

Identification and Characterization of *pptA*: a Gene Involved in the Phase-Variable Expression of Phosphorylcholine on Pili of *Neisseria meningitidis*

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Pili of pathogenic *Neisseria* are major virulence factors associated with adhesion, cytotoxicity, twitching motility, autoaggregation, and DNA transformation. Pili are modified posttranslationally by the addition of phosphorylcholine. However, no genes involved in either the biosynthesis or the transfer of phosphorylcholine in *Neisseria meningitidis* have been identified. In this study, we identified five candidate open reading frames (ORFs) potentially involved in the biosynthesis or transfer of phosphorylcholine to pilin in *N. meningitidis*. Insertional mutants were constructed for each ORF in *N. meningitidis* strain C311#3 to determine their effect on phosphorylcholine expression. The effect of the mutant ORFs on the modification by phosphorylcholine was analyzed by Western analysis with phosphorylcholine-specific monoclonal antibody TEPC-15. Analysis of the mutants showed that ORF NMB0415, now defined as *pptA* (pilin phosphorylcholine transferase A), is involved in the addition of phosphorylcholine to pilin in *N. meningitidis*. Additionally, the phase variation (high frequency on-off switching of expression) of phosphorylcholine on pilin is due to changes in a homopolymeric guanosine tract in *pptA*.

The genus *Neisseria* consists of gram-negative pathogenic and nonpathogenic bacteria (generally diplococci) that colonize mucosal surfaces. The two major pathogenic species are *Neisseria gonorrhoeae* and *N. meningitidis*, which are closely related and colonize only humans. Pili of pathogenic *Neisseria* are long filamentous structures of the type IV fimbria family that extend from the bacterial surface and consist primarily of the monomer pilin (27). Pili play a major role in disease and have been associated with adhesion, cytotoxicity, twitching motility, autoaggregation, and DNA transformation (15, 17, 22, 39). Phosphorylcholine (ChoP) is involved in the posttranslational modification of pili of pathogenic *Neisseria* (14, 42). ChoP is expressed by a range of organisms of the respiratory tract, including *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae* (11). In *S. pneumoniae*, ChoP is attached to teichoic acid and lipoteichoic acid (5, 10, 20). In *H. influenzae* and commensal *Neisseria*, ChoP is attached to lipopolysaccharide (LPS) (25, 33, 46). ChoP increases the adherence of pneumococci and nontypeable *H. influenzae* (7, 35) and aids in the colonization of the nasopharynx by *H. influenzae* (35). Thus, it seems that ChoP is of general importance in the colonization of the respiratory tract (35). ChoP expression is phase variable in *S. pneumoniae*, *H. influenzae*, *N. meningitidis*, and commensal *Neisseria*, suggesting the existence of functional and/or immunological selection for the switching expression of ChoP (33, 42, 44, 46).

In both nontypeable *H. influenzae* and commensal *Neisseria*, ChoP is important in receptor binding and signal cascades,

resulting in increased adherence to and invasion of epithelial cells via the platelet-activating factor receptor (32, 35). C-reactive protein binds to the ChoP epitope on teichoic acid of *S. pneumoniae* (36) and on LPS of *H. influenzae* and commensal *Neisseria* (33, 45). This binding results in decreased attachment via binding to the platelet-activating factor receptor (12). Also, C-reactive protein binding acts as an opsonin, activating complement via the classical pathway and resulting in a bactericidal effect (45). Therefore the phase-variable expression of ChoP seems to be a critical mechanism in respiratory bacterial colonization and pathogenesis. Variants expressing ChoP may be better adapted to colonization of the nasopharynx, while ChoP-negative variants may be able to avoid complement-mediated killing in serum (45).

In *S. pneumoniae*, *H. influenzae*, and commensal *Neisseria*, ChoP is attached to sugar moieties (teichoic acid, lipoteichoic acid, and LPS) (5, 10, 20, 25, 33, 46). In pathogenic *Neisseria*, ChoP is attached to the pilus subunit protein, pilin (42). To determine the gene(s) responsible for the biosynthesis, transfer, and phase-variable expression of ChoP on pilin, we analyzed homologues of known biosynthetic and transferase genes in both the ChoP-positive strain *N. meningitidis* C311#3 (42) and its ChoP-negative variant 26A (24a). This study describes the characterization of a gene involved in the addition of ChoP to pili in *N. meningitidis* and the mechanism responsible for the phase variation of ChoP expression.

MATERIALS AND METHODS

Bacterial strains and media. The meningococcal strains used in this study were *N. meningitidis* C311#3 (40) and its phase variant 26A (which lacks ChoP) (Power et al., submitted) or clinical isolates (6). Meningococcal strains were grown on brain heart infusion agar (Oxoid) at 37°C with 5% CO₂ for 16 to 18 h. Brain heart infusion agar plates were made with 1% agar and supplemented with 10% Levinthal base (1). All recombinant plasmids were replicated in *Escherichia*

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TABLE 1. List of primers

Primer name	Sequence (5'-3')
NMB0415ForGAAATCGTCGGACAGGCTTTGTAACTCG
NMB0415RevCACAGATTATTTCCCATTCATTCCGGC
NMB1638ForCCAACCCCTTATCCGACACATTCCA
NMB1638RevATGTCATCAGCCCAATACCGTGCTG
PmtAForTTGCGGGCAATAGTGCCTTCA
PmtARevTGAGGGGTGCTTTTCGTTGTAA
BetTForGGTTTAACCGTGCCGGATCA
BetTRevCTCTGCCAATTCACCTGCTC
NMB0939ForCCGTGCAGTATATCGAAGTCAA
NMB0939RevCTGTCTTACCGACGCGGTAA
KanUpOutTCCCGTTGAATATGGCTCA
NMB0415PolyGForTTACGTCCGGCACAAACCCCTCA
NMB0415PolyGRevCAACAACAGGCCGCATCAGGT

coli DH5 α and grown on Luria-Bertani media (29). Luria-Bertani agar plates were supplemented with 1.5% agar (Difco). Ampicillin and kanamycin were used in media at a final concentration of 100 μ g/ml each.

DNA manipulation and analysis. Routine DNA manipulations were carried out essentially as described by Sambrook et al. (29). Nucleotide sequencing was carried out by using a Big Dye Terminator kit (29). The homopolymeric tract of NMB0415 was sequenced with primers NMB0415PolyGFor and NMB0415PolyGRev; the primer sequences are shown in Table 1. PCR was done essentially as previously described (28). Nucleotide analysis was performed by using the MacVector program (Oxford Molecular).

Southern blotting and hybridization. Restriction endonuclease (*S*yl)-digested DNA was separated on a 1% agarose gel and blotted onto a GeneScreen polyvinylidene difluoride membrane (Perkin-Elmer) essentially as previously described (29). Primers NMB0415For and NMB0415Rev (primer sequences are shown in Table 1) were used to amplify the DNA fragment, which was then purified by using a QIAquick gel extraction kit (Qiagen). This product was labeled by using a DIG-High Prime DNA labeling kit (Roche), and hybridization was performed according to the manufacturer's instructions.

Construction of knockout mutants of the various ChoP biosynthetic and transferase homologues. Candidate ChoP biosynthetic genes were PCR amplified with the primers shown in Table 2 (primer sequences are shown in Table 1) and cloned into vector pGEM-T-Easy (Promega). Plasmids containing the cloned genes were digested with a restriction endonuclease (Table 2) that cut only once within the open reading frame (ORF) and not in the cloning vector. A kanamycin resistance cassette was inserted into the various ORFs by ligation of the *H*incII 1.3-kb restriction fragment from plasmid pUC4Kan (Amersham Biosciences). The constructs of the knockout alleles were linearized with restriction enzyme *Not*I and transformed into *N. meningitidis* C311#3 as previously described (24). Kanamycin-resistant colonies were screened for the insertion of the kanamycin resistance cassette by PCR with various forward primers and KanUpOut (relevant primers and primer sequences are shown in Tables 1 and 2).

Western immunoblotting. Pilin from parent strain C311#3, 26A, and the various C311#3 mutants constructed in this study was isolated as previously described (24). These samples were analyzed by Western blotting with monoclonal antibody (MAb) SM1 (specific for pilin) (41) and MAb TEPC-15 (specific for ChoP) (Sigma Chemical Co.) (42).

RESULTS

ChoP is not linked to the pilin-linked trisaccharide. In respiratory pathogens, ChoP is usually attached to sugar moieties. In *S. pneumoniae*, ChoP is attached to teichoic acid and lipoteichoic acid (5, 10, 20), and in *H. influenzae* and commensal *Neisseria*, it is attached to LPS (25, 33, 46). As the pilin in *N. meningitidis* is modified by the pilin-linked trisaccharide, it is possible that ChoP could be attached to meningococcal pilin via this glycan. However, Western analysis of previously described pilin glycosylation mutants (*pglA* to *pglE*) (13, 24, 24a) showed there was no difference in the binding of MAb TEPC-15 to these mutants and to the wild type (results not shown).

System	ORF name	Homologue	%		Proposed function of homologue	Effect of mutant on TEPC-15 reactivity with pilin	Forward primer	Reverse primer	Insertion site	Construct name
			Identity ^a	Similarity ^a						
Biosynthesis or uptake	NMB1270	<i>pmtA</i>	32	47	Methyltransferase of PEA of <i>R. sphaeroides</i>	-	PmtAFor	PmtARev	<i>Cla</i> I	pGEM-PmtAKan
	NMB1277	<i>betT</i>	35	62	Choline uptake of <i>E. coli</i>	-	BetTFor	BetTRev	<i>Bsm</i> II	pGEM-BetTKan
	NMB0939	<i>pmtA</i>	28	42	Methyltransferase of PEA of <i>S. meliloti</i>	-	NMB0939For	NMB0939Rev	<i>Bsm</i> II	pGEM-NMB0939Kan
Transferase	NMB0415	<i>lpt-3</i>	27	44	PEA transferase of <i>N. meningitidis</i>	+	NMB0415For	NMB0415Rev	<i>Sma</i> I	pGEM-NMB0415Kan
	NMB1638	<i>lpt-3</i>	26	44	PEA transferase of <i>N. meningitidis</i>	-	NMB1638For	NMB1638Rev	<i>Bst</i> III	pGEM-NMB1638Kan

^a Percent identity and similarity relative to the following proteins (GenBank accession numbers in parentheses) *pmtA* (*R. sphaeroides*, L07247); *betT* (X52905); *pmtA* (*S. meliloti*, AF201699); and *lpt-3* (AE002550).

TABLE 2. Genes knocked out and methods used

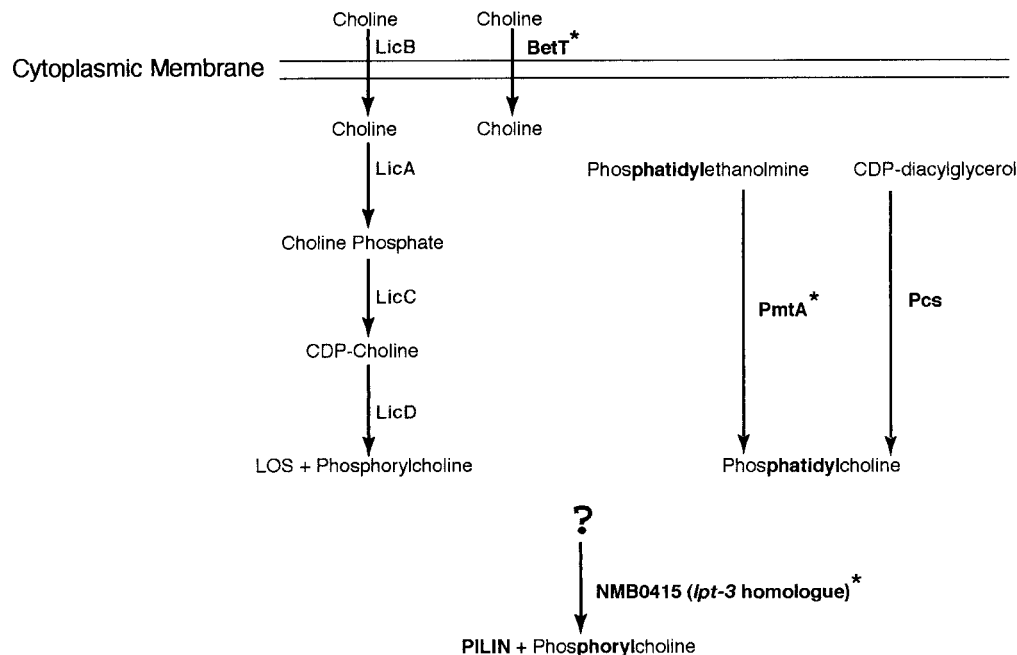


FIG. 1. Phosphatidylcholine and phosphorylcholine biosynthetic pathways showing biosynthetic genes and intermediates. The three described pathways are the *lic-1* pathway (in grey), found in commensal *Neisseria* and *H. influenzae* (33, 43)—previous work showed that there are no homologues of this pathway in pathogenic *Neisseria* (32, 33); the *pmtA* pathway, found in *Pseudomonas*, *Bradyrhizobium*, *Rhodobacter*, and *Sinorhizobium* (4, 8, 19, 47); and the *pcs* pathway, found in *Sinorhizobium* and *Pseudomonas* (8, 47). Genes and proteins identified and characterized in this study are marked by asterisks. LOS, lipooligosaccharide.

Identification and analysis of ORFs potentially involved in ChoP biosynthesis in *N. meningitidis*. The mechanisms responsible for the phase variation of ChoP expression on pilin, the biosynthesis or acquisition of ChoP, and the transfer of ChoP to pili of *Neisseria* have not been described. In other bacteria, there are three known pathways for the biosynthesis of ChoP and phosphatidylcholine. These pathways are the *lic-1* pathway (found in *H. influenzae*, *S. pneumoniae*, and commensal *Neisseria*) (33, 43, 48), the *pmtA* pathway (found in *Rhodobacter sphaeroides*, *Sinorhizobium meliloti*, *Bradyrhizobium japonicum*, and *P. aeruginosa*) (4, 8, 19, 47), and the *pcs* pathway (found in *S. meliloti* and *P. aeruginosa*) (8, 47) (Fig. 1 shows the details of these pathways). In commensal *Neisseria*, ChoP is synthesized by a pathway homologous to the *lic-1* pathway of *H. influenzae*; however, the genes for this pathway are absent in pathogenic *Neisseria* (32, 33). The *pmtA* gene is responsible for three sequential methylations of phosphatidylethanolamine, resulting in phosphatidylcholine in *P. aeruginosa*, *B. japonicum*, *R. sphaeroides*, and *S. meliloti* (4, 8, 19, 47). Phosphatidylcholine could be a substrate for phosphorylcholine transfer.

TBLAST-X (2) homology searches were used to identify ORFs in the *N. meningitidis* MC58 genome (37) that were homologous to candidate ChoP biosynthetic genes. The homologues identified in this study are listed in Table 2. In addition to ChoP biosynthetic gene homologues, we investigated ORFs potentially involved in ChoP uptake. A previous study with ^{14}C -labeled choline suggested that ChoP is not taken up from the medium and attached to pilin in pathogenic *Neisseria* (33); however, we identified an ORF that is homologous to choline transporter gene *betT* in the *N. meningitidis* genome (Table 2).

In *E. coli*, the *betT* gene product is a choline transporter responsible for the uptake of choline used for glycine betaine synthesis (3).

To determine whether the ORFs identified were involved in ChoP expression, each of the candidate genes was inactivated by the insertion of a kanamycin resistance cassette into the unique restriction endonuclease site as described in Table 2. These constructs were transformed into *N. meningitidis* strain C311#3 to recombine the inactive allele into the chromosome. The presence of the inactivated allele was confirmed by PCR or Southern hybridization. The phenotypes of the mutants were investigated by Western immunoblotting; MAb TEPC-15 was used to determine whether ChoP was present on pilin expressed by the various mutants. MAb SM1 was used to confirm that there were similar concentrations of pilin in all samples and to detect any apparent changes in migration. This analysis showed there was no change in the migration or reactivity of pilin from the NMB0939, NMB1270 (*pmtA* homologue), and NMB1277 (*betT* homologue) mutant strains compared to that from the parent strain with MAbs SM1 and TEPC-15 (Fig. 2). As there was no change in the binding of MAb TEPC-15, which is specific for ChoP (42), no role for these ORFs in ChoP biosynthesis or addition could be assigned.

A homologue of the *lpt-3* gene product is involved in the addition of ChoP to pilin. The *lpt-3* gene was recently identified as encoding the transferase responsible for the addition of phosphoethanolamine (PEA) to position 3 of the β -chain heptose of the inner core of LPS in *N. meningitidis* (16). As PEA is similar to ChoP, we hypothesized that homologues of the

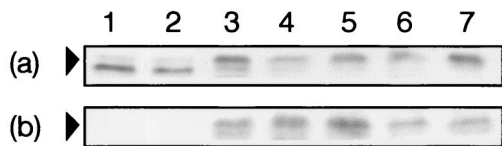


FIG. 2. Analysis of pilin from *N. meningitidis* C311#3 ChoP biosynthetic mutants. Western blotting of pilin was carried out to investigate the effects of mutations on the presence of ChoP. Pilin was isolated from C311#3 phase variant 5 (lane 1), C311#3 phase variant 6 (lane 2), C311#3 (lane 3), C311#3 *betT* (lane 4), C311#3 NMB0939 (lane 5), C311#3 *pmtA* (lane 6), and C311#3 NMB1638 (lane 7). Arrowheads represent the 20.5-kDa molecular mass marker. (a) Western blot with MAb SM1 (specific for class I pili of *Neisseria*) (41) showing the presence of pilin in all samples. (b) Western blot with MAb TEPC-15 (specific for ChoP) (42) showing the presence of ChoP on pilin in lanes 3 to 7.

lpt-3 gene product could be transferases involved in the addition of ChoP to pilin. TBLAST-X (2) homology searches showed there were two homologues of this gene in *N. meningitidis* MC58 (37), ORFs NMB1638 and NMB0415.

Pilin was isolated from the putative NMB1638 and NMB0415 ChoP transferase mutants and analyzed as described above. These experiments showed that pilin from the NMB1638 mutant strain had the same migration and reactivity with MAb TEPC-15 as the parent strain, indicating that this ORF is not involved in the transfer of ChoP to pilin in *N. meningitidis* (Fig. 2). Conversely, inactivation of NMB0415 resulted in an increased migration of pilin (similar to that seen with ChoP-negative strain 26A), and the pilin no longer bound MAb TEPC-15 (Fig. 3). As TEPC-15 is specific for ChoP (42) and ChoP is absent from pilin in the NMB0415 mutant strain, we conclude that NMB0415, now called *pptA* (pilin phosphorylcholine transferase A), is involved in the addition of ChoP to pilin.

Changes in the homopolymeric tract of *pptA* mediate the phase variation of ChoP. Phase variation of surface-exposed epitopes is a common feature of host-adapted pathogens (9, 18, 21). ChoP is a phase-variable modification of pilin in *N. meningitidis* (42). The *pptA* gene contains a homopolymeric guanosine tract located approximately 420 bp from the start of the ORF. Previous studies showed that this homopolymeric tract is of variable lengths in different strains, suggesting that the ORF is potentially phase variable (34).

In this study, we expanded the survey of the homopolymeric

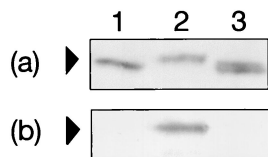


FIG. 3. Analysis of pilin from *N. meningitidis* C311#3 ChoP transferase mutants. Western blotting of pilin was carried out to investigate the effects of mutations on the presence of ChoP. Pilin was isolated from C311#3 26A (lane 1), C311#3 (lane 2), and C311#3 *pptA* (lane 3). Arrowheads represents the 20.5-kDa molecular mass marker. (a) Western blot with MAb SM1 (specific for class I pili of *Neisseria*) (41) showing the presence of pilin in all samples. (b) Western blot with MAb TEPC-15 (specific for ChoP) (42) showing the presence of ChoP on pilin in the parent strain only.

tract of *pptA* to include *N. meningitidis* isolates from patients, additional *N. meningitidis* strains, and three independently identified ChoP phase variants. The three independently identified ChoP phase variants are the previously identified 26A (24a) and two spontaneous ChoP phase variants identified in this study, C311#3 phase variant 5 and C311#3 phase variant 6.

Our survey revealed that *pptA* was present in all 13 *N. meningitidis* strains analyzed. Sequencing of the homopolymeric repeat from these strains showed that the guanosine repeat length varied from 8 to 11 bp in the analyzed strains (Fig. 4). In the three independently isolated ChoP phase variants, 26A, phase variant 5, and phase variant 6, either the gain or the loss of a repeat in the homopolymeric tract of *pptA* resulted in the ORF being out of frame (Fig. 4). Therefore, the phase variation of ChoP on pilin in *N. meningitidis* is controlled by a frameshift of the homopolymeric repeat of *pptA*.

There have been reports of phase variation of ChoP in *N. gonorrhoeae* (42). However, sequencing of the homopolymeric guanosine repeat of *pptA* in several *N. gonorrhoeae* strains showed that the tract was shortened to 5 bp and had an additional 3 nucleotides, TGT, in the repeat region. As repeats of less than 6 bp are not considered phase variable (30), it would appear that ChoP phase variation in *N. gonorrhoeae* is not due to the homopolymeric tract of *pptA*.

In this study, eight sets of clinical isolates from different body compartments within an individual patient were also analyzed. Typically, these sets included isolates from the blood and cerebrospinal fluid of the patient. This analysis showed that *pptA* was present in all patient isolates and that isolates from different body compartments in a patient all had the same number of repeats (Fig. 4).

DISCUSSION

Phosphorylcholine is a covalently linked posttranslational modification found on several surface-exposed moieties of many different pathogens of the respiratory tract (11). In *H. influenzae* and commensal *Neisseria*, ChoP is attached to LPS; in *S. pneumoniae*, ChoP is attached to teichoic and lipoteichoic acids, and the biosynthetic and attachment pathways are well understood (33, 43, 48). The biosynthetic pathway, mechanism of attachment, phase variation, phenotypic effects, and location of attachment for commensal *Neisseria* and *H. influenzae* are very similar (32, 33). The addition of ChoP to LPS in commensal *Neisseria* is mediated by homologues of the *lic-1* pathway (including the potential ChoP transferase gene *licD*), which is absent in pathogenic *Neisseria* (32, 33). In pathogenic *Neisseria*, ChoP is covalently attached to surface-exposed pilin but is not found attached to LPS (42). The expression of ChoP on pathogenic *Neisseria* is phase variable (42). Prior to this study, the mechanisms of ChoP biosynthesis, attachment, and phase variation were not understood.

ChoP is linked via a sugar moiety in *H. influenzae*, *S. pneumoniae*, and commensal *Neisseria* (33, 43, 48). *N. meningitidis* has a pilin-linked glycan, suggesting that ChoP could be attached to pilin via this pilin-linked trisaccharide. However, Western analysis of previously described pilin glycosylation mutant strains showed that they all have reactivities with MAb TEPC-15 similar to that of the parent strain. This finding

		Poly-Guanosine Repeat		Gene
<i>Ng</i> FA1090	ACAGTTTGGAAATATTGTGTATC	TGTGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Ng</i> 1291	ACAGTTTGGAAATATTGTGTATC	TGTGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Ng</i> MS11	ACAGTTTGGAAATATTGTGTATC	TGTGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> FAM18	ACAGTTTGGAGATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> MPJ03	ACAGTTTGGAGATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> MPJ06	ACAGTTTGGAGATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> MPJ11	ACAGTTTGGAGATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> MPJ18	ACAGTTTGGAGATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> MPJ35	ACAGTTTGGAGATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> MPJ50	ACAGTTTGGAAATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> MPJ53	ACAGTTTGGAAATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> MPJ60	ACAGTTTGGAGATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> C311#3 PV6	ACAGTTTGGAAATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	Off
<i>Nm</i> C311#3 PV5	ACAGTTTGGAAATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	Off
<i>Nm</i> C311#3	ACAGTTTGGAAATATTGTGTATC	GGGGGGGG---	TATTTGCTGACGTAAAAA	On
<i>Nm</i> C311#3 26A	ACAGTTTGGAAATATTGTGTATC	GGGGGGGGGG--	TATTTGCTGACGTAAAAA	Off
<i>Nm</i> Z2491	ACAGTTTGGAGATATTGTGTATC	GGGGGGGGGG--	TATTTGCTGACGTAAAAA	Off
<i>Nm</i> MC58	ACAGTTTGGAAATATTGTGTATC	GGGGGGGGGG--	TATTTGCTGACGTAAAAA	Off
<i>Nm</i> 8013	ACAGTTTGGAGATATTGTGTATC	GGGGGGGGGGGG	TATTTGCTGACGTAAAAA	On

ThrValTrpArgTyrCysVal Ser [Gly]_n Val PheAlaAspValLysAsnTyr

FIG. 4. Sequencing of the poly(G) tracts in various pathogenic *Neisseria* strains. *Ng*, *N. gonorrhoeae*; *Nm*, *N. meningitidis*. MPJ laboratory strains are patient isolates (see reference 6 for details). Only one sequence is displayed for each of the patient isolate sets, as no difference was observed in the homopolymeric tracts within each set.

demonstrates that ChoP is not linked to pilin via this glycan. However, it does not eliminate the possibility that ChoP is attached to pilin via another, as-yet-uncharacterized pilin-linked glycan. Therefore, the site of ChoP addition to pilin in *N. meningitidis* is still unclear.

The precursors and intermediates involved in the biosynthesis of ChoP in *N. meningitidis* are unknown. A previous study suggested that there is no uptake of ChoP from the environment in *N. meningitidis*. That study showed that ¹⁴C-labeled choline added to defined medium could not be detected on pilin by autoradiography, suggesting that *N. meningitidis* endogenously produces ChoP and its precursors rather than acquiring them from the environment (33).

The inactivation of several potential ChoP biosynthetic ORFs and one potential choline uptake system in this study resulted in no obvious change in the presence of ChoP on pilin in *N. meningitidis*. Thus, the mechanism by which *N. meningitidis* makes ChoP is still unclear. However, there could be multiple pathways for the biosynthesis of ChoP in *N. meningitidis*. This redundancy would allow ChoP to still be made if one pathway were inactivated. Alternatively, ChoP biosynthesis could occur either by an unknown pathway or by a pathway similar to the *pcs* pathway of *Sinorhizobium* and *Pseudomonas*, or the uptake of choline from the medium could be important (8, 47). The mechanism of ChoP biosynthesis in *N. meningitidis* remains to be determined.

Analysis of *lpt-3* suggested that its product is a transferase responsible for the addition of PEA to position 3 of the β-chain heptose of the inner core of LPS in *N. meningitidis* (16). This assignment of function is based on the phenotype of the *lpt-3* mutant, which showed a loss of PEA from only one site of the LPS molecule, suggesting that *lpt-3* is responsible for the transfer of PEA to this particular position and not for PEA biosynthesis (16). The results presented here demonstrate that the inactivation of *pptA* results in the loss of ChoP from pilin

in *N. meningitidis*. These results, combined with the homology of this ORF to *lpt-3*, suggest that the *pptA* gene product is the ChoP transferase responsible for the addition of ChoP to pilin in *N. meningitidis*. *pptA* is found in a region of the chromosome that is specific for pathogenic *Neisseria* (23), within the cell wall biosynthesis cluster (34).

This study also demonstrates that all three independently identified ChoP phase variants resulted from changes in the homopolymeric repeat of *pptA*. Phase-variable expression of ChoP is important in the colonization of different microniches and contributes to the pathogenicity of nontypeable *H. influenzae* (38, 45). These data suggest that the phase variation of *pptA* also may be important in the pathogenesis of *N. meningitidis*. In contrast, our work suggests that this gene may not be phase variable in *N. gonorrhoeae* due to the short homopolymeric tract. It is interesting that there have been reports of *N. gonorrhoeae* strains that exhibit phase variation of ChoP expression (42). As these same strains do not contain long homopolymeric tracts, it seems that an alternative mechanism of phase variation must be operating. Changes at the site of ChoP addition, mediated by changes in the amino acid sequence at this site, may occur at a high frequency (9, 18, 26, 31). Alternatively, the phase variation seen in *N. gonorrhoeae* could occur through alterations in as-yet-uncharacterized pilin-linked glycans or through the phase variation of other ORFs involved in either the biosynthesis or the uptake of ChoP in *N. gonorrhoeae*.

The results presented in this communication demonstrate that *pptA* is a phase-variable ORF involved in the addition of ChoP to pilin in *N. meningitidis*. It is interesting that there are homologues of this ORF in many other bacteria (Table 3). As the expression of ChoP on surface oligosaccharides has been described for many of these bacteria, the presence of *pptA* homologues suggests that the modification of proteins by ChoP also may occur in these organisms.

TABLE 3. Homologues of *pptA* found in other pathogens

Organism	ORF name	%	
		Identity ^a	Similarity ^a
<i>H. influenzae</i>	HI 1005	38	53
<i>E. coli</i> (O157:H7)	NP308920	32	47
<i>Shigella flexneri</i>	NP706693	31	46
<i>Salmonella enterica</i> serovar Typhimurium	NP459811	31	45
<i>P. aeruginosa</i>	NC002156	26	44

^a Relative to *N. meningitidis pptA*.

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