

Genetic and Functional Analyses of PptA, a Phospho-Form Transferase Targeting Type IV Pili in *Neisseria gonorrhoeae*^{∇†}

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The PilE pilin subunit protein of *Neisseria gonorrhoeae* undergoes unique covalent modifications with phosphoethanolamine (PE) and phosphocholine (PC). The pilin phospho-form transferase A (PptA) protein, required for these modifications, shows sequence relatedness with and architectural similarities to lipopolysaccharide PE transferases. Here, we used regulated expression and mutagenesis as means to better define the relationships between PptA structure and function, as well as to probe the mechanisms by which other factors impact the system. We show here that *pptA* expression is coupled at the level of transcription to its distal gene, *murF*, in a division/cell wall gene operon and that PptA can act in a dose-dependent fashion in PilE phospho-form modification. Molecular modeling and site-directed mutagenesis provided the first direct evidence that PptA is a member of the alkaline phosphatase superfamily of metalloenzymes with similar metal-binding sites and conserved structural folds. Through phylogenetic analyses and sequence alignments, these conclusions were extended to include the lipopolysaccharide PE transferases, including members of the disparate Lpt6 subfamily, and the MdoB family of phosphoglycerol transferases. Each of these enzymes thus likely acts as a phospholipid head group transferase whose catalytic mechanism involves a *trans*-esterification step generating a protein-phospho-form ester intermediate. Coexpression of PptA with PilE in *Pseudomonas aeruginosa* resulted in high levels of PE modification but was not sufficient for PC modification. This and other findings show that PptA-associated PC modification is governed by as-yet-undefined ancillary factors unique to *N. gonorrhoeae*.

Zwitterionic phospho-forms are increasingly being recognized as influential substituents of pathogenic and commensal microbe cell surfaces. Phosphoethanolamine (PE) modification of lipopolysaccharide (LPS) has been documented in a number of important pathogens (3, 13, 23, 38, 57) and has been implicated in resistance to cationic microbial peptides (24, 53) and recognition by complement component C4b (34). Phosphocholine (PC) modification of LPS is documented in *Haemophilus influenzae* and commensal *Neisseria* species (25, 42), while in *Streptococcus pneumoniae*, PC is found covalently linked to teichoic/lipoteichoic acid, as well as to some capsular polysaccharides (55). Activities ascribed to the PC moiety found in LPS, teichoic/lipoteichoic acids, and complex oligosaccharides include enhancing epithelial and endothelial cell adherence through binding to the receptor for platelet-activating factor (7, 41, 51) and acting as a ligand for C-reactive protein (41, 60) and PC-recognizing antibodies (4). The PilE protein subunit of the *Neisseria gonorrhoeae* type IV pilus (Tfp) colonization factor can be uniquely modified by the covalent addition of PE, as well as PC, to select serine residues (1, 14). This situation thus contrasts with all other examples noted, in which PE and PC are exclusively linked through carbohydrate moieties.

Given their increasing recognition as biologically active substituents, heightened attention has been focused on characterizing biosynthetic pathways involved in zwitterionic phospho-form modifications. Phospholipid headgroups represent abundant and conveniently localized donor sources for such phospho-form modifications. The *Escherichia coli* EptB protein (formerly called YhjW) has been shown to be directly responsible for modifying the inner core of LPS with PE, using phosphatidylethanolamine as a precursor (35). Most gram-negative species possess multiple genes whose products show structural homology to EptB. They include the *Salmonella enterica* gene *cptA* (53) and *pmrC* (24), as well as *lptA* (6) and the Lpt3 (26) and Lpt6 (70) genes from *Neisseria meningitidis*. Although phosphatidylcholine is found in significant amounts in diverse groups of bacteria, there are as yet no known instances in which its head group serves as a precursor. Rather, PC modifications of both LPS and teichoic/lipoteichoic acids involve a CDP-choline-type pathway utilizing enzymes encoded by the *lic* genes (61, 71) that requires exogenous choline or choline-containing compounds as precursors. Although present in commensal *Neisseria* species, the *lic* genes are absent in both *N. gonorrhoeae* and *N. meningitidis*. We recently identified PptA as a putative pilin phospho-form transferase required for modification of *N. gonorrhoeae* PilE with both PE and PC (1). The orthologous *N. meningitidis* PptA protein was implicated early on in PC modification of pilin, as high-frequency frame-shifting events within *pptA* correlated with phase (on-off) variation of the PilE PC epitope (58). PptA shares multiple structural features with *E. coli* EptB and related proteins implicated in LPS PE modification, which are all grouped

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together in the so-called YhjW/YjdB/YijP family, which comprises a subfamily of the larger alkaline phosphatase superfamily. The members of this superfamily have conserved core structures and active-site residues, which has led to the proposal that these enzymes involve catalytic cycles of phosphorylation, sulfation, or phosphonation of conserved Ser/Cys/Thr residues (12). It has also been suggested that these enzymes have the same reaction scheme as was originally proposed for *E. coli* alkaline phosphatase (AlkP) (21).

The structural relatedness of PptA with EptB and other LPS PE transferases likely utilizing phosphatidylethanolamine as a donor strongly suggests similar modes of action. However, phosphatidylcholine has been documented only once in *N. gonorrhoeae* (49), while a more recent study of gonococcal phospholipids using fast atom bombardment-mass spectrometry (MS) and gas liquid chromatography-MS technologies failed to detect its presence (33). Two bacterial pathways for phosphatidylcholine synthesis have been characterized in bacteria: one in which endogenous phosphatidylethanolamine undergoes methylation by phospholipid *N*-methyltransferases and another in which it is synthesized via direct condensation of exogenous choline with CDP-diacylglyceride mediated by the activity of phosphatidylcholine synthase (46). To date, no phospholipid *N*-methyltransferases or phosphatidylcholine synthase orthologs are readily identifiable within neisserial genome sequences.

An additional confounding factor in pilin phospho-form modification involves the pilin-like PilV protein. Pilin PC modification has been seen only in *pilV* null mutants, a background that is also associated with PE hypermodification while PilV overexpression is associated with PE hypomodification (1). The mechanisms by which PilV impacts each of these aspects of Tfp biology remain enigmatic.

The goal of this study was to characterize *pptA* in regard to its genetic organization and to probe the structure-function relationships of PptA, as well as to gain insight into the relationships between PE and PC modifications mediated by PptA.

MATERIALS AND METHODS

Bacterial strains, vectors, and culture conditions. The bacterial strains used in this study are described in Table 1. *N. gonorrhoeae* strains were grown on conventional GC medium as described previously (9) unless otherwise described as grown on defined, choline-free medium (30). *E. coli* DH5 α or HB101 was used for plasmid propagation and cloning experiments and was grown on Luria-Bertani (LB) medium (37). The antibiotics used for selection of transformants and transconjugants were at the following concentrations: in *N. gonorrhoeae*, chloramphenicol, 10 μ g/ml, and erythromycin, 8 μ g/ml; in *E. coli*, chloramphenicol, 30 μ g/ml; erythromycin, 300 μ g/ml; kanamycin, 50 μ g/ml; ampicillin, 100 μ g/ml; tetracycline, 15 μ g/ml; and streptomycin, 100 μ g/ml; in *Pseudomonas aeruginosa* PAK, carbenicillin, 1,000 μ g/ml, and kanamycin, 1,000 μ g/ml. For growth of *P. aeruginosa* PAK, the concentrations of carbenicillin and kanamycin were reduced to 300 μ g/ml. Isolation and purification of plasmid DNA were performed by using QIAprepSpin Miniprep columns (no. 27106) according to the manufacturer's specifications (Qiagen, Chatsworth, CA). The nucleotide sequences of all clones and constructs described were determined from plasmid DNA or directly from PCR products derived from *N. gonorrhoeae* mutant strains at GATC Biotech AG (Konstanz, Germany).

Construction of an *N. gonorrhoeae pptA* null mutant and strains carrying site-specific mutations. The *pptA::kan* allele carried on plasmid pLS3 (44) was introduced into the wt strain N400 and into GV1 [*pilV*(Fs) (a null allele of *pilV* with a frameshift mutation at the G₋₁ codon (65)), by transformation and selection for kanamycin-resistant transformants, to generate the *pptA* null mutants designated KS9 (*pptA::kan*) and KS10 [*pilV*(Fs) *pptA::kan*]. The strains carrying the *pptA*_{LS1}::*kan* allele carried on plasmid pLS1 (44) have been previously described (1).

The *pptA* gene was amplified from genomic DNA from the *N. gonorrhoeae* strain N400 with the primers *pptA*5' and *pptA*3'. The resulting PCR fragment was digested with restriction endonucleases cutting at EcoRI and BamHI (see Table S1 in the supplemental material) and cloned into the polylinker of the plasmid pUP6 (67), yielding plasmid pUP6 *pptA*. Point mutations in *pptA* were made by using a modified version of the PCR-based technique termed splicing by overlap extension (SOE). The SOE PCR products were subcloned into pUP6 *pptA*, and new restriction sites created by the point mutation were used for screening. For the point mutations listed in Table S1 in the supplemental material, the two PCR products for each site-specific mutation were subjected to SOE using the flanking primers *pptA*5'1150 and *pptA*3'1927 and cut with BstEII and NsiI (521 bp). The resulting fragment was then ligated with the NsiI/NheI (2,009-bp)- and the BstEII/NheI (2,509-bp)-cut fragments of pUP6 *pptA*. For *pptA*(G239A), the upstream region was amplified using primers G239A5' and *pptA*3', whereas the downstream primers were *pptA*5' and G239A3'. The two PCR products were spliced together using the flanking primers *pptA*5'747 and *pptA*3'. The resulting PCR fragment was cut with XmnI and BstEII and ligated with the XmnI/NheI- and the BstEII/NheI-cut fragments of pUP6 *pptA*, resulting in pUP6 *pptA*(G239A). The G239A mutation created a unique DdeI restriction site. The following point mutations were made by subcloning SOE PCR products into pUP6 *pptA*(G239A) and screening for loss of the DdeI restriction site: pUP6 *pptA*(S241A) was made using PCR products from S241A5' plus *pptA*3' and *pptA*5' plus S241A3'; pUP6 *pptA*(Δ 241A) was made using PCR products from Δ S241_5' plus *pptA*3' and *pptA*5' plus Δ S241_3'.

All resulting plasmids were introduced into strain GV1 [*pilV*(Fs)] for further analysis. pUP6 *pptA*(804) (see below) was cut with the restriction endonucleases AdhI and NotI, and the fragment was transformed into KS25 and KS22 in order to rescue the phenotype of the *pptA*(H436) and *pptA*(H379) point mutations.

Expression of *pptA* from an inducible promoter. To create an isopropylthiogalactoside (IPTG)-regulatable *pptA* construct, the NotI-blunted fragment of pVD300 *recA6* carrying *lacI*^q, the tandem *lac* operator promoter sequences *tacOP* and *UV5OP*, and the *tetM* gene (39) was inserted into the HpaI site of pUP6 *pptA*. The resulting plasmid was cut with XhoI and BamHI, and the 3' recessed ends were filled in using Klenow (New England BioLabs Inc.). The resulting fragment containing *lacIOP pptA* was inserted into the SacI site (filled in using Klenow) of p2/16/1 (67), resulting in p2/16/1 *pptA*(Ind).

Transposon mutagenesis of the *pptA* locus. The two target plasmids, pUP6 *pptA*(1315) and pUP6 *pptA*(804), were constructed by amplifying the *pptA* gene in overlapping fragments from *N. gonorrhoeae* strain N400. The first 894 bp of *pptA*, along with 421 bp upstream of the gene, were amplified using the primers *pptA*5' and *pptA*1315_3'. The terminal 1,327 bp of *pptA*, along with 227 bp downstream of the gene, were amplified using primers *dca804_5'* and *pptA*3'. The resulting PCR fragments were digested with unique flanking EcoRI and BamHI sites (see Table S1 in the supplemental material) and cloned into the polylinker of the plasmid pUP6, yielding plasmids pUP6 *pptA*(1315) and pUP6 *pptA*(804), respectively. Transposon mutagenesis was performed on pUP6 *pptA*(1315) and pUP6 *pptA*(804) as described previously (40). Transposon insertions were isolated, sequenced, and transformed into the gonococcal strain N400 as previously described (9).

***P. aeruginosa* strains and construction of mutants.** The *P. aeruginosa* mutants used in this study were derived from the wt strain PAK (52). Construction of the *pilA* null allele was previously described (18), as were constructs for the *pilT* and *pilB* mutations (50). The *pilY1* null allele was assembled by removing an internal fragment of coding sequence (amino acids 7 to 1094) from the targeted gene by SOE PCR to generate a nonpolar in-frame deletion. Specific primer sequences are available upon request. Deletion alleles were cloned into the suicide vector pEXGmGW (68) and introduced onto the chromosome of strain PAK as described previously (16). All deletions were confirmed by PCR and sequencing of the relevant chromosomal regions.

Conjugation of plasmids into *P. aeruginosa* PAK. *E. coli* S17.1 (Table 1) was used as a donor for conjugation of plasmids into *P. aeruginosa* PAK strains. Donor and recipient strains were grown in LB broth at 37°C to an optical density at 600 nm (OD₆₀₀) of ~0.4 to 0.8, and 1 ml of each was mixed in a centrifuge tube and pelleted. The cells were resuspended in 100 μ l LB medium and deposited as a drop on LB agar overnight at 37°C. The resulting growth was diluted in 6 ml of LB medium, and 10 \times to 1,000 \times dilutions were plated onto pseudomonas isolation agar (Difco) with the appropriate antibiotics.

Expression of PilE and PptA in *P. aeruginosa* PAK and *E. coli*. To express gonococcal PilE in *E. coli* and *P. aeruginosa*, a Bsu36I/SmaI-blunted fragment from pPilE2 (1) was ligated into pMMB67EH (10), cut with SmaI, and transformed into *E. coli* DH5 α and S17.1, respectively. The plasmid in S17.1 was transferred to KS32 by conjugation. To express gonococcal PptA in *E. coli* and KS32, an NdeI restriction site was fused to the ATG start codon of *pptA* by SOE

TABLE 1. Strains used in this study

Strain	Parental strain	Relevant genotype	Reference
<i>N. gonorrhoeae</i>			
VD300 ^a	MS11		22
N400	VD300	<i>recA6</i> ^b	56
GV1	N400	<i>pilV</i> (Fs)	65
GE68	N400	<i>pilE</i> (S68A)	1
GE68V	GE68	<i>pilE</i> (S68A) <i>pilV</i> (Fs)	1
KS1	GV1	<i>pilV</i> (Fs) <i>pptA</i> ::Tn5	This study
KS2	GV1	<i>pilV</i> (Fs) <i>pptA</i> ::Tn9	This study
KS3	GV1	<i>pilV</i> (Fs) <i>pptA</i> ::Tn11	This study
KS4	GV1	<i>pilV</i> (Fs) <i>pptA</i> ::Tn22	This study
KS5	N400	<i>pptA</i> ::Tn5	This study
KS6	N400	<i>pptA</i> ::Tn9	This study
KS7	N400	<i>pptA</i> ::Tn11	This study
KS8	N400	<i>pptA</i> ::Tn22	This study
KS9	N400	<i>pptA</i> :: <i>kan</i>	44
KS10	GV1	<i>pilV</i> (Fs) <i>pptA</i> :: <i>kan</i>	This study
GD2	N400	<i>pptA</i> _{LS1} :: <i>kan</i>	44
GD3	GV1	<i>pilV</i> (Fs) <i>pptA</i> _{LS1} :: <i>kan</i>	1
KS11	KS10	<i>pilV</i> (Fs) <i>pptA</i> :: <i>kan</i> <i>iga</i> :: <i>pptA</i> (Ind)	This study
KS12	KS5	<i>pptA</i> ::Tn5 <i>iga</i> :: <i>pptA</i> (Ind)	This study
KS13	KS1	<i>pilV</i> (Fs) <i>pptA</i> ::Tn5 <i>iga</i> :: <i>pptA</i> (Ind)	This study
KS14	KS11	<i>pilV</i> (Fs) <i>pptA</i> :: <i>kan</i> <i>iga</i> :: <i>pptA</i> (Ind)::Tn7	This study
KS15	KS11	<i>pilV</i> (Fs) <i>pptA</i> :: <i>kan</i> <i>iga</i> :: <i>pptA</i> (Ind)::Tn23	This study
KS16	GV1	<i>pilV</i> (Fs) <i>pptA</i> (S241A)	This study
KS17	GV1	<i>pilV</i> (Fs) <i>pptA</i> (ΔS241)	This study
KS18	GV1	<i>pilV</i> (Fs) <i>pptA</i> (G239A)	This study
KS19	GV1	<i>pilV</i> (Fs) <i>pptA</i> (H374A)	This study
KS20	GV1	<i>pilV</i> (Fs) <i>pptA</i> (G377A)	This study
KS21	GV1	<i>pilV</i> (Fs) <i>pptA</i> (S378A)	This study
KS22	GV1	<i>pilV</i> (Fs) <i>pptA</i> (H379A)	This study
KS23	GV1	<i>pilV</i> (Fs) <i>pptA</i> (S434A)	This study
KS24	GV1	<i>pilV</i> (Fs) <i>pptA</i> (D435A)	This study
KS25	GV1	<i>pilV</i> (Fs) <i>pptA</i> (H436A)	This study
KS26	GV1	<i>pilV</i> (Fs) <i>iga</i> :: <i>pptA</i>	This study
KS27	KS10	<i>pilV</i> (Fs) <i>pptA</i> :: <i>kan</i> <i>iga</i> :: <i>pptA</i>	This study
KS28	N400	<i>iga</i> :: <i>pptA</i> (Ind)	This study
KS29	KS9	<i>pptA</i> :: <i>kan</i> <i>iga</i> :: <i>pptA</i> (Ind)	This study
KS30	GE68	<i>pilE</i> (S68A) <i>iga</i> :: <i>pptA</i> (Ind)	This study
KS31	GE68V	<i>pilV</i> (Fs) <i>pilE</i> (S68A) <i>iga</i> :: <i>pptA</i> (Ind)	This study
<i>P. aeruginosa</i>			
PAKΔ <i>pilA</i>		<i>pilA</i>	18
KS32	PAKΔ <i>pilA</i>	<i>pilA pilT</i>	This study
KS33	KS32	<i>pilA pilT</i> pMMB67EH <i>pilE</i>	This study
KS34	KS33	<i>pilA pilT</i> pMMB67EH <i>pilE</i> pJT19 <i>pptA</i>	This study
KS35	KS32	<i>pilA pilT</i> pMMB67EH <i>pilE</i> (S68A)	This study
KS36	KS32	<i>pilA pilT pilY1</i>	This study
KS37	KS36	<i>pilA pilT pilY1</i> pMMB67EH <i>pilE</i>	This study
KS38	KS37	<i>pilA pilT pilY1</i> pMMB67EH <i>pilE</i> pJT19 <i>pptA</i>	This study
KS39	KS32	<i>pilA pilT pilB</i>	This study
KS40	KS39	<i>pilA pilT pilB</i> pMMB67EH <i>pilE</i>	This study
KS41	KS40	<i>pilA pilT pilB</i> pMMB67EH <i>pilE</i> pJT19 <i>pptA</i>	This study
<i>E. coli</i>			
S17.1		RP4 2-Tc::Mu-Km::Tn7 <i>pro res mod</i> ⁺	43
KS42	DH5α	pMMB67EH <i>pilE</i>	This study
KS43	KS42	pMMB67EH <i>pilE</i> pJT19 <i>pptA</i>	This study

^a VD300 is an Opa⁻ derivative of MS11.
^b *recA6* is an IPTG-inducible allele of *recA*.

PCR, using pUP6 *pptA* as a template. The upstream region was amplified using primers pUP6_5' and *pptA*_NdeI_3', whereas the downstream primers were *pptA*_NdeI_5' and *pptA*3'1000. The two PCR products were subjected to SOE together, using flanking primers pUP6_5' and *pptA*3'1000. The resulting PCR fragment was digested with restriction endonucleases recognizing the unique EcoRI and BsgI sites and subcloned into pUP6 *pptA* cut with the same restriction

enzymes, yielding plasmid pUP6 *pptA*_{M1}NdeI, which was then cut at the unique NdeI and BamHI restriction sites. The resulting fragment was cloned into the polylinker of pJT19 (64), yielding plasmid pJT19 *pptA*. This plasmid was transformed into *E. coli* DH5α and S17.1, respectively, using S17.1 to transfer the plasmid into KS32 by conjugation. For induction of PilE and PptA in *E. coli* and KS32, the bacteria were grown to an OD₆₆₀ of 0.8 and induced with 0.4 mM

IPTG and 2 mM *m*-toluate, respectively, for 2 hours. The cells were spun at 4,000 × *g* for 20 min, and the cell pellets were used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and MS (see below).

Immunofluorescence microscopy, SDS-PAGE, and immunoblotting. For immunofluorescence microscopy, immobilization of bacteria was performed by incubating 100 μl of gonococcal cells at an OD₆₆₀ of 0.1 for 1 h at 37°C in GC medium on poly-L-lysine-coated glass coverslips and fixing them for 1 h with 2% formaldehyde in phosphate-buffered saline. After being washed three times for 5 min each time in phosphate-buffered saline, the gonococci were labeled using a 1:10 dilution of MicroTrak *Neisseria gonorrhoeae* Culture Conformation Test according to the manufacturer's specifications (Trinity Biotech Plc) and mounted in the supplied mounting fluid before being viewed in a Nikon Eclipse C400 fluorescence microscope.

The procedures for SDS-PAGE and immunoblotting have been described previously (9). PC-decorated proteins were detected by using a 1:1,000 dilution of the monoclonal antibody (MAb) TEPC-15 (Sigma) and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin A (Sigma) (14). PilE, PilV, and PptA were detected by immunoblotting of whole-cell lysates, using rabbit polyclonal antibodies and alkaline phosphatase-coupled goat anti-rabbit antibodies (Tago Inc.). PilV-specific sera have been described previously (65). PilE-specific antisera were generated against isolated pili from *N. gonorrhoeae* strain N400 (Invitrogen [lot 904]). PptA-specific sera were generated and affinity purified against recombinant His-tagged protein (Agrisera AB, Sweden). The recombinant His-tagged protein encompassed amino acids 230 to 548 of PptA and was amplified from *N. gonorrhoeae* strain N400 with primers pptA:sulfatase5' and pptA:sulfatase3'. The resulting PCR fragment was digested using restriction endonucleases cutting at the unique, flanking BamHI and HindIII sites (see Table S1 in the supplemental material) and cloned into the polylinker of the plasmid pQE30 (Qiagen), resulting in the plasmid pQE30 *pptA*, which was transformed into *E. coli* strain M15[pREP4]. For protein purification, 6 liters of LB medium was inoculated with M15[pREP4] pQE30 *pptA* and was first incubated for 4 h to approximately an OD₆₀₀ of 1.3 before induction with 1 mM IPTG and further incubation for 2 h to approximately an OD₆₀₀ of 1.9. The cells were harvested at 4,000 × *g* for 20 min and frozen in liquid nitrogen. The frozen cells were lysed using an X-press French press (AB BIOX, Sweden) and dissolved in 100 ml 20 mM NaH₂PO₄·2H₂O, 20 mM Na₂HPO₄, 5 mM DNase I added. The suspension was agitated for 10 min, and urea was directly added to a final concentration of 8 M and stirred until it completely dissolved. Cellular debris was removed by centrifugation at 20,000 rpm in a JA25-50 rotor for 90 min, and the supernatant was filtered through a 0.22-μm filter (Millipore). The filtered solution was applied to a 5-ml Hitrap Chelating Histrap column mounted on an ÄKTApurifier FPLC (Amersham Biosciences). The column was washed twice with wash buffer 1 (20 mM NaH₂PO₄, 20 mM Na₂HPO₄, 8 M urea) and wash buffer 2 (20 mM NaH₂PO₄, 20 mM Na₂HPO₄, 8 M urea, 10 mM imidazole). The protein was then removed from the column by using an elution buffer (20 mM NaH₂PO₄, 20 mM Na₂HPO₄, 8 M urea, 0.5 M imidazol). The fractions containing the recombinant PptA protein were concentrated through an Amicon Ultra 12 MWCO 30,000 centrifugal filter (Millipore) and applied to a Tricorn Superdex high-performance column (Superdex 200 10/300 GL; Amersham Biosciences) with baseline separation. The fractions with PptA were concentrated through a Centricon 10 centrifugal filter (Millipore) for 3 h at 5,000 × *g* and diluted in 20 mM Tris, pH 7.4, 4 M urea.

Quantitative real-time RT-PCR. RNA was isolated from *N. gonorrhoeae* using Trizol (Invitrogen) according to the manufacturer's instructions. The RNA was purified using an RNeasy minikit (Qiagen) with the RNase-free DNase set (Qiagen) according to the manufacturer's instructions. Ten nanograms of RNA was reverse transcribed and used as a template for quantitative real-time reverse transcription (RT)-PCR on a Light Cycler (Roche) in the same reaction, using a Quantitect Sybr green RT-PCR kit (Roche). The cyclor conditions were the same for all reactions; 57°C was used as the annealing temperature, and fluorescence was read at 81°C. The primers used as the loading control were *tbpA5'* and *tbpA3'*. Transcription of *murF* and *pptA* was carried out using the primer pairs *murF5'*-*murF3'* and *pptA5'*-*pptA3'*, respectively. Negative controls without reverse transcriptase were performed in parallel.

Sample preparation and infusional MS analysis of intact PilE. Pilus purification was carried out as described previously (66), and preparations were further processed using a methanol/chloroform precipitation procedure (1, 62). All data were acquired on a quadrupole time-of-flight mass spectrometer (Q-ToF micro; Micromass, Manchester, United Kingdom) equipped with the standard Z-spray electrospray ionization (ESI) source as described previously (1).

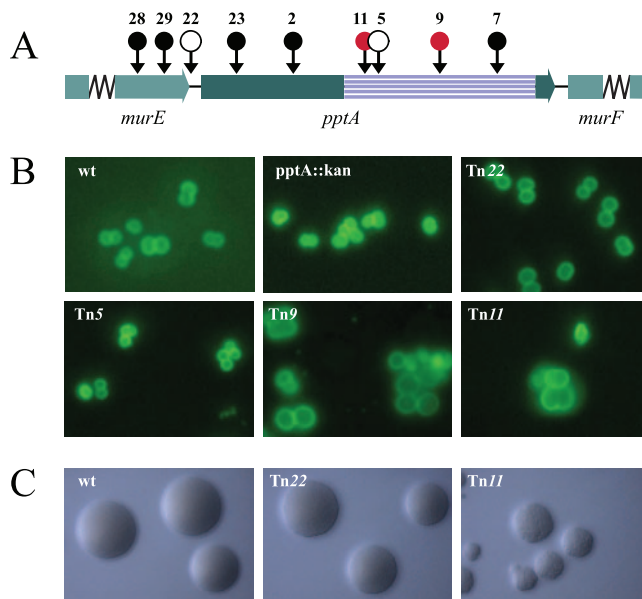


FIG. 1. Mapping and phenotypic characterization of *pptA*-linked transposon insertion mutants. (A) Physical map of the gonococcal *pptA* locus with transposon insertions indicated by circles. ●, transposon insertions that could not be recovered in *N. gonorrhoeae*; ○, insertions with wt growth phenotype. The red circles represent transposon insertions that resulted in aberrant cell septation and coccal morphology. The precise sites of transposon insertion are shown in Fig. S1 in the supplemental material. The striated box defines the Pfam PF00884 sulfatase domain of PptA. (B) Cell septations and coccal morphologies of transposon mutants detected by immunofluorescence microscopy. Gonococci were detected using fluorescein-labeled MABs (green). (C) Gonococcal colonies on conventional medium showing the different growth phenotypes of transposon mutants. The strains used were wt (N400), *pptA::kan* (KS9), Tn22 (*pptA::Tn22*; KS8), Tn5 (*pptA::Tn5*; KS5), Tn9 (*pptA::Tn9*; KS6), and Tn11 (*pptA::Tn11*; KS7).

RESULTS

Genetic characterization of *pptA*. *pptA* maps upstream of *murF*, whose product is required for synthesis of UDP-MurNAc pentapeptide, a precursor essential to peptidoglycan synthesis (45). When the genetic organization of this locus was examined by transposon mutagenesis, only four of the nine insertions constructed in *E. coli* were recoverable in strain N400 (Fig. 1A). Moreover, transposon insertion mutants 9 and 11 uniquely displayed aberrant cell septation and “giant” cell morphology phenotypes that were paralleled by unusual colony morphologies and poor growth properties (Fig. 1B and C). Steady-state levels of *murF* mRNA were significantly reduced in the Tn9 and Tn11 backgrounds, slightly less diminished in the Tn22 mutant, and surprisingly elevated in the Tn5 background (Fig. 2A). Unlike the kanamycin resistance gene cassette insertion mutant (in which the level of *murF* mRNA was indistinguishable from the wt background), all four of the transposon insertion mutants displayed abnormal levels of *murF* mRNA.

PilE phospho-form modification was abolished in backgrounds in which transposon insertions disrupted the *pptA* open reading frame (ORF) (Tn5, -9, and -11) (data not shown) and reduced in that carrying the insertion upstream of the *pptA* ORF (Tn22) (Fig. 2B and C). These findings were consistent

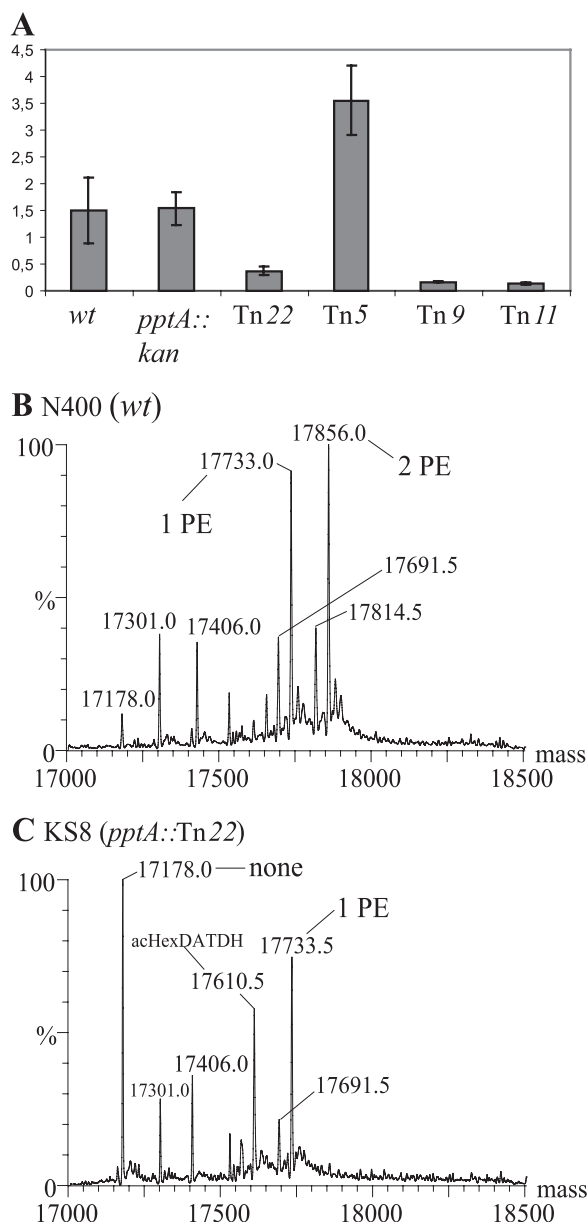


FIG. 2. PptA-linked transposon insertion mutations perturb *murF* expression and PptA activity. (A) Real-time RT-PCR analysis of *murF* mRNA levels in mutants carrying transposon insertions. Amplification of *tbpA* (5) served as an internal reference and control. Negative controls were performed without reverse transcriptase treatment (not shown). The values are means \pm standard errors of the mean; $n = 3$. The strains used were wt (N400), *pptA::kan* (KS9), Tn22 (*pptA::Tn22*; KS8), Tn5 (*pptA::Tn5*; KS5), Tn9 (*pptA::Tn9*; KS6), and Tn11 (*pptA::Tn11*; KS7). The Tn22 mutation reduces PilE phospho-form modification. (B and C) Intact mass analysis of PilE N400 (wt) (B) and KS8 (*pptA::Tn22*) (C). Species labeled as bearing PE/PC modifications also carry the hexose-2,4-diacetamido-2,4,6-trideoxyhexose (Hex-DATDH) disaccharide in its acetylated form. Peaks in the MS spectra that are not labeled are listed in Table S3 in the supplemental material.

with our earlier work documenting the requirement for PptA in phospho-form modification (1). However, complementation there employed an ectopic, intact *pptA* gene copy (encompassing the *pptA* ORF and 420 bp of upstream sequence) that

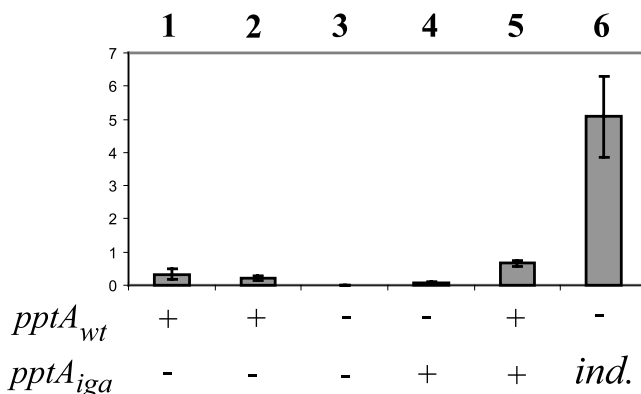


FIG. 3. Relative *pptA* mRNA levels in defined isogenic backgrounds. Real-time RT-PCR amplification of *tbpA* mRNA (5) served as an internal reference/control. Negative controls included reactions performed without reverse transcriptase; no products were detected under these conditions (not shown). The values are means \pm standard errors of the mean; $n = 3$. Bars: 1, wt (N400); 2, *pilV*(Fs) (GV1); 3, *pilV*(Fs) *pptA::kan* (KS10); 4, *pilV*(Fs) *pptA::kan iga::pptA* (KS27); 5, *pilV*(Fs) *iga::pptA* (KS26); 6, *pilV*(Fs) *pptA::kan iga::pptA* (Ind) (KS11) induced with 0.25 mM IPTG. *pptA_{wt}* and *pptA_{iga}* refer to *pptA* allele status, with the gene at either the endogenous locus (*wt*) or the ectopic locus (*iga*). *ind.*, ectopic, inducible *pptA* in its derepressed state.

failed to restore phospho-form modification to levels seen in the wt background. Based on a real-time RT-PCR assay, complementation with the ectopic *pptA* gene copy restored *pptA* mRNA detection in a *pptA::kan* background, but not to levels seen in the wt background (Fig. 3). These studies were carried out in a *pilV* null background that enhanced PptA-mediated modification and led to PC decoration in order to facilitate phospho-form analyses and quantification. It is important to note, however, that *pptA* mRNA levels in a *pilV* background were not significantly altered from those seen in a wt background (Fig. 3, lane 2).

To provide higher levels of *pptA* for complementation, an inducible *pptA* allele carrying a tandem *lac* operator-promoter sequence was placed in the *iga* locus. In its repressed state, no mRNA was detected (data not shown), while when maximally derepressed, mRNA levels 17-fold higher than those seen for the wt strain were observed (Fig. 3, lane 6). Using a *pptA::kan pilV* background (KS11), a clear correlation was observed between levels of inducer, PptA protein, and the PilE PC epitope (as assessed by reactivity with the PC epitope recognizing TEPC-15 MAb) to a point where PC epitope levels plateaued while PptA levels increased (Fig. 4A). MS analyses of intact PilE protein from purified Tfp confirmed that increasing *pptA* expression was paralleled by concomitant increases in PE and PC site occupancy (Fig. 4B to D). Despite the high levels of *pptA* mRNA seen when maximally derepressed, detection of the pilin PC epitope remained dependent on the absence of PilV and was not seen under any conditions in the presence of the wt *pilV* allele (data not shown).

While transformants bearing Tn7 and Tn23 were not recoverable in a wt background, they could be established in a *pptA* diploid background (data not shown), and in all these instances, the insertions mapped to the ectopic gene copy. Together, these results are consistent with the hypotheses that (i) *pptA* and *murF* are part of a single transcriptional unit whose

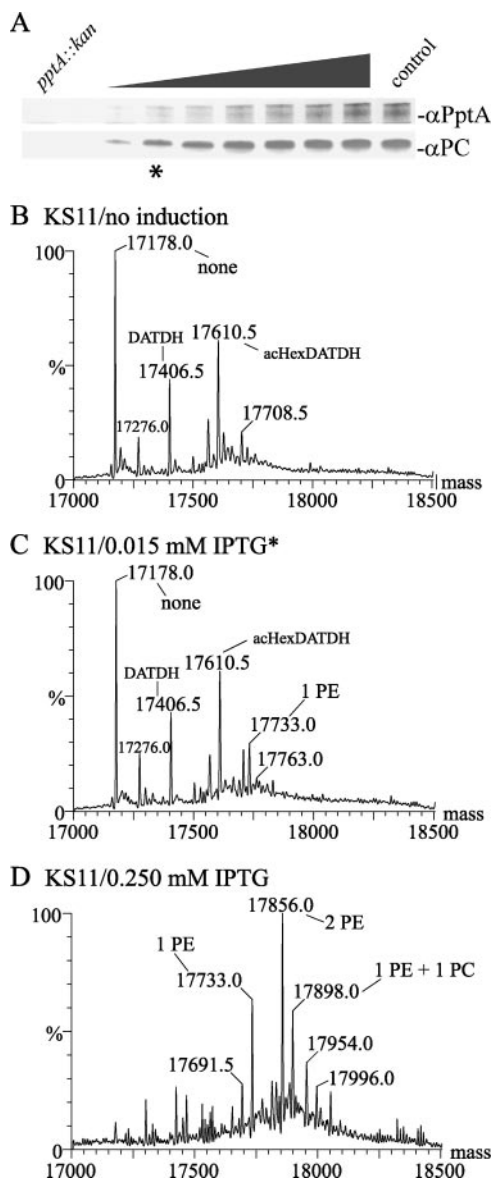


FIG. 4. PiliE PC modification levels parallel regulated expression of PptA. (A) Lane 1 (from left), KS9 (*pptA::kan*); lane 2, KS11 without IPTG [*pilV(Fs pptA::kan iga::pptA(Ind)*]. Lanes 3 to 9, KS11 with IPTG in the following concentrations: lane 3, 0.01 mM; lane 4, 0.015 mM; lane 5, 0.02 mM; lane 6, 0.03 mM; lane 7, 0.04 mM; lane 8, 0.05 mM; lane 9, 0.1 mM; lane 10, control (GV1). (B to D) Deconvoluted molecular weight spectra from intact PiliE ESI MS analyses in strains KS11 without IPTG [*pilV(Fs pptA::kan iga::pptA(Ind)*] (B), KS11 with IPTG (0.015 mM) (C), and KS11 with IPTG (0.250 mM) (D). Species labeled as bearing PE/PC modifications (HexDATDH) disaccharide in its acetylated (ac) form. A complete list of *m/z* forms and corresponding species of PiliE can be found in Table S3 in the supplemental material. Asterisks indicate samples in which *pptA* was induced with 0.015 mM IPTG (A and C).

promoter maps at least 400 bp upstream of the *pptA* ORF, (ii) the growth defects seen in some *pptA* transposon mutants are the result of perturbed cell wall metabolism ensuing from reduced levels of MurF and are not directly related to altered

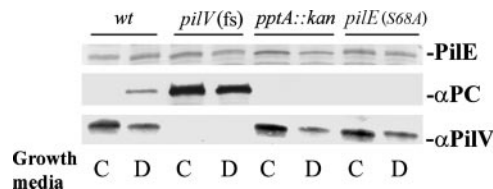


FIG. 5. PiliE PC modification occurs in a wt background when propagated in defined, choline-free medium. (Top) Immunoblot of whole-cell lysates using polyclonal antibodies specific for PiliE. (Middle) MAb TEPC-15 specific for PC. (Bottom) Polyclonal antibodies specific for PilV. *N. gonorrhoeae* strains were grown either on solid conventional medium (C) or on solid defined, choline-free medium (D). Lanes: 1 and 2, wt (N400); 3 and 4, *pilV(Fs)* (GV1); 5 and 6, *pptA::kan* (KS9); 7 and 8, *pilE(S68A)* (GE68).

PptA expression, and (iii) the *pilV* null allele does not lead to increased *pptA* transcription.

PiliE PC modification does not require exogenous choline.

Both the phosphatidylcholine synthase- and *lic* gene-based pathways require exogenous choline as a precursor (46). Although neither pathway has been documented in pathogenic *Neisseria* species, we nonetheless tested if the pilin PC modification occurred in the absence of exogenous choline by growing the bacteria on chemically defined, choline-free medium (30). As seen by pilin reactivity with the TEPC-15 MAb for a *pilV* strain, PC modification occurred independently of an exogenous choline source (Fig. 5). Surprisingly, PC modification of pilin was seen for the first time in a wt background when propagated in defined, choline-free medium. Moreover, PC modification was PptA dependent under these conditions and was not detectable when a strain expressing PiliE carrying an alanine substitution in place of serine at position 68 (the major residue for phospho-form modification) was used. To examine pilin phospho-form modifications occurring under these growth conditions in more detail, MS analysis of intact pilin was performed. In contrast to the sample derived from growth in conventional medium, one of the major species detected corresponded to PiliE bearing the modified disaccharide and no phospho-form (Fig. 6A and B). Along with signals corresponding to glycosylated PiliE carrying either one or two PE moieties, species corresponding to PiliE bearing the modified disaccharide and a single PC and PiliE bearing the modified disaccharide, one PE, and one PC were found in this sample. The signals for PiliE derived from the *pilE(S68A)* strain grown in choline-free medium were not notably different from those observed previously for that mutant propagated in conventional medium (Fig. 6C). Given the unique finding of PC modification in a wt background, the status of PilV expression was assessed. In all backgrounds, PilV levels in cells cultured in defined, choline-free medium were reduced two- to threefold relative to levels seen in samples from conventional medium (Fig. 5, bottom). These findings demonstrate that the PiliE PC moiety can be endogenously synthesized and corroborated earlier data showing that PC modification is associated with diminished PilV expression.

Comparative modeling of the putative catalytic domain of PptA. PptA shares basic structural features with LPS PE transferases, including an N-terminal contiguous region containing five consecutive transmembrane domains and a large sulfatase

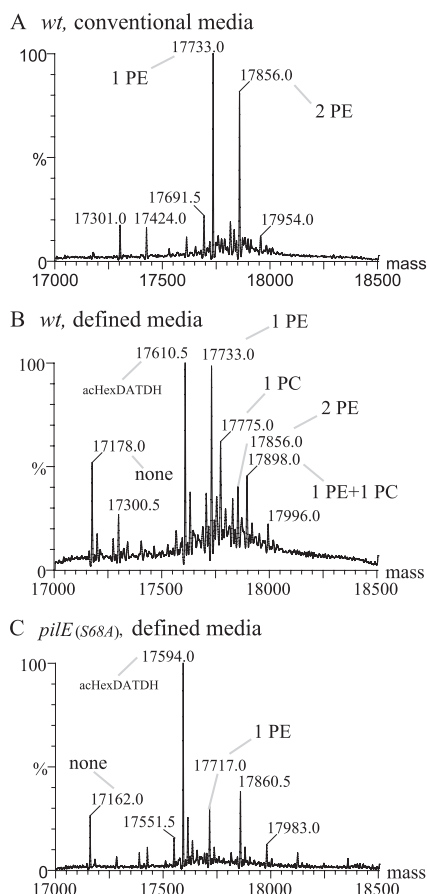


FIG. 6. MS analysis of intact PilE from *N. gonorrhoeae* strains grown in defined, choline-free medium. (A) Wt (N400) grown on solid conventional medium. (B) Wt (N400) grown on solid defined, choline-free medium. (C) *pilE(S68A)* (GE68) grown on solid defined, choline-free medium. The species labeled as bearing PE/PC modifications also carry the hexose-2,4-diacetamido-2,4,6-trideoxyhexose disaccharide (HexDATDH) in its acetylated (ac) form. The peaks in the MS spectra that are not labeled are listed in Table S3 in the supplemental material.

domain of the Pfam PF00884 family. Based on topological studies of PmrC, the sulfatase domain is predicted to be oriented in the periplasm (24). Included within the PF00884 domain family are the catalytic sites of members of the alkaline phosphatase superfamily of metalloenzymes as defined on the basis of biochemical and X-ray structural studies (2, 21, 36). We structurally modeled the PptA sulfatase domain to assess the similarities of its potential catalytic core to known protein structures. Although modeling with the CPHmodels and Swiss-Model programs failed to give significant hits, two models of significance were found using the ModWeb Modeler server (32) (Fig. 7A and B). These high-quality models derived from the human arylsulfatases ArsA and *N*-acetylgalactosamine-4-sulfatase both predicted prototypic α/β topology consisting of a central core of β -strands circumferentially flanked by α -helices. Moreover, putative metal-binding residues predicted previously (11, 12) (H^{374} , H^{379} , D^{435} , and H^{436}) were well colocalized, and there was remarkable conservation in their spatial positioning relative to the active-site structures of other alkaline phosphatase superfamily members. While not pre-

cisely superimposable on the structures of the other enzymes, the results strongly suggest that PptA possesses the same highly conserved catalytic core structure and therefore acts through a similar catalytic mechanism. If true, a nucleophile would likely be present in the vicinity of the metal coordination residues, and here, the hydroxyl of S^{241} is a good candidate. It is important to specify here that despite their overall congruence, the two models differ, with the ArsA-based model being comprised of six central β -strands while the *N*-acetylgalactosamine-4-sulfatase-based model has five central β -strands. This discrepancy influences the positioning of another putative active-site nucleophile (T^{278}), which coordinates only with the other residues of the putative catalytic domain in the model based on ArsA.

Site-directed mutagenesis of putative active-site residues in PptA. The modeled structures of the PptA active site and the inferred mechanism of activity predicted that a number of amino acid residues were likely to be of significant importance to enzyme catalysis. We also assessed the degrees of conservation of these candidate residues within the LPS PE transferase family (previously described as the YhjW/YjdB/YijP family), as found in Prodom entries PD005703 and PD461453, which encompass S^{241} , H^{374} , H^{379} , D^{435} , and H^{436} (Fig. 8). PptA, all known LPS PE transferases (excluding those related to Lpt6-type enzymes), and over 100 other uncharacterized proteins possess the PD005703 defined domain. All of these proteins carry readily identifiable, highly conserved residues corresponding to S^{241} (serine or threonine) and conserved residues corresponding to H^{374} and H^{379} . Likewise, PptA and all known LPS PE transferases (excluding those related to Lpt6-type enzymes) and over 100 other uncharacterized proteins possess the PD461453 defined domain, including conserved residues corresponding to D^{435} and H^{436} . Other absolutely conserved residues are seen in proteins bearing the PD005703 and PD461453 defined domains, and very often, these map adjacent to the residues noted above. To test the relevance of the PptA models and the significance of putative active-site residues, alanine substitution mutations for those residues modeled as being in the active site, along with certain conserved flanking residues, were constructed and introduced into a *pilV* null background. This background facilitated screening of the mutants for PptA function by virtue of PilE reactivity with the TEPC-15 MAb recognizing the PC epitope and the reduction in PilE mobility associated with phospho-form modification. As shown in Fig. 9, three of the nine *pptA* missense mutations (H^{379} , D^{435} , and H^{436}) resulted in the *pptA* null phenotypes seen as loss of reactivity with the TEPC-15 MAb and increased PilE mobility in SDS-PAGE. MS analysis of intact PilE confirmed the absence of PC and PE modified forms (see Fig. S2 in the supplemental material and data not shown). In contrast, mutation of neighboring residues (G^{377} , S^{378} , and S^{434}) had no observed effect on these PptA phenotypes. Mutants with alanine substitutions at either S^{241} or H^{374} retained TEPC-15 MAb reactivity and retarded PilE migration (Fig. 9). However, MS analyses of intact pilin from these two backgrounds were consistent with phospho-form hypomodification and showed pilin subunits exclusively modified with a single PC moiety (see Fig. S2 in the supplemental material). Finally, deleting residue S^{241} resulted in a *pptA* null phenotype, as seen by MS analysis of intact pilin and reaction to TEPC-15 MAb, although expression of PptA was normal (Fig. 9 and data not

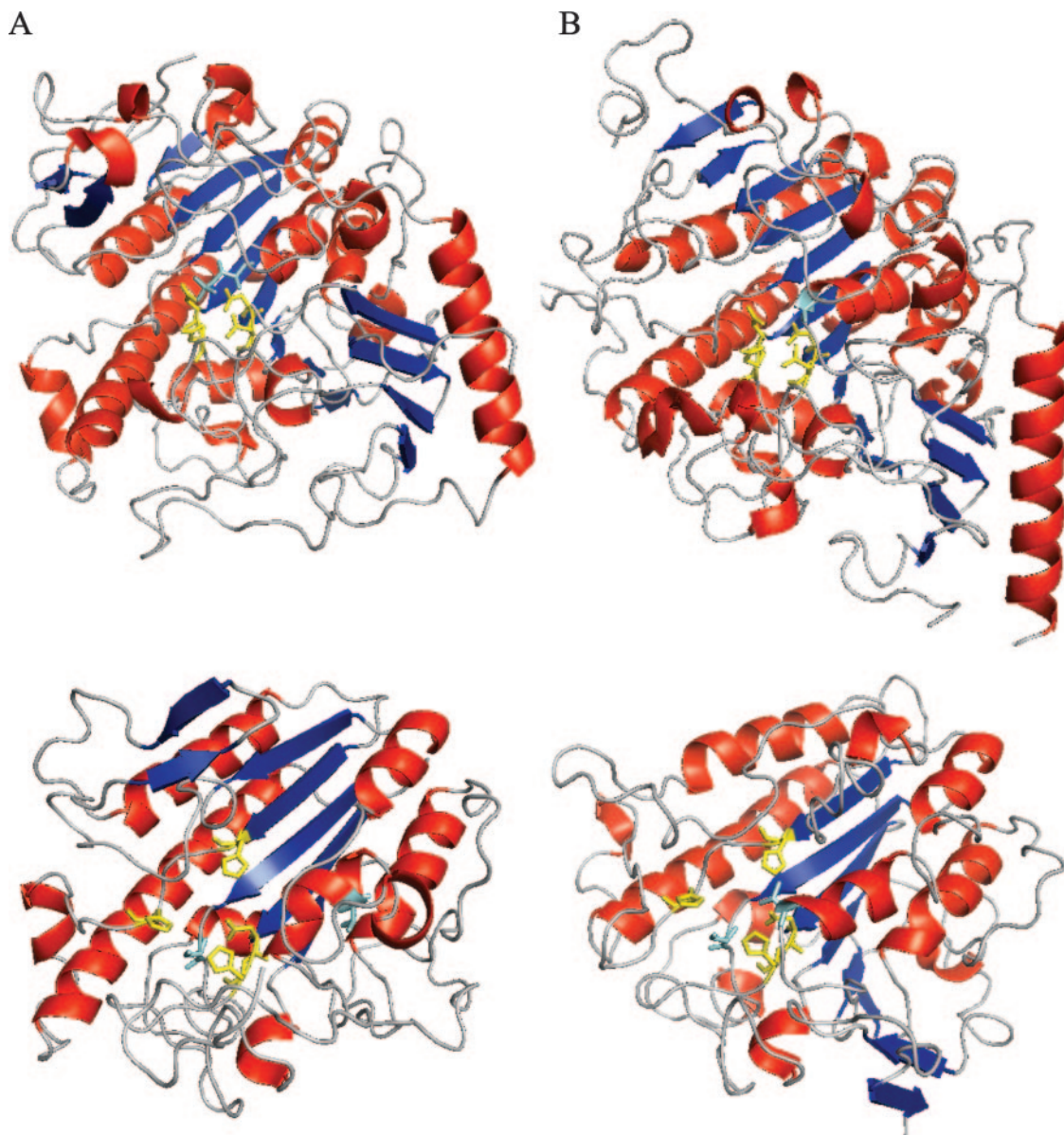


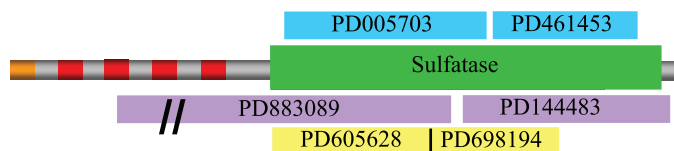
FIG. 7. Comparative modeling of the catalytic domain of PptA. (A) (Top) Crystallographic structure of human *N*-acetylgalactosamine-4-sulfatase (residues 43 to 533; Protein Data Bank [PDB] no. 1fsu). (Bottom) Three-dimensional structure of the sulfatase domain of PptA (residues 230 to 540) based on comparative modeling with 1fsu (sequence identity, 15%; E value, $1e-16$; model score, 1.00). (B) (Top) Crystallographic structure of human arylsulfatase A (residues 19 to 503; PDB no. 1auk). (Bottom) Three-dimensional structure of the sulfatase domain of PptA (residues 230 to 547) based on comparative modeling with 1auk (sequence identity, 14%; E-value, $6e-42$; model score, 0.90). Color coding of the protein backbone is as follows: red, α -helices; blue, β -strands; gray, loop regions. Color coding for residues is as follows: yellow, residues involved in metal ion coordination (human *N*-acetylgalactosamine-4-sulfatase, D⁵³, D⁵⁴, D³⁰⁰, and N³⁰¹; arylsulfatase A, D²⁹, D³⁰, D²⁸¹, and N²⁸²); cyan, active-site residues (human *N*-acetylgalactosamine-4-sulfatase, C⁹¹; arylsulfatase A, C⁶⁹). Yellow, analogous residues in PptA, i.e., putative residues involved in metal ion coordination (H³⁷⁴, H³⁷⁹, D⁴³⁵, and H⁴³⁶); cyan, putative active-site residues (S²⁴¹ and T²⁷⁸).

shown). The residues shown here to impact on PptA activity are illustrated in the context of the modeled active-site structures based on arylsulfatase A and *N*-acetylgalactosamine-4-sulfatase (see Fig. S3 in the supplemental material).

Potential implications for the Lpt6 subfamily of LPS PE transferases and MdoB. PE modifications at position 6 of inner core heptose II of LPS in *H. influenzae* and some strains of *N. meningitidis* involve a unique family of putative transferases termed Lpt6 (19, 70). Despite their high degree of

identity to one another, *H. influenzae* and *N. meningitidis* Lpt6 were reported to have an extremely limited degree of relatedness to PptA and the other putative LPS PE transferases. This situation is mirrored in the fact that although the Lpt6 proteins share the sulfatase domain of the Pfam PF00884 family and analogous transmembrane domains of other LPS PE transferases, they lack the PD005703 and PD461453 defined domains (Fig. 8). A previous sequence comparison of the two Lpt6 proteins against Lpt3 and LptA aligned only 3 residues

A Graphical view



B Prodom family PD005703

PptA	239	GESARSYMNVYGFPLPDTFEL (12) STAHANNLSL	282	344	SAGLSRLL (17) LIVMLMGSH	379	
LptA	239	GETTRAAWGLNGYSRQTPELL (14) SCGTSIAHSL	284	357	LLTKFDEVL (07) VLILHTIGSH	383	
Lpt3	221	GESESAHLKLFYGERSTFEL (16) SAGFMIAVSL	268	346	LLPLFDKIN (06) FIVLHQKSH	370	
EptB	255	GETTRWDHMGIFGYERNTPKLI (12) SCDTAKKLSL	298	366	GKPVDDMLL (16) LILHLTKGSH	400	
PmrC	235	GETSRGDDFSLGGYPRDTNPERL (12) SCGTAAIAISV	286	352	DGECYDEVL (15) VIVLHTIGSH	385	
CptA	236	GETQRGRMSLYGYPRETTFEL (17) TSRPYLIEIL	292	360	SAREYDSNV (15) FIVLHLGSH	393	
Consensus GE[ST]x(9)Gx(26-37)T				Consensus Dx(21-49)HxxGxH			
Lpt6	217	MESLIDDPHCFDFAAAKIDTLK (16) TFGGATWKSE	264	342	PMGKNLWHI (27) FVYVLTMKSH	387	
MdoB	169	GESLERTYFDNEAFDILTPELG (15) PGTDXITAGM	215	300	DWGFYDITV (18) SLFTLTVDTSH	336	

C Prodom family PD461453

PptA	402	CYVSTIAQTKF (17) VYFSDHGLM (18) YGVVELV	464
LptA	417	TYDNTVLYVDQF (18) HYVSDHGES (20) THIPMV	483
Lpt3	392	KYDNTIHKTDQM (18) AYFSDHGQY (15) YLVVELV	451
EptB	433	SYDNSVTYVDHF (15) FYAADHGES (19) FRVEMM	493
PmrC	420	TYDNTVLYVDYI (19) VYLSDHGES (20) KHVPML	485
CptA	428	DYDNANLYNDYV (17) LYFSDHGEE (21) YTVVELV	492
Consensus Yx(7)Dx(21-24)YxxDHGx(22-26)P			
Lpt6	427	VESFDRY (10) GYFGDHQVP	459
MdoB	365	SCSQENI (18) VVSSDHLAM	398

FIG. 8. Alignment of sulfatase domains from the PptA/LPS PE transferase protein family. (A) Graphical overview of the domain structure of PptA. Color codes: orange, signal peptide (residues 1 to 33) with a hidden transmembrane region (residues 13 to 32); red, transmembrane regions (residues 42 to 61, 68 to 90, 122 to 144, and 156 to 175); green, sulfatase domain PF00884 (residues 230 to 516); blue, Prodom families PD005703 and PD461453; pink, Prodom families PD883089 and PD144483 (found in Lpt6); yellow, Prodom families PD605628 and PD698194 (found in MdoB). The domain structures are similar for all the proteins belonging to Prodom families PD005703 and PD461453. (B) Juxtaposed alignment of the sulfatase domain-containing proteins from Prodom family PD005703. Color codes: red, absolutely conserved residues; green, either S or T; blue, residues conserved only between characterized proteins. Lpt6 is part of the Prodom families PD883089 and PD144483, as indicated by the pink coloring of the residues. MdoB is part of the Prodom families PD605628 and PD698194, as indicated by the yellow coloring of the residues. (C) Alignment of characterized proteins from Prodom family PD461453. The color coding is the same as in panel B. The aligned proteins are as follows: PptA, Q9RMJ3 NEIGO; LptA, Q7DD94 NEIMB; Lpt3, Q9JXJ7 NEIMB; EptB, Q38J80 ECOLI (formerly YhjW); PmrC, YjdB SALTY; CptA, Q7CPC0 SALTY; Lpt6, Q9JWE8 NEIMA; MdoB, P39401 (OPGB) ECOLI.

common to all four (corresponding to Y⁴³², D⁴³⁵, and H⁴³⁶ of PptA) out of the total of 14 that are conserved in all proteins with the PD005703 and PD461453 defined domains (70). Another work concluded that Lpt6 “had no significant amino acid

homology with the Lpt3 family proteins” (19). Rather, Lpt6 proteins are characterized by the PD883089 and PD144483 entries that nonetheless map within their Pfam PF00884 domains. By juxtaposing the PD005703 and PD883089 domains in reference to the common PF00884 sulfatase domain, it becomes obvious that the Lpt6 proteins have well-conserved, similarly positioned residues corresponding to those identified in the PptA/LPS PE transferases (Fig. 8). For example, 33 of 34 proteins possessing the PD144483 domain have a histidine residue juxtaposable to PptA H³⁷⁹, and all 34 have the DH residues (equivalent to PptA D⁴³⁵ H⁴³⁶) 60 to 70 residues distal to it. Parenthetically, it is intriguing to note that over half of the PD144483 domain-bearing proteins are from gram-positive organisms. This finding may give some insight into the evolutionary origins of Lpt6 PE transferases.

The same analysis used for Lpt6 was applied to MdoB, a member of the periplasmically localized phosphoglycerol transferase family that catalyzes the addition of phosphoglycerol to membrane-derived oligosaccharides from phosphatidylglycerol (17). This protein and its orthologues also possess the Pfam PF00884 domain but are uniquely defined by the

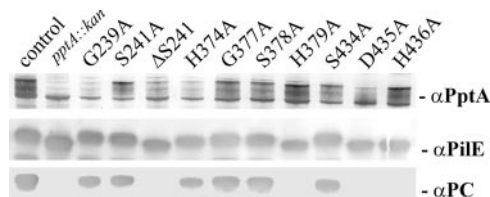


FIG. 9. PptA activities in structure-based, site-directed PptA mutants. (Top) Immunoblot of whole-cell lysates using polyclonal antibodies specific for PptA. (Middle) Polyclonal antibodies specific for PilE. (Bottom) MAB TEPC-15 specific for PC. Lanes: control, *pilV*(Fs) (GV1); *pptA::kan*, *pilV*(Fs) *pptA::kan* (KS10); G239A, G239A (KS18); S241A, S241A (KS16); ΔS241, ΔS241 (KS17); H374A, H374A (KS19); G377A, G377A (KS20); S378A, S378A (KS21); H379A, H379A (KS22); S434A, S434A (KS23); D435A, D435A (KS24); H436A, H436A (KS25). As controls for all mutants, immunoblotting was used to ensure that reduced or absent phospho-form modification did not result from decreased PptA stability.

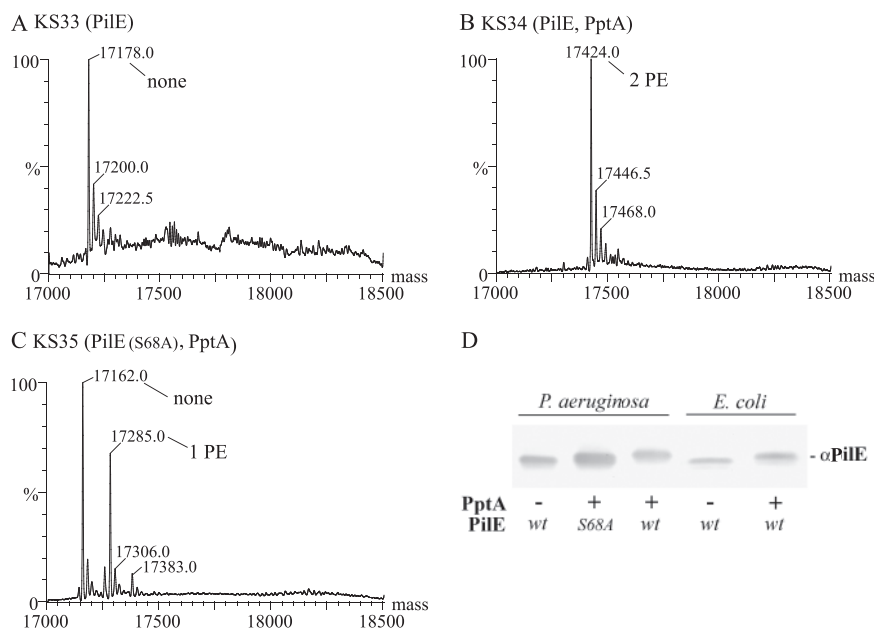


FIG. 10. PptA-mediated PilE modification in *P. aeruginosa* and *E. coli*. (A) MS analysis of intact PilE expressed in *P. aeruginosa*. (B) MS analysis of intact PilE coexpressed with PptA in *P. aeruginosa*. (C) Intact mass analysis of a PilE(S68A) mutant coexpressed with PptA in *P. aeruginosa*. (D) Immunoblot of whole-cell lysates using polyclonal antibodies specific for PilE. Lanes 1 to 3 (from left), *P. aeruginosa pilA pilT* expressing *N. gonorrhoeae* proteins: lane 1, PilE; lane 2, PilE(S68A); lane 3, PilE and PptA. Lanes 4 and 5, *E. coli* DH5 α expressing the proteins: lane 4, PilE; lane 5, PilE and PptA. Peaks in the MS spectra that are not labeled are listed in Table S3 in the supplemental material.

PD605628 and PD698194 domains. By juxtaposing these domains in reference to the shared sulfatase domain, MdoB and related proteins have conserved, similarly positioned residues corresponding to those identified in the PptA/LPS PE transferases. Here, 29 of 33 proteins possessing the PD698194 domain have a histidine residue juxtaposable to PptA H³⁷⁹, while 13 of 33 have the DH residues (equivalent to PptA D⁴³⁵ H⁴³⁶) 60 to 70 residues distal to it. The latter value is misleadingly low due to the fact that the DH signature is at the very end of the domain, and manual inspection shows that over half of the PD698194 domain-bearing proteins actually have similarly positioned DH residues. The striking conservation of putative active-site residues for all these likely phospholipid head group transferases strongly suggests that each involves the same reaction mechanisms defined for better-characterized members of the phospho-/sulfo-coordinating metalloenzymes.

PilE phospho-form modification in heterologous host species. As another approach to probing the relationship between PE and PC PilE modifications, we expressed both *N. gonorrhoeae* PptA and PilE in *P. aeruginosa*. Unlike the case in pathogenic *Neisseria* species, phosphatidylcholine is an established phospholipid constituent in *P. aeruginosa*, with a well-characterized phosphatidylcholine synthase biosynthetic pathway (63). Here, the relaxed specificity of the *P. aeruginosa* pilus assembly machinery made it possible to carry out MS analyses of intact PilE from purified Tfp. In the presence of PptA, the sole species observed in the mass spectrum of intact PilE corresponded to the mature protein modified with two PE moieties, while in its absence, a single signal reflecting unmodified PilE was found (Fig. 10A and B). Coexpression of PptA and PilE with an alanine substitution at S⁶⁸ showed two signals

corresponding to unmodified PilE and PilE with a single PE attached (Fig. 10C). Save for the absence of glycan-related species, these findings were remarkably similar to those seen for this altered PilE derived from *N. gonorrhoeae*. Moreover, given the stoichiometry of PE site occupancy in the wt and S⁶⁸ mutant PilE evidenced by the signals seen from *P. aeruginosa*-derived samples, the heterologous system recapitulates the hierarchical nature of PE modification seen in *N. gonorrhoeae*, with modification at S⁶⁸ significantly increasing the likelihood that a second site will be modified (1). The hierarchical nature of PilE PE modification is thus an intrinsic property of the substrate and enzyme.

To confirm that the PilE species seen in the top-down MS analysis reflected the major forms expressed in *P. aeruginosa*, immunoblotting of whole-cell lysates was performed. As seen in Fig. 10D, migration of wt PilE expressed in conjunction with PptA was clearly retarded relative to its unmodified form, while the mobility of the S⁶⁸ mutant PilE was intermediate between those of unmodified and modified forms of wt PilE. The mobilities of wt PilE coexpressed with and without PptA in *E. coli* were also examined by immunoblotting, and again, a significant PptA-dependent reduction in mobility was seen. Finally, these samples were also probed with the PC epitope recognizing TEPC-15 MAb with negative results (data not shown). Thus, PptA alone is sufficient for pilin modification with PE in *P. aeruginosa* and appears to have a similar activity when expressed in *E. coli*. However, there was no evidence for PilE PC modification in *P. aeruginosa* under the same conditions, despite the availability of the potential precursor phosphatidylcholine, as well as functional pathways for phosphatidylcholine biosynthesis.

DISCUSSION

Accumulating evidence suggests that the decoration of microbial surfaces with PE and PC moieties is a surprisingly common and important factor in host-parasite interactions. However, the biosynthetic pathways and enzymes that generate the modified PE and PC molecules presented on microbial surfaces are poorly understood. In this study, we addressed several outstanding questions regarding PptA and pilin phospho-form modification biology. Lacking *in vitro* assays for pilin phospho-form modification activity, we focused on genetic characterization of *pptA* as a means to probe the relationships between PptA expression, structure, and function, as well as the mechanisms by which other factors impact the system. In addition to confirming the role of PptA in pilin phospho-form modification, phenotypes associated with transposon mutations mapping upstream and in its ORF show that *pptA* expression is tightly coupled to that of *murF* and that MurF is undoubtedly an essential protein. Taken together with the low levels of *pptA* mRNA and PptA activity imparted by an ectopic allele containing 420 bp 5' of the ORF, *murE*, *pptA*, and *murF* are likely part of a single operon whose promoter precedes *mraZ* (28). This finding is interesting because *pptA* expression in *N. meningitidis* has been shown to undergo phase variation due to frameshifting within a monotonous stretch of nucleotides (58). Such a *pptA* regulatory system is one of the few, if not the only, genetic control mechanisms compatible with its polycistronic organization that would not disturb distal *murF* expression. Based on the unperturbed *murF* expression seen for the *pptA::kan* allele, this background was used to show a dose-dependent relationship between PptA and pilin phospho-form modification and full stoichiometric restoration of phospho-form site occupancy. It is important to note here that although PC substitution may be an indirect readout of PptA activity (see below), assays to detect the PC epitope are more sensitive and quantitative for monitoring pilin modification at low to moderate PptA levels than MS detection of the PE substituent.

Galperin et al. and Galperin and Jedrzejas (11, 12), first noted the pattern of sequence similarities between phosphotransferases, cofactor-independent phosphoglycerate mutases, alkaline phosphatases, and sulfatases defining the alkaline phosphatase superfamily. Encompassed within the catalytic core structures for each are similarly located serine and threonine (and cysteine in sulfatases) residues shown to form the covalent intermediates and residues implicated as metal-binding and coordinating sites. Included in that early analysis were EptB, PmrC, CptA, and orthologous proteins from *H. influenzae*, but at that time, their functions were not known. The relevance of those findings to establishing structural and catalytic similarities between PptA and the other alkaline phosphatase superfamily members was first demonstrated here by molecular modeling, with high-quality models derived using human lysosomal sulfatases predicting a striking colocalization of putative active-site and metal-coordinating residues. The significance of these findings was further validated by mutagenesis with individual substitution mutations of three residues implicated in metal coordination abolishing PptA activity, whereas that of a fourth such residue diminished activity. Alanine substitution at a highly conserved serine potentially cor-

responding to the catalytic residue (S²⁴¹) reduced PptA activity. While this result might imply that S²⁴¹ is not the site of formation of a covalent intermediate, the loss of the phosphorylatable hydroxyl group nucleophile might be compensated for by a water molecule, as shown for catalytic residue S¹⁰² substitution mutants of AlkP (48). The idea that active-site architecture (together with an appropriate nucleophile source) might contribute to catalysis is further attested to by the complete lack of activity seen for the S^{A241} mutant. Nonetheless, at this point, we cannot rule out the importance of a highly conserved T²⁷⁸ as an active-site residue. Potentially highly conserved residues synonymous with those implicated in PptA catalysis are readily identifiable within the Prodom PD005703 and PD461453 domains defining the LPS PE transferase family members, the Prodom PD883089 and PD144483 domains in the Lpt6 LPS PE transferase family members, and even the Prodom PD605628 and PD698194 domains defining MdoB phosphoglycerol transferase family members. Accordingly, the conserved structure and modular organization of these enzymes clearly attest to common sites of action and conserved reaction mechanisms.

The bulk of evidence supports the hypothesis that PptA is a PE transferase that utilizes the phospholipid head group of phosphatidylethanolamine as a donor. To date however, utilization of phosphatidylethanolamine as a precursor for PE has been demonstrated only for EptB (35), *N. gonorrhoeae* Lpt3 (31), and modifications of membrane-derived oligosaccharides (17). The enzyme responsible for the last activity is unknown, although the enzyme MdoB (which catalyzes the addition of phosphoglycerol derived from phosphatidylglycerol to membrane-derived oligosaccharides) has been ruled out (29). Assuming phosphatidylethanolamine is the precursor for PptA-mediated modifications, the question of what the precursor for PC is remains a perplexing problem. We previously proposed two plausible scenarios, one in which PptA might utilize phosphatidylcholine as a head group donor and another in which PptA would decorate PilE with PE that would be modified *in situ* into PC by the action of PE methyltransferase(s) (1). The phosphatidylcholine donor model is challenged by a number of observations. First, the presence of phosphatidylcholine in *N. gonorrhoeae* remains unsubstantiated. Second, the activities and active-site architecture of alkaline phosphatase superfamily enzymes are quite specific, and the ability of PptA to accommodate PE, as well as PC, with its additional trimethylammonium group, would likely require a high degree of relaxed specificity. Third, PC modification is not seen, even in the phosphatidylcholine possessing *P. aeruginosa* background despite high levels of PptA-mediated PE site occupancy. Finally, well-characterized structural modules and domains implicated in PC recognition or binding, such as those seen in PC esterase (15), CRP (54), phospholipase C (27), and pneumococcal choline binding proteins (8), are not readily recognizable in PptA (data not shown). An alternative model invoking methylation of PE bound to pilin is also problematic, as this would undoubtedly have to occur in the periplasm and the methyl donor S-adenosylmethionine is unlikely to be available at this site. Nonetheless, we have targeted potential *N. gonorrhoeae* PE methyltransferase homologs identified using the criteria detailed previously (46) for mutagenesis. However, the results are equivocal, since in some cases it has not been possible to

recover null mutants, while in those cases where knockouts were viable, PC modification was not altered. Further evaluation of the alternative models would be dramatically facilitated by high-resolution structural analysis of the PptA sulfatase domain. Likewise, the availability of the PE pilin modification system in *P. aeruginosa* and *E. coli* should provide unique opportunities for both biochemical and genetic screens for PC-generating activities.

The relationships between PE and PC modifications are further complicated by the influence of the PilV pilin-like protein. Both PE hypermodification and PC modification are associated with *pilV* null backgrounds, and conversely, phospho-form hypomodification has been demonstrated in *pilV*-overexpressing backgrounds. However, levels of *pptA* mRNA are not discernibly elevated in a *pilV* null background, and overexpression of *pptA* does not in and of itself lead to PC modification in either *N. gonorrhoeae* or *P. aeruginosa*. Conversely, PC modification is seen even at low levels of *pptA* expression, provided that PilV is not present. Therefore, PilV does not impact on the system by modulating levels of PptA but rather may influence PptA activity or PC donor availability. It is interesting to note here that there is evidence in related periplasmic, phospholipid head group transferase systems that enzyme activity can be dramatically influenced by environmental signals. For example, the activity of *E. coli* EptB requires elevated levels of Ca^{2+} in a heptose-deficient mutant (35), PE modification of *N. meningitidis* lipid A by LptA is reported to be significantly influenced by growth conditions (6), and the activity of MdoB phosphoglycerol transferase is regulated directly by osmolarity (20). Even in the case of the seemingly unrelated system of *lic1*-mediated PC modification of LPS in *H. influenzae*, it has been suggested that levels of PC modification are influenced by growth conditions in a manner that cannot be solely accounted for by alterations in *lic1* mRNA levels (69). It is therefore not without precedent that PptA activity might be altered by environmental conditions, although how PilV might be involved here is open to speculation.

Despite the findings made here, a considerable number of facets of the systems remain to be explored. Is PptA dedicated solely to pilin modification, or are other proteins modified? This is a particularly daunting task, as protein PE modifications are only recognizable through MS analyses. Likewise, do other members of the PptA/LPS PE transferase family possess protein-modifying activity? And what structural features, domains, and residues dictate the seemingly exclusive propensities for most family members to target LPS while PptA modifies pilin? Finally, there remains a serious gap in our knowledge of the enzymes directly engaged in PC modification of molecules elaborated on bacterial cell surfaces. In the case of PC modifications of LPS and teichoic acid associated with *lic* genes, the role of LicD as a PC transferase is inferred from genetic data indicating that *licD* allele status influences the sites of PC modifications in the target moieties (59, 71). Furthermore, the enzymes involved in the incorporation of PC into certain pneumococcal capsular polysaccharides are undefined (47). Formally, then, the enzymes that carry out the committed step of PC modification have yet to be unambiguously identified in any of these systems.

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