taq Extender PCR additive (Stratagene, La Jolla, Calif.) was used to increase efficiency and reliability. The oligonucleotide primers used are shown in Table 1. Amplified products were visualized by 1.2% agarose gel electrophoresis and UV detection after ethidium bromide staining. PCR products were purified by passage through QIAquick PCR purification spin columns (Qiagen, Chatsworth, Calif.) prior to further manipulations. Chromosome walking via single-specific-primer (SSP) PCR was performed by the technique described by Shyamala and Ames (34) and previously used by us (37).

**Primer extension and RT-PCR.** We used the AMV Reverse Transcriptase Primer Extension System (Promega, Madison, Wis.) according to the manufacturer's directions. A reverse transcriptase (RT) PCR assay developed in our laboratory and previously described was used for these studies (37).

**Colony PCR.** A single colony from a plated culture was collected with a sterile loop and was resuspended in 20 µl of sterile distilled water. The colony suspension was then subjected to two rounds of freeze-thawing using a dry-ice-ethanol bath and a 37°C water bath. One microliter of the freeze-thaw mixture was then used as the template in standard PCR.

**Cloning of PCR products.** DNA products amplified by standard PCR or SSP-PCR were cloned with the TA Cloning Kit (Invitrogen) or the pGEM-T Vector System (Promega).

**Nucleotide sequencing.** Purified plasmid DNA and PCR products were sequenced by both manual and automated means. For manual sequencing we used the AmpliCycle Cycle Sequencing Kit (Perkin-Elmer) according to the manufacturer's directions. Automated DNA sequencing was performed with the Prism Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc. [ABI], Foster City, Calif.), and completed reactions were run on an ABI model 377 Automated DNA Sequencer.

Nucleotide and amino acid sequence analysis was performed with either the DNASTAR (Madison, Wis.) sequence analysis package or the Genetics Computer Group (GCG) sequence analysis software package, version 7.3.1-UNIX (11). We also used search tools and nucleotide and amino acid databases available on the Internet; sites visited included the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>), the FA1090 gonococcal genome database at the University of Oklahoma's Advanced Center for Genome Technology (<http://www.genome.ou.edu>), and the Institute for Genomic Research (<http://www.tigr.org>).

**DNA transformation procedures.** Serogroup A meningococcal strain F8229 was transformed by the semiquantitative transformation assay of Janik et al. (18). Chemical transformation of *E. coli* was performed by the method described by Chung et al. (6).

**Southern DNA hybridization.** We used the Genius nonradioactive DNA labeling and detection system (Boehringer Mannheim, Indianapolis, Ind.). Specific DNA probes were PCR amplified, labeled with digoxigenin, and used to probe

Southern DNA blots (35) under high-stringency conditions according to the manufacturer's protocols.

**Polar and nonpolar insertional mutagenesis.** Polar mutagenesis of defined genes was conducted by insertion of an  $\Omega$ -spectinomycin resistance cassette derived from pHP45. Briefly, the genetic region to be interrupted was amplified by PCR from chromosomal DNA and then cloned into *E. coli*  $\alpha$ InvF'. The plasmid containing the cloned PCR product was then linearized at a unique, blunt-ended restriction site present in the insert. A blunt *Sma*I fragment derived from pHP45, containing the entire  $\Omega$ -spectinomycin resistance cassette, was then ligated into the cloned product and transformed into *E. coli* with selection for spectinomycin resistance. Putative transformants were checked by colony PCR to confirm assembly of appropriate constructs. Plasmid DNA was prepared from confirmed colonies and was used to transform serogroup A strain F8229 with selection for spectinomycin resistance. Putative meningococcal transformants were checked by colony PCR and Southern DNA hybridization to confirm acquisition of the polar  $\Omega$ -insertion mutation by homologous recombination. Primers JS102 and JS103 (Table 1) were used to amplify a 600-bp PCR fragment from the 5' end of the F8229 open reading frame 1 (ORF1) which was subsequently cloned in *E. coli*. This product contained a unique *Sma*I restriction site located 356 bp downstream of the predicted ORF1 start codon. A *Sma*I fragment from pHP45, encoding the  $\Omega$ -spectinomycin resistance cassette, was inserted into the unique *Sma*I site, and the resulting recombinant plasmid was used to transform wild-type serogroup A strain F8229. Spectinomycin-resistant transformants were selected, and acquisition of the  $\Omega$ -insertion was confirmed by colony PCR and Southern hybridization.

A similar approach was used to introduce  $\Omega$ -spectinomycin resistance cassettes into ORF2, ORF3, and ORF4. To inactivate ORF2, a 451-bp DNA fragment derived from ORF2 was PCR amplified from strain F8229 with primers JS104 and JS105 (Table 1). An  $\Omega$ -fragment was inserted into a unique *Hinc*II site present in the cloned PCR product (located 729 bp from the putative ORF2 start codon), and the resulting plasmid was transformed into strain F8229. Primers SE57 and SE61 (Table 1) were used to amplify an 858-bp product from ORF3, containing a unique *Ssp*I site located 507 bp downstream of the ORF3 start codon. Again, an  $\Omega$ -fragment was inserted into this cloning site, and the construct was transformed into F8229. Finally, a 765-bp product was amplified from ORF4 with primers SE63 and SE56 (Table 1). The unique *Ssp*I cloning site in this product was located 159 bp from the putative ORF4 start codon. An  $\Omega$ -fragment was inserted into the cloning site, and the construct was transformed into F8229.

Nonpolar mutants were created by the allelic exchange technique described above; however, instead of a polar  $\Omega$ -fragment, a nonpolar *aphA-3* kanamycin resistance cassette derived from pUC18K (27) was inserted into the genetic region to be mutated. The orientation of the *aphA-3* insertion was checked by colony PCR and direct DNA sequencing of the product to ensure that the ATG start site at the end of the cassette was in frame to the downstream sequence.

**Serum selection for capsule revertants.** Strain F8229-43 was exposed to normal human serum (NHS) in order to screen for serogroup B capsule revertants. A 100-µl aliquot containing 25% NHS and  $3.75 \times 10^4$  meningococci, diluted in HEPES-minimal essential medium, was incubated at 37°C for 30 min. The entire aliquot was then plated on a GC agar plate containing 5 µg of tetracycline/ml, and survivors (e.g., putative capsule revertants) were collected after overnight growth. Revertants were studied for serogroup-specific capsule phenotype by using colony immunoblots and whole-cell enzyme-linked immunosorbent assay (ELISA), as described below.

**Capsule quantitation by colony immunoblot and whole-cell ELISA.** Colony immunoblots were performed as previously described (36) by using the anti-serogroup A monoclonal antibody 14-1-A (45) (generously provided by Wendell Zollinger, Walter Reed Army Institute of Research). Whole-cell ELISA was performed by the method of Abdillahi and Poolman (1). Briefly, strains to be assayed were grown overnight on GC agar plates. Plate growth was then harvested and suspended in 5 ml of phosphate-buffered saline containing 0.02% sodium azide (Sigma Chemical Co.). The cells were heat inactivated at 56°C for 30 min, then adjusted to an  $A_{650}$  of 0.1 and stored at 4°C to be used in assays within 1 week. To perform the ELISA, 100 µl of the cell suspension was added to a flat-bottom microtiter plate (Maxi-sorp or Poly-sorp [Nalge Nunc International, Naperville, Ill.]) and evaporated overnight at 33°C. The plate was then washed three times with a 0.05% solution of Tween 80 (Sigma Chemical Co.) in sterile water. One hundred microliters of monoclonal antibody 14-1-A (diluted 1:30,000 in phosphate-buffered saline containing 0.01% Tween 80 and 0.3% Casamino Acids [Difco Laboratories]) was added to each well, and the plate was incubated at 33°C for 1 h. After three washes, 100 µl of goat anti-mouse immunoglobulin A (IgA), IgG, and IgM alkaline phosphatase-conjugated antibody (ICN Pharmaceuticals, Inc., Costa Mesa, Calif.) was added (diluted 1:10,000 in the above buffer), and the plate was incubated for 90 min at 33°C. The plate was washed three times, 200 µl of substrate (1 mg of *p*-nitrophenyl phosphate [Sigma Chemical Co.] dissolved per ml of 0.5 M diethanolamine buffer containing 0.5 mM MgCl<sub>2</sub> [pH 9.8]) was added, and the plate was left to stand at room temperature for 20 to 45 min. The reaction was stopped by the addition of 50 µl of 3 N NaOH, and the  $A_{405}$  of each well was read with a BIO-TEK Instruments (Winooski, Vt.) model EL 312e Automated Plate Reader. Mean values were averages of 3 to 10 optical density values for individual wells  $\pm$  standard deviations (SD).

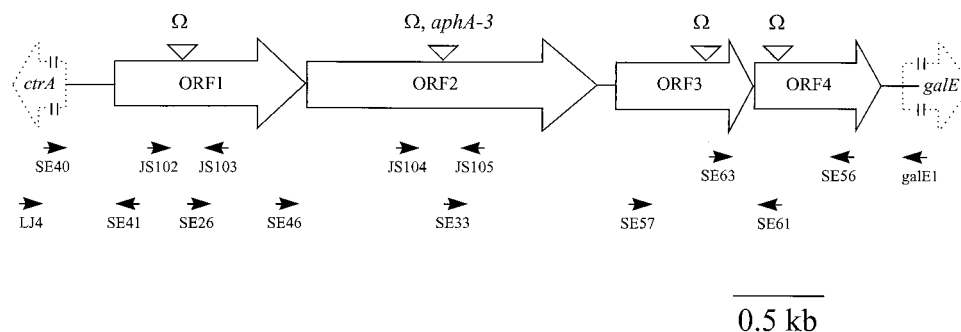


FIG. 1. Genetic organization of the ~4.7-kb region separating *ctrA* and *galE* in serogroup A meningococcal strain F8229. Open arrows, drawn to scale, represent the locations and orientations of ORF1 to -4. Dotted open arrows indicate the flanking genes *ctrA* and *galE* only, portions of which are shown. Open triangles point to the locations of insertion mutations created in ORF1 to ORF4 (see text). Locations of primers (Table 1) are shown by solid arrows.

**Nucleotide sequence accession number.** The nucleotide sequences and predicted amino acid translations described in this article have been submitted to GenBank and are available under accession no. AF019760.

## RESULTS

**Serogroup A *ctrA*-to-*galE* nucleotide sequence.** The nucleotide sequence spanning the region between *ctrA* and *galE* in the encapsulated serogroup A meningococcal strain F8229 was determined by a combination of standard PCR and SSP-PCR. Primer LJ4, which anneals to a sequence complementary to the 5' end of *ctrA*, and primer SE26, which anneals to a sequence upstream of *ctrA* (Table 1; Fig. 1), were used to "chromosome walk" 2.2 kb upstream of *ctrA* in strain F8229 by SSP-PCR. Sequence obtained from these products was used to construct primer SE33, designed to anneal to the 3' end of the 2.2-kb region. Primer SE33 and primer GalE1, designed to anneal to a sequence complementary to the 5' end of *galE*, were used to PCR amplify the additional 2.5 kb of DNA separating *ctrA* and *galE*. The double-stranded sequence of the 4,701-bp stretch separating *ctrA* from *galE* in serogroup A *N. meningitidis* was determined from these products by a combination of manual and automated DNA sequencing methods. The sequence was further confirmed by analysis of overlapping PCR products (Fig. 1).

Computer analysis of the 4.7-kb sequence indicated the presence of four ORFs transcribed in the opposite orientation from *ctrA* (Fig. 1). The first ORF (ORF1) was separated from *ctrA* by a 218-bp intergenic region. ORF1 was 1,119 nucleotides and was predicted to encode a 372-amino acid (aa) protein. ORF1 was separated by a single base pair from the second ORF, designated ORF2, which was 1,638 bp and was predicted to encode a 545-aa protein. ORF2 was in turn separated by 72 bp from a 744-bp ORF, designated ORF3, predicted to encode a 247-aa protein. Finally, ORF3 was separated by a single nucleotide from an 864-bp ORF, designated ORF4, which was predicted to encode a 287-aa protein.

In addition to the sequence derived from the encapsulated wild-type strain F8229, we also sequenced the first 2,330 bp of the region between *ctrA* and *galE* in the unencapsulated serogroup A variant strain F8239. Comparison of the nucleotide sequences derived from F8239 and F8229 indicated that they were nearly identical (11 nucleotide differences [7 deletions or additions, 2 transversions, and 2 transitions]) over the entire 2.2-kb stretch. However, in strain F8239, ORF1 was predicted to be only 744 nucleotides (encoding a 247-aa predicted protein). Computerized alignment of the putative amino acid translation of the F8239 and F8229 ORF sequences indicated

that in F8239, ORF1 was prematurely truncated by a frameshift mutation.

**Analysis of serogroup A *ctrA*-to-*galE* ORFs.** Nucleotide and predicted amino acid sequences of the putative ORFs were compared to the GenBank/EMBL and other genome project databases, including the FA1090 gonococcal genome database (see Materials and Methods for Internet addresses). The ORF1 product showed homology (57.8% amino acid identity and 73.9% similarity) with a cytoplasmic *E. coli* protein designated NfrC. The 1,131-bp *nfrC* gene encodes a 377-aa protein predicted to be a UDP-*N*-acetyl-D-glucosamine 2-epimerase (21). ORF2 demonstrated nucleotide and predicted amino acid homology with three separate ORFs of unknown function, the 1,632-bp *cpsY* of *Mycobacterium leprae* (30.6% identity and 51.7% similarity) (GenBank/EMBL accession no. U15182), a similar ORF in the *Mycobacterium tuberculosis* genome, and a 1,125-bp ORF (ORF5) found downstream of *galE/rfbBCD* in serogroup B *N. meningitidis* (GenBank/EMBL accession no. L09188) (16). ORF3 did not exhibit nucleotide or predicted protein homology with any genes or proteins currently in the databases. ORF4 exhibited distant homology with the XcpS protein of *Pseudomonas putida* (19.4% identity over a 129-aa overlap [GenBank/EMBL accession no. X81085] [10]) and appeared to share an amino acid motif (aa 123 to 168) with the presenilin (8) class of eukaryotic proteins. Except for the homology of ORF2 with the unknown ORF5 in serogroup B *N. meningitidis*, ORF1 to -4 were not found in the genomes of other meningococcal serogroups or *Neisseria gonorrhoeae* by database search or Southern hybridizations (data not shown).

**ORF1 to -4 are transcribed as an operon.** To determine whether ORF1 to ORF4 were organized as an operon, we performed RT-PCR experiments on whole-cell RNA obtained from strain F8229. The results, shown in Fig. 2, indicate that ORF1 to ORF4 are cotranscribed on the same mRNA message. The start site of transcription of the ORF1 to ORF4 operon, as defined by primer extension (Fig. 3), was located within the 218-bp intergenic region separating *ctrA* and ORF1 (Fig. 4). This transcriptional start site was preceded by a putative  $\sigma$ -70 promoter sequence (17). The -35 region of the ORF1 promoter, TTTATT, had a three-of-six match to the consensus  $\sigma$ -70 -35 promoter binding sequence and was 18 bp from the -10 region (Pribnow box) sequence, TATATT. The serogroup A *ctrA* transcriptional start site was also present in the 218-bp intergenic region, as shown by primer extension (Fig. 4). The serogroup A *ctrA* promoter overlapped the ORF1 promoter on the complementary DNA strand. However, the

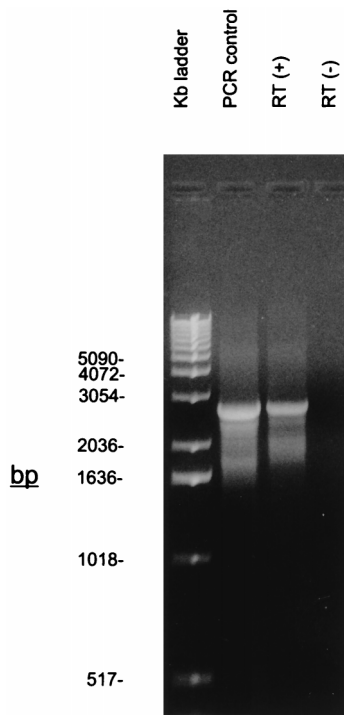


FIG. 2. RT-PCR of mRNA prepared from wild-type serogroup A strain F8229 to detect an ORF1 to ORF4 polycistronic transcript. Primer SE56 (Table 1; Fig. 1), which is complementary to the 3' end of ORF4, was used as the RT extension primer to generate cDNA in the RT reaction [RT (+)]. A control without RT added [RT (-)] was included under otherwise identical conditions. Primers SE46 and SE61 were used for PCR amplification of an approximately 2.8-kb ORF1-to-ORF4 product. Lane 1, 1-kb ladder (Gibco-BRL); lane 2, positive control using F8229 chromosomal DNA as the template; lane 3, PCR result using the RT (+) template; lane 4, negative control using the RT (-) template. DNA size standards (in base pairs) are indicated.

-35 region of the *ctrA* promoter, TTGTGT, had less resemblance to the  $\sigma$ -70 consensus -35 promoter binding sequence.

**Mutations of ORF1 to ORF4.** To confirm the role of ORF1 to ORF4 in serogroup A capsule expression, we created insertion mutations in each of the ORFs in the wild-type encapsulated strain F8229 (Fig. 1). Strains F8229-ORF1 $\Omega$ , F8229-ORF2 $\Omega$ , F8229-ORF3 $\Omega$ , and F8229-ORF4 $\Omega$  were created by  $\Omega$ -spectinomycin insertional mutagenesis of each of the ORFs. PCR and Southern hybridizations (data not shown) confirmed the correct insertion of the  $\Omega$ -spectinomycin fragments in each of the mutants (Fig. 1). Immunoblots, shown in Fig. 5, demonstrated that polar mutagenesis of all of the four ORFs in wild-type strain F8229 resulted in a reduction or loss of encapsulation. These data were confirmed by a quantitative capsule whole-cell ELISA in which the wild-type encapsulated strain F8229 was used as the 100% standard ( $A_{405} = 0.939 \pm 0.16$  [mean  $\pm$  SD]). Strains F8239, F8229-ORF1 $\Omega$ , and F8229-ORF2 $\Omega$  demonstrated a 100% reduction versus the wild-type strain ( $A_{450} = 0$ ), while F8229-ORF4 $\Omega$  demonstrated an 89% reduction ( $A_{450} = 0.101 \pm 0.007$ ).

We also attempted to create nonpolar interruptions of ORF1 and ORF2 by integrating an *aphA-3* cassette into the same unique sites used for the  $\Omega$ -cassette mutagenesis, but, despite repeated attempts, we were able to integrate this fragment only into ORF2, producing F8229-ORF2*aphA-3*. Like the polar  $\Omega$ -spectinomycin mutants, the nonpolar interruption of ORF2 also resulted in the complete loss of group A capsule expression, as shown by whole-cell ELISA. To further confirm

the function of this cassette in serogroup A capsule production, we repaired the F8229-ORF1 $\Omega$  mutation with the 4.7-kb PCR product generated by primers SE40 and GalE1. Capsule-expressing transformants were selected by survival in 25% NHS, and serogroup capsule A expression was confirmed by colony immunoblot. Repair of the ORF1- $\Omega$  mutation in these transformants was further confirmed by PCR with primers JS102 and JS103.

**Capsule switching.** We attempted to determine whether replacement of the *ctrA-galE* capsule cassette in serogroup A strain F8229 with a serogroup B cassette was sufficient to switch capsule expression. Chromosomal DNA from the serogroup B Tn916 meningococcal mutant strain 43 (37) was prepared and used to transform encapsulated serogroup A strain F8229. Strain 43 contains a class I "intact" Tn916 insertion in the *synD* capsule polymerase gene of the group B capsule biosynthetic gene cassette. Tetracycline-resistant transformants of strain F8229 were obtained at a frequency of  $\sim 1 \times 10^{-7}$ . The transformants were checked by colony immunoblot for loss of serogroup A capsule production, and one unencapsulated mutant, designated F8229-43, was saved for further analysis. Strain F8229-43 was screened by passage through 25% NHS to identify capsule revertants resulting from the precise excision of the Tn916 insertion from *synD* (predicted to occur at a frequency of  $\sim 10^{-4}$ ), resulting in restoration of capsule production and serum resistance. A serum survivor, designed F8229-43<sup>R</sup>, was tested by colony immunoblot using monoclonal anti-serogroup A and anti-serogroup B antibodies. The F8229-43<sup>R</sup> strain no longer expressed the serogroup A capsule, but instead expressed serogroup B capsular polysaccharide. PCR confirmed that the F8229-43<sup>R</sup> strain did not contain a serogroup A biosynthetic gene cassette but instead harbored an intact serogroup B cassette (data not shown).

## DISCUSSION

The biosynthesis of the serogroup A capsule of *N. meningitidis* requires genes that are not found in other meningococcal serogroups. However, the overall genomic organizations of the capsule transport and biosynthesis regions of serogroup A meningococci and of the sialic acid-containing capsular sero-

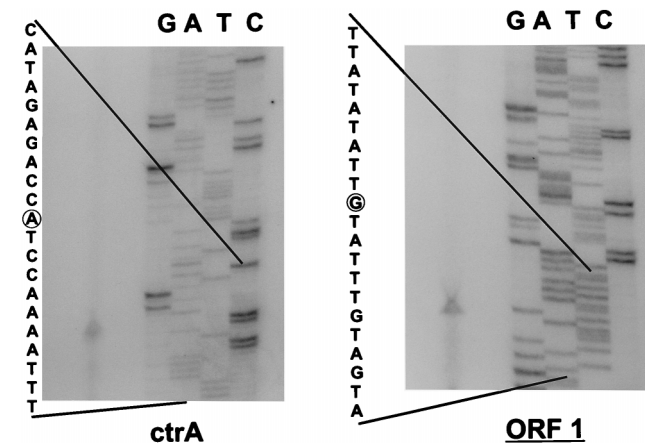


FIG. 3. Autoradiographs showing primer extension products for the serogroup A meningococcal genes *ctrA* and ORF1. Primer extension reactions were loaded alongside standard double-stranded DNA-sequencing reactions (load orientation of G, A, T, C) obtained by sequencing *ctrA* and ORF1 control DNA templates with the extension primers SE40 (*ctrA*) and SE41 (ORF1). The DNA sequence surrounding the primer extension bands has been expanded. The nucleotides corresponding to the putative start points of transcription are circled.

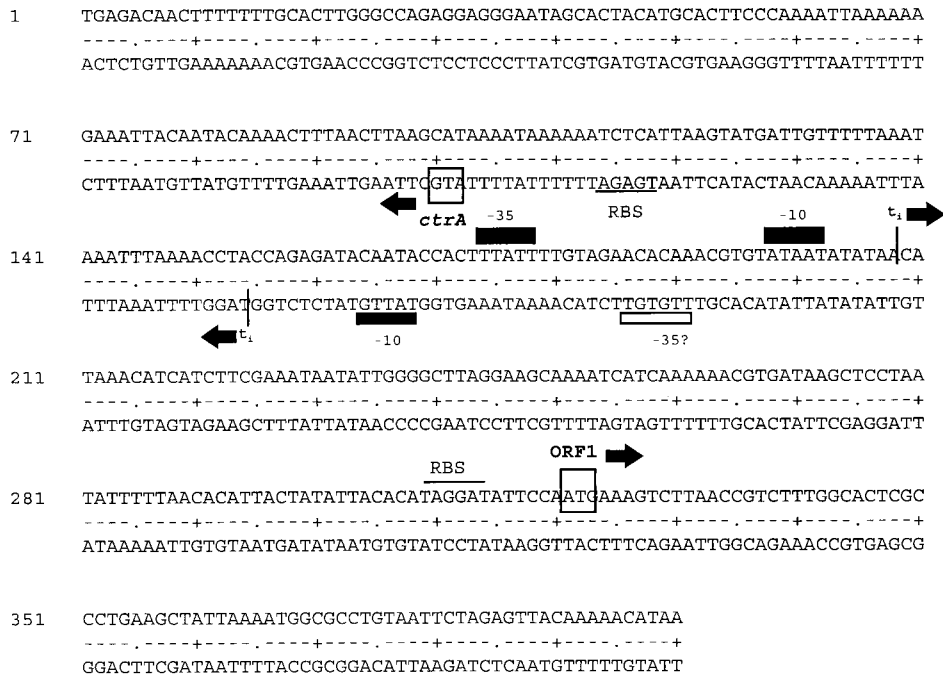


FIG. 4. Nucleotide sequence of the 218-bp intergenic region separating the start codons for the serogroup A *ctrA* and ORF1 loci. The start points and directions of transcription of the ORF1 and *ctrA* mRNAs are indicated by  $t_i$  and arrows, respectively. Predicted -10 and -35 promoter binding sequences are indicated, as well as the putative Shine-Dalgarno ribosome binding sites (RBS). The predicted initiation codons for *ctrA* and ORF1 are shown in boxes.

groups (B, C, Y, and W-135) are similar. In all serogroups, the genes of the *ctr* capsule transport operon are preceded by an intergenic region which separates this operon from divergently transcribed operons required for capsule biosynthesis. The capsule biosynthetic operons are distinct genetic cassettes located between *ctrA* and *galE* encoding the UDP-glucose-4-epimerase involved in lipooligosaccharide biosynthesis (24).

Based on the data presented here, previous studies of the biosynthetic cassettes of serogroups B, C, Y, and W-135 (38), and epidemiologic observations, the meningococcal biosynthetic gene cassettes can undergo recombinational exchange resulting in capsule switching. The genetic mechanism by which cassette exchange occurs is not defined but may be the result of homologous recombination between identical regions in the *ctr* operon and *galE*. Replacement of the capsule cassette and, possibly, of adjacent genes (e.g., *ctrA*) appears to be sufficient for capsule switching in *N. meningitidis*. *E. coli*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* also contain type-specific capsule gene cassettes which may be transferred by transformation and recombination events, resulting in a switching of the type of capsule expressed (5, 7, 20, 22).

In meningococcal serogroups B, C, Y, and W-135, the intergenic region separating *ctrA* from the biosynthesis operon (*synA* to -D [E, F, G]) is 134 bp and contains the *ctrA* to -D promoter, the divergent *synX* to -D (E, F, G) biosynthesis operon promoter, and other transcriptional regulatory elements (37, 38). The 218-bp intergenic region of serogroup A *N. meningitidis* does not have any homology with the 134-bp region found in the sialic acid capsular serogroups. However, both intergenic regions contain overlapping promoters that initiate transcription of the capsule transport operon and the divergent biosynthetic operons, suggesting that encapsulation of all the serogroups may be coordinately regulated through transcriptional control of promoters located in the intergenic regions. Both the serogroup A and the sialic acid biosynthetic

operon promoters (37) resemble  $\sigma$ -70 promoters and thus may be constitutively transcribed. However, the *ctrA* promoters are different, do not resemble  $\sigma$ -70 promoters, and may require activators.

For *N. meningitidis* producing sialic acid-containing capsules, the first three genes of the biosynthetic cassette, *synA*, -B, and -C, convert *N*-acetyl-D-glucosamine-6-phosphate (GlcNAc-6-P) into CMP-*N*-acetylneuraminic acid. The fourth gene, *synD* (E, F, G), is the distinct capsule polymerase which links the capsule sugar monomers together, thereby defining the serogroup (38). In the serogroup A *N. meningitidis* biosynthetic cassette, ORF1 has significant homology with the *E. coli* gene *nfrC*, which encodes a cytoplasmic protein required for bacteriophage N4 adsorption (21), and with *rffE* (o389) (23), which encodes a UDP-*N*-acetyl-D-glucosamine 2-epimerase responsible for converting UDP-*N*-acetyl-D-glucosamine (UDP-GlcNAc) into UDP-*N*-acetyl-D-mannosamine (UDP-ManNAc). Immediately downstream of *rffE* in *E. coli* is *rffD* (o379), which encodes a UDP-*N*-acetyl-D-mannosamine dehydrogenase (9, 25). Both *rffE* and *rffD* are required for the biosynthesis of the enterobacterial common antigen, a tricyclic, polysaccharide structure present on the cell surfaces of most enterobacterial species (23). ORF1 also has amino acid homology with other UDP-GlcNAc 2-epimerases from *H. influenzae* (13) and humans (GenBank/EMBL accession no. AA210747). Thus, ORF1 likely encodes the epimerase which converts UDP-GlcNAc to UDP-ManNAc.

UDP-ManNAc, in addition to its role in enterobacterial common antigen biosynthesis in gram-negative species (23), is a common biosynthetic precursor in the formation of several cell wall structures in gram-positive organisms such as *Micrococcus luteus* (19) and numerous *Bacillus* species (44), including *Bacillus pumilus* (41). Thus, in contrast to the sialic acid capsule pathway, which utilizes GlcNAc-6-P (40), the serogroup A capsule biosynthetic pathway utilizes UDP-GlcNAc

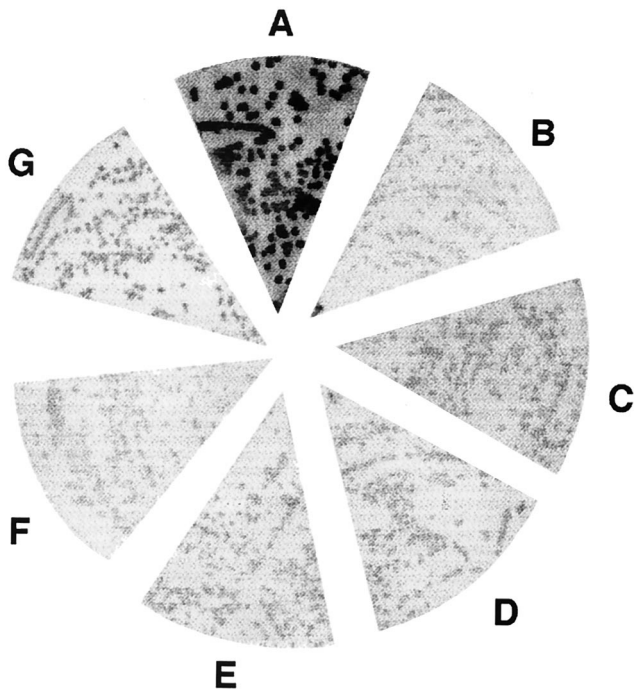


FIG. 5. Colony immunoblots of wild-type serogroup A meningococcal strain FA8229, its ORF1 to -4 mutants, and unencapsulated variant F8239. Strains were grown overnight on GC base agar, transferred to nitrocellulose, and probed with anti-serogroup A monoclonal antibody 14-1-A (45). (A) Wild-type encapsulated strain F8229; (B) unencapsulated variant F8239; (C) F8229-ORF1 $\Omega$ ; (D) F8229-ORF2 $\Omega$ ; (E) F8229-ORF2 $\Delta$ phA-3; (F) F8229-ORF3 $\Omega$ ; (G) F8229-ORF4 $\Omega$ .

as the starting substrate. Interestingly, the first genes of all meningococcal capsule biosynthetic gene cassettes encode 2-epimerases converting either UDP-GlcNAc or GlcNAc-6-P to UDP-ManNAc or *N*-acetyl-D-mannosamine-6-phosphate, respectively.

ORF2 has the greatest amount of homology with a pair of uncharacterized mycobacterial genes, in terms of both amino acid identity and protein length, and with another gene of unknown function, designated ORF5, that has been identified downstream of *galE* and *rfbBCD* in serogroup B meningococci (16). The homology of ORF2 with the mycobacterial gene products and ORF5 is concentrated towards the C termini of the predicted proteins. We hypothesize that ORF2 may be the polymerase linking individual UDP-ManNAc monomers together. This is likely to occur through the release of UMP from UDP-ManNAc, which provides the requisite energy for the creation of a phosphodiester bond between the 1 and 6 positions of individual monomers (26). The large size of the ORF2 gene may support this proposed function. In the sialic acid-producing serogroups, the ORFs encoding the capsule polymerases are significantly larger than the other biosynthetic genes (38).

Polar and nonpolar insertional mutagenesis of ORF1-4 demonstrated that each of these genes is involved in the production of the serogroup A capsule. However, the functions of ORF3 and ORF4 are unclear. ORF3 does not have homology with other genes or predicted proteins in the computerized nucleotide and amino acid databases but is transcribed as part of the serogroup A biosynthetic cassette operon. In studies of the composition and chemical properties of the group A capsule, Liu et al. (26) noted that the polysaccharide was not completely *N*-acetylated and that the 1 $\rightarrow$ 6 phosphodiester

bond was not the only linkage present. Cross-linking of the polysaccharide chains was predicted from their analyses. ORF3 may be involved in these modifications to the serogroup A polysaccharide.

ORF4 demonstrated distant homology with the XcpS protein of *Pseudomonas aeruginosa* (4) and *P. putida* (10). ORF4 also exhibited homology with a 46-aa motif shared by members of the presenilin class of eukaryotic proteins (8). Presenilins contain multiple transmembrane domains and are thought to be involved in sensory transduction or in the formation of ion channels. The 46-aa presenilin motif found in ORF4 is thought to contain two transmembrane domains separated by a hydrophilic loop (8). Interestingly, the insertion mutation affecting ORF4 resulted in only an 89% reduction in serogroup capsule production compared with the complete abrogation seen with interruptions of the other ORFs. This suggests that ORF4 is not directly required for the biosynthesis of the ( $\alpha$ 1 $\rightarrow$ 6)-linked *N*-acetyl-D-mannosamine-1-phosphate polymers. Considering the homology to membrane-associated proteins, the ORF4 gene product may be involved in membrane transport, assembly, or cross-linking of the serogroup A capsule to the meningococcal cell surface.

In summary, an operon of four genes, ORF1 to -4, located between *ctrA* and *galE* in the serogroup A *N. meningitidis* genome, is required for the production of serogroup A ( $\alpha$ 1 $\rightarrow$ 6)-linked *N*-acetyl-D-mannosamine-1-phosphate capsule. We propose that ORF1 to ORF4 be designated *mynA*, *mynB*, *mynC*, and *mynD* (for mannosamine capsule biosynthesis genes), respectively. The first biosynthetic step in the pathway is likely the production of UDP-ManNAc from UDP-GlcNAc, a function that is likely performed by the gene product of *mynA*. The *mynB* gene may encode the ( $\alpha$ 1 $\rightarrow$ 6) UDP-ManNAc polymerase, and *mynC* and *mynD* may be involved in further modification and assembly of the serogroup A capsule.

The data also support the model that meningococcal capsular serogroups are determined by specific biosynthesis genetic cassettes that insert between the *ctrA* operon and *galE*. We have previously shown (38) that the cassettes determining the sialic acid serogroups can recombine to switch the type of capsule expressed. In this report we have shown that the serogroup A capsule can be switched by a similar recombinational exchange of biosynthetic cassettes. Our data should prove useful in the design of improved meningococcal vaccines, in development of rapid diagnostic approaches for the detection of serogroup A disease, and for understanding of the molecular basis of serogroup A meningococcal pathogenesis.

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