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Duplicate Copies of *lic1* Direct the Addition of Multiple Phosphocholine Residues in the Lipopolysaccharide of *Haemophilus influenzae*▽

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The genes of the *lic1* operon (*lic1A* to *lic1D*) are responsible for incorporation of phosphocholine (PCho) into the lipopolysaccharide (LPS) of *Haemophilus influenzae*. PCho plays a multifaceted role in the commensal and pathogenic lifestyles of a range of mucosal pathogens, including *H. influenzae*. Structural studies of the LPS of nontypeable *H. influenzae* (NTHI) have revealed that PCho can be linked to a hexose on any one of the oligosaccharide chain extensions from the conserved inner core triheptosyl backbone. In a collection of NTHI strains we found several strains in which there were two distinct but variant *lic1D* DNA sequences, genes predicted to encode the transferase responsible for directing the addition of PCho to LPS. The same isolates were also found to express concomitantly two PCho residues at distinct positions in their LPS. In one such NTHI isolate, isolate 1158, structural analysis of LPS from *lic1* mutants confirmed that each of the two copies of *lic1D* directs the addition of PCho to a distinct location on the LPS. One position for PCho addition is a novel heptose, which is part of the oligosaccharide extension from the proximal heptose of the LPS inner core. Modification of the LPS by addition of two PCho residues resulted in increased binding of C-reactive protein and had consequential effects on the resistance of the organism to the killing effects of normal human serum compared to the effects of glycoforms containing one or no PCho. When bound, C-reactive protein leads to complement-mediated killing, indicating the potential biological significance of multiple PCho residues.

Phosphocholine (PCho) has been detected on the cell surface of a range of bacteria, predominantly bacteria residing in the human respiratory tract, such as *Haemophilus influenzae* (5, 25, 39). In *H. influenzae*, PCho is a substituent of the lipopolysaccharide (LPS) molecule (24, 25, 39), the main glycolipid on the bacterial cell surface that is a target for host immune responses and influences both the commensal and pathogenic behavior of the organism (38). *H. influenzae* LPS comprises a membrane-anchoring lipid A (endotoxin) linked to oligosaccharide chains that extend from the bacterial cell surface. The LPS of a number of different strains have been analyzed and have been shown to be composed of a common L-*glycero*-D-*manno*-heptose-containing inner-core trisaccharide unit attached to the lipid A via a phosphorylated 2-keto-3-deoxyoctulosonic acid (Kdo) residue (20, 23). Each of the heptose (Hep) residues can provide a point for addition of a hexose (Hex) residue, which in turn can lead to oligosaccharide chain extension (for a review, see reference 28). The nature of the oligosaccharide chains and the degree to which they contain noncarbohydrate substituents, such as PCho, show intra- and interstrain variation that can affect the virulence of the organism. PCho in *H. influenzae* LPS plays a role in persistence of the bacterium on the mucosal surface of the nasopharynx, at least in part by mediating bacterial adherence to, and invasion of, the host epithelia (38), which has been shown to occur through the receptor for platelet-activating factor on bronchial epithelial cells (30). PCho expressed on the LPS of the organism is the target for the serum component C-reactive protein (CRP), which, when bound, mediates killing of the bacteria via activation of complement (37, 38). PCho expression on the LPS of *H. influenzae* has also been associated with increased resistance to host antimicrobial peptide killing (18).

Several of the surface-exposed epitopes of *H. influenzae* LPS are subject to high-frequency phase variation, an adaptive mechanism that is advantageous for survival of bacteria confronted by the different microenvironments and immune responses of the host (22). Tetranucleotide repeat tracts in a number of LPS biosynthesis genes mediate this phase variation through slippage mechanisms (9, 11, 34, 36). The genes responsible for incorporation of PCho on the LPS of *H. influenzae* are the genes of the *lic1* operon, which consists of four open reading frames (*lic1A* to *lic1D*) (33). A 5′CAAT tetranucleotide repeat is located just within the 5′ end of the *lic1A* reading frame, making addition of PCho to the LPS subject to phase variation (34). Choline incorporation in *H. influenzae* occurs via the transport of choline from the host environment by Lic1B, phosphorylation of choline by Lic1A, activation of PCho via a pyrophosphorylase, Lic1C, and transfer of the activated PCho to the LPS by Lic1D (39). PCho is also present on the cell surface of a number of other major pathogens residing in the human respiratory tract, such as *Streptococcus pneumoniae* and *Neisseria meningitidis* (13, 21, 32), suggesting that

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TABLE 1. Primers used in this study for PCR amplification and DNA sequencing of pAD and for verification of *lic1* mutant strains and the *lic1* tandem duplication

Primer	Sequence	Open reading frame containing primer binding site	Orientation of primer binding site with respect to open reading frame
LIB1	5′TAACGCAGTATATTCAC3′	*lic1B*	Reverse
1158lic1DA	5′GGATCATTAAACCAGCCTATTGAC3′	*lic1D*	Forward
1158lic1DB	5′TTGTATTGTTTGAATTGTGGGAAG3′	*lic1D*	Reverse
1158lic1Dr	5′GAAGTTGCATATAGTCGCCGTA3′	*lic1D*	Reverse
lic1DA	5′GTGATGATATATTTGAAATG3′	*lic1D*	Forward
lic1Dr	5′TGGAAGCTTCATATAATCTCCATAA3′	*lic1D*	Reverse

this structure may contribute to the ability of these species to occupy their niche on the host mucosal surface. Phase-variable expression of PCho is also common in other bacteria (12, 29, 32, 35).

Structural studies of *H. influenzae* LPS have demonstrated that PCho can be linked to hexoses attached to any one of the three heptoses (19, 25, 27). Lysenko and colleagues have shown that the *lic1D* gene directs this addition and that the location of the PCho molecule can alter the susceptibility of the bacteria to CRP-mediated bacterial killing (17). In this study we found that the presence of two distinct PCho epitopes located in the same LPS molecule in a limited subset of nontypeable *H. influenzae* (NTHI) strains is due to the presence of two distinct copies of the *lic1* operon in these strains. Further, the *lic1D* sequence in each operon is responsible for directing PCho addition to unique sites in the LPS. One of the additions, in which PCho is added to a heptose that is part of the oligosaccharide extension from the proximal heptose of the LPS inner core, is novel.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Twenty-six NTHI isolates used in this study, designated isolates 162, 176, 285, 375, 432, 477, 486, 667, 723, 981, 1003, 1008, 1124, 1158, 1159, 1180, 1181, 1200, 1207, 1209, 1231, 1232, 1233, 1247, 1268, and 1292, were obtained from the Finnish Otitis Media Study Group. Isolates were obtained from the middle ear and were selected as organisms that are representative of the genetic diversity of *H. influenzae* after ribotyping analysis (2). Isolate 176, as described in this study, is distinct from that described in reference 27, which actually referes to isolate 1292. *H. influenzae* was grown at 37°C in brain heart infusion (BHI) broth supplemented with hemin (10 μg/ml) and NAD (2 μg/ml). BHI medium plates were prepared with 1% agar, and they were supplemented with 10% Levinthals base (1) and, when appropriate, tetracycline (4 μg/ml) or chloramphenicol (2 μg/ml). *Escherichia coli* strain DH5α was used to propagate plasmids and was grown at 37°C in LB broth (26) supplemented, when appropriate, with ampicillin (100 μg/ml), tetracycline (12 μg/ml), or chloramphenicol (30 μg/ml).

Cloning of *lic1 and mutation of *lic1D*.*** The novel *lic1* locus (*lic1**) of isolate 1158 was identified as a *lic1D*-hybridizing 1.9-kb band and a *lic1A*-hybridizing 2.9-kb band in MfeI-digested DNA by Southern analysis. The 1.9-kb fragment of *lic1** was included in a 1.5- to 2.3-kb size fraction of DNA extracted and purified from the agarose gel and then cloned into plasmid pUC18. *E. coli* transformants were colony blotted and hybridized with a *lic1D*-specific probe to identify plasmid pAD. The insert from the plasmid was confirmed to contain *lic1*-specific sequences by PCR and Southern analyses (Table 1). The 2.9-kb MfeI *lic1**-hybridizing fragment was similarly cloned from 2.5- to 3.5-kb size-fractionated chromosomal DNA to obtain plasmid pAE.

The cloned *lic1D** gene from NTHI isolate 1158, in plasmid pAD, was disrupted by replacement of a 663-bp EcoRV restriction fragment (spanning bp 46 to 709 in the 807-bp *lic1D** gene) with a blunt-ended chloramphenicol resistance cassette released from pACYC184 (14), resulting in plasmid pAD1cat1 (Fig. 1).

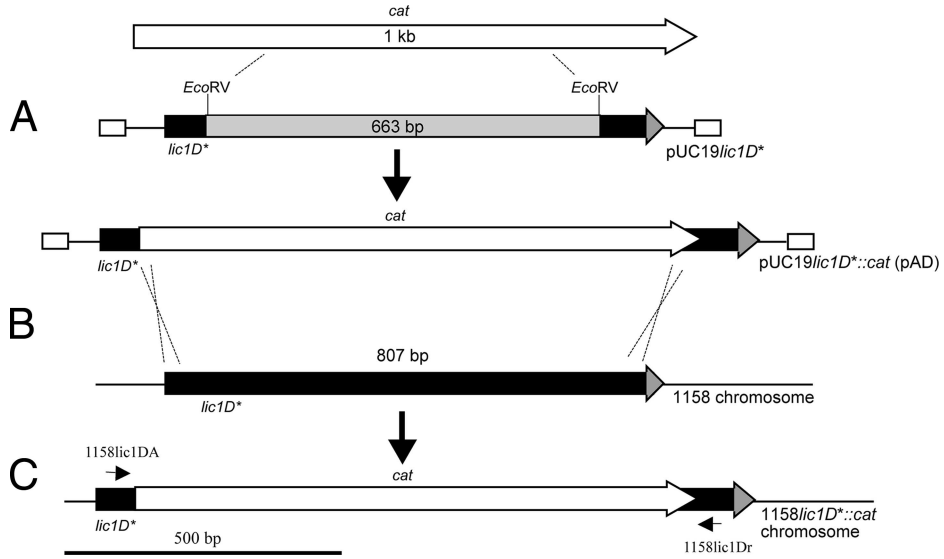

FIG. 1. Schematic representation of the construction of pUC19*lic1D**::*cat* and subsequent transformation into *H. influenzae*. (A) Insertion of the chloramphenicol resistance (*cat*) cassette into the *lic1D** open reading frame. (B) Transformation into NTHI isolate 1158. (C) Homologous recombination results in exchange of the plasmid insert with *lic1D* on the 1158 chromosome, producing 1158*lic1D**::*cat* mutants. The binding sites for primers used for screening transformants are shown in panel C.

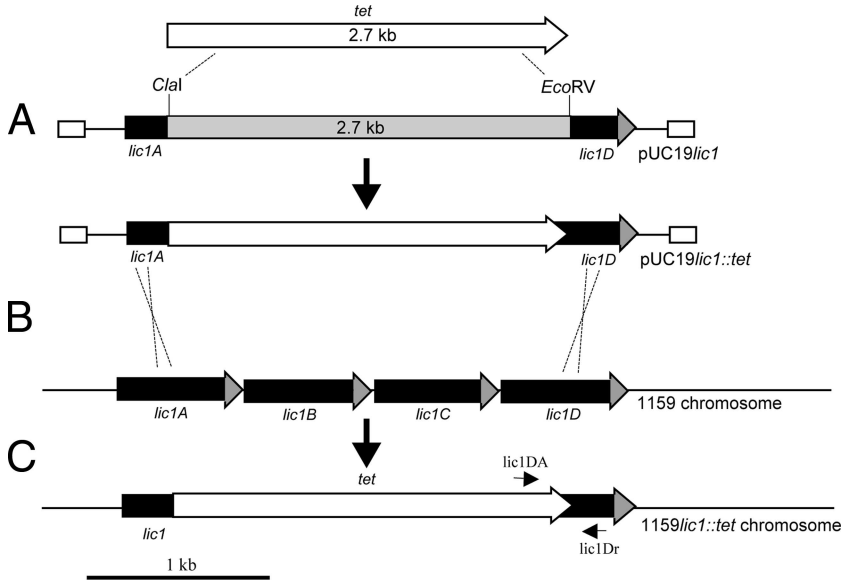

FIG. 2. Schematic representation of the construction of pUC19*lic1*::*tet* and subsequent transformation into *H. influenzae*, adapted from reference 40 with permission. (A) Insertion of the *tet* cassette into the *lic1* locus. (B) Transformation into NTHI isolate 1159. (C) Homologous recombination results in exchange of the plasmid insert with *lic1* on the 1159 chromosome, producing 1159*lic1*::*tet* mutants. The binding sites for primers used in screening transformants are shown in panel C.

After transformation into *E. coli*, mutant strains were confirmed by restriction and PCR analyses (Table 1).

Construction of *lic1* mutant strains of NTHI. *lic1* mutants strains of NTHI isolates 176, 1158, and 1159 were constructed by transformation using DNA in which the whole *lic1* operon was disrupted by deletion of part or all of *lic1A* through *lic1D* and replacement with a tetracycline resistance cassette (40) (Fig. 2). A mutant strain in which the *lic1D** gene had been specifically targeted and disrupted in NTHI isolate 1158 was constructed by transformation with linearized plasmid pAD1cat1 described above. All *H. influenzae* transformations were carried out using the MIV procedure (8). Transformants were confirmed by culturing on the appropriate selective media and by PCR analyses using primer sets specific for each copy of the *lic1D* gene (Tables 1 and 2).

Analysis of LPS by electrophoresis. Bacterial lysates were prepared from cells grown overnight on BHI medium plates and then suspended in phosphate-buffered saline (PBS) at an absorbance at 260 nm of 1. Lysates were analyzed by Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (16) and staining with silver (Quicksilver; Amersham Pharmacia).

Immunoblot analysis using MAbs TEPC15 and 12D9. A single *H. influenzae* colony was resuspended in PBS, serially diluted, and plated to obtain approximately 1×10^3 and 5×10^3 colonies per plate. Either single colonies or streaks of bacterial growth were transferred onto a Protran nitrocellulose filter (Schleicher & Schuell) and air dried. Nonspecific binding sites were blocked with PBS–0.05% (vol/vol) Tween 20 and 2% (wt/vol) bovine serum albumin. The filter was then incubated with monoclonal antibody (MAb) TEPC15 or 12D9. Primary antibody was detected with either an immunoglobulin G (IgG)-specific antibody for MAb 12D9 or an IgA-specific antibody for MAb TEPC15 conjugated to alkaline phosphatase. Bound antibody was detected with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium (NBT) (Perkin Elmer Life Sciences).

TABLE 2. PCR analysis of *lic1* mutants in NTHI isolates 176, 1158, and 1159, using primer sets specific for each copy of the *lic1D* gene

Strain	Presence of PCR product with primers[a]:	
	lic1DA and lic1Dr (*lic1* specific)	1158lic1DA and 1158lic1Dr (*lic1** specific)
176	+	+
176*lic1*	−	+
1158	+	+
1158*lic1*	+	−
1159*lic1*	−	+
1158*lic1D**	+	+ (1.1 kb)

[a] PCR amplification products were ~800 bp long unless indicated otherwise.

Western blot analysis of LPS using MAb TEPC15. Bacterial lysates were boiled in dissociation buffer and then separated by 16.5% Tricine-SDS-PAGE. After transfer from the gel to a nitrocellulose membrane (Schleicher & Schuell), membranes were blocked and then incubated with MAb TEPC15. Primary antibody was detected using an IgA antibody conjugated to alkaline phosphatase and developed using BCIP and NBT (Perkin Elmer Life Sciences).

Structural analysis of LPS. The structure of LPS was elucidated using procedures described previously (41). Briefly, reduced core oligosaccharide material was obtained after mild acid hydrolysis (1% aqueous acetic acid [pH 3.1] 100°C, 2 h) and simultaneous reduction of LPS following centrifugation and purification by gel chromatography. Oligosaccharide was dephosphorylated using 48% hydrogen fluoride and then was permethylated using methyl iodide in dimethyl sulfoxide in the presence of lithium methylsulfinylmethanide. O deacylation was performed using 1% aqueous ammonia at 20 to 22°C for 10 h. Liquid chromatography (LC)–multiple-step tandem electrospray ionization mass spectrometry (ESI-MS^n) in the positive-ion mode was performed with a Waters 2690 high-performance liquid chromatography system (Waters, Milford, MA) coupled to a Finnigan LCQ ion trap mass spectrometer (Finnigan-MAT, San Jose, CA) as described previously (7). Capillary electrophoresis (CE)-ESI-MS experiments were carried out with a Prince CE system (Prince Technologies, The Netherlands) coupled to either an API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) or a 4000 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) as described previously (41).

Binding of CRP to *H. influenzae* strains expressing different amounts of PC. *H. influenzae* strains were grown to an optical density at 620 nm of 0.4, washed in PBS, and resuspended in 2 volumes of normal human serum which had been heat inactivated by incubation at 56°C for 30 min, and 1 μg of purified human CRP (Sigma) per 10^8 cells was added. After incubation with agitation for 15 min at 37°C, the cells were washed in PBS and resuspended in gel loading buffer. Samples were boiled for 5 min and separated by 16% Tricine-SDS-PAGE (Invitrogen). After transfer from the gel to nitrocellulose membranes (Protran), the membranes were blocked, and then bound CRP was detected using an MAb against human CRP (Sigma). Primary antibody was detected using an IgG anti-mouse antibody conjugated to alkaline phosphatase and developed using BCIP and NBT (Perkin Elmer Life Sciences).

Serum bactericidal assay. Bacteria cultured on BHI medium plates were resuspended in PBS, and the concentration was adjusted to ~10^4 cells/ml. A total of 5×10^2 organisms were then added to doubling dilutions of pooled normal

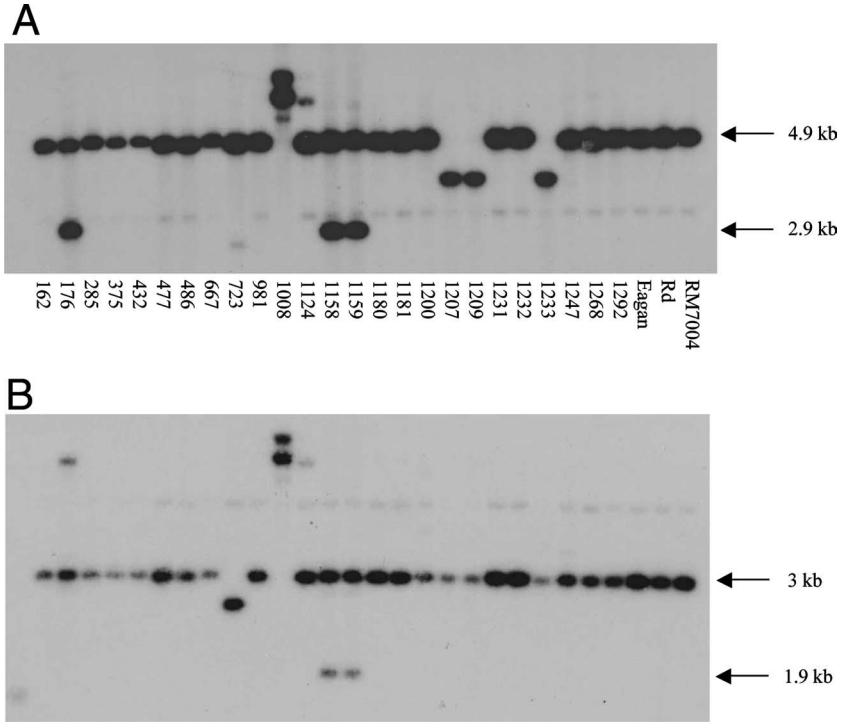

FIG. 3. Southern blot of MfeI-digested chromosomal DNA isolated from 25 NTHI isolates and then probed with DNA fragments of the *lic1* locus. The blot contained (from left to right) the 25 NTHI isolates in ascending numerical order and our control *H. influenzae* strains, Eagan, Rd, and RM7004. The probes used were *lic1A* (A) and *lic1D* (B). The positions of *lic1A*- and *lic1D*-hybridizing bands of interest are indicated by arrows.

human serum (0.15 to 10%) in PBS containing 0.1% glucose, as previously described (10). After 45 min of incubation at 37°C, the survival of bacteria at each serum concentration was determined by plating and culturing. CRP was removed from the pooled human serum using PCho-coated beads as described previously (17).

Adhesion to airway epithelial cells. The adherence of *H. influenzae* strains to cultured human bronchial epithelial cells (16HBE14o$^-$) was studied by using a method similar to a method described previously (10, 31).

RESULTS

Some strains of *H. influenzae* have duplicated sequences of the *lic1* operon. In a recent survey of tetranucleotide repeat tracts in *H. influenzae*, Southern blots of restriction endonuclease-digested DNA from 25 NTHI isolates were probed with 20-base oligonucleotides representing all possible tetranucleotide repeat sequences. Seven to 16 hybridizing bands were found for each isolate (4). Repeat tracts were investigated further by stripping the blots and reprobing them with sequences representing the various genes that are known to be associated with tetranucleotide repeats; in this way it was possible to identify novel candidate genes associated with repeats. In the case of the oligonucleotide probe $(5'CAAT)_5$, three previously reported LPS-related genes, *lic1A*, *lic2A*, and *lic3A*, were identified in each strain, but up to five $(5'CAAT)_5$-hybridizing bands were obtained for a minority of NTHI strains. An additional DNA band was identified for four NTHI isolates, isolates 176, 1008, 1158, and 1159, and this band cohybridized to a $(5'CAAT)_5$ repeat oligonucleotide and to separate probes containing *lic1A* and *lic1D* gene sequences (Fig. 3). Isolates 1158 and 1159 were chosen for closer analysis since the detailed structure of the LPS was available for these isolates and two PCho residues are present at distinct locations. Isolates 1158 and 1159 are believed to be independent isolates of the same strain (2).

Cloning of the duplicated *lic1* sequence shows that it is novel and differs from the original *lic1* gene. Two copies of *lic1*-specific sequences were identified in NTHI isolate 1158 by Southern hybridization. An MfeI digest of chromosomal DNA from NTHI isolate 1158 contained 4.9- and 2.9-kb fragments which hybridized to a $(5'CAAT)_5$ and *lic1A* probe; 3- and 1.9-kb fragments hybridized to a *lic1D* probe (Fig. 3). Southern analysis of MfeI-digested chromosomal DNA from strain Rd revealed a 4.9-kb band that hybridized to *lic1A* and a 3-kb band that hybridized to *lic1D*, consistent with fragment sizes predicted from the genome sequence. Our interpretation of this was that the 2.9-kb *lic1A*-hybridizing band and the 1.9-kb *lic1D*-hybridizing band observed with isolate 1158 likely corresponded to the second "non-Rd-like" *lic1* copy of interest. The second copy of *lic1* was designated *lic1**.

To investigate these sequences further, we cloned what was hypothesized to be the second, or duplicate, sequence. The 1.9-kb fragment of *lic1** was cloned in *E. coli* to obtain plasmid pAD. The DNA sequence of the cloned insert of pAD was obtained and compared to the sequence in the *H. influenzae* genome database using the BLAST algorithm (3). The cloned insert of pAD was found to contain sequences with homology to *lic1B*, *lic1C*, and *lic1D* (HI1538 to HI1540, respectively) and, unexpectedly, to *sppA* (HI1541), the gene adjacent to *lic1D* in the strain Rd genome sequence. From these sequence comparisons two possible conclusions could be drawn: either the copy of *lic1* cloned from 1158 was equivalent to the copy found

TABLE 3. Primers used in this study for PCR amplification of genes flanking *lic1*, which were used as probes in Southern analysis

Gene amplified	Primer	Sequence
HI1535	5′HI1535	5′TTCGTTTCACGAAACCCAAT3′
	3′HI1535	5′TATCCACATCTTGCGTCCAA3′
HI1536	5′HI1536	5′ATCTCGCTCGTGTAGGCATT3′
	3′HI1536	5′TACCCAAGCTCGGTTAGCTG3′
sppA	sppA1	5′GTGCATGCAGATGGTGTTTC3′
	3′sppA	5′GGCAAACCAAGCCATTTAAC3′
HI1542	5′HI1542	5′TGCACCTAATGCAGAACAGC3′
	3′HI1542	5′CGATAACTCGGCATTCACG3′
HI1543	5′HI1543	5′TCCAACAGATAAAGTACTCGCTGA3′
	3′HI1543	5′GCCAATATTTCTCGTGTAAGAAGG3′

in strain Rd due to an "Rd-like" gene arrangement, or it represented a second copy in which the duplication extended further than the *lic1* operon itself. To further examine the DNA flanking this copy of the *lic1* operon, the Southern blot of MfeI-digested chromosomal DNA was hybridized using a 3′ segment of the *sppA* gene as a probe. This probe hybridized to both *lic1D* bands in DNA from isolates 1158 and 1159. Since a segment of *sppA* appeared to be associated with both copies of *lic1* in isolate 1158, adjacent genes both upstream and downstream of *lic1* were used as hybridization probes (Table 3) to examine the extent of the duplication.

First, a 5′ segment of the *sppA* gene (HI1541) was used as a probe. There is an MfeI site in this region of DNA in the Rd genome sequence, so two hybridizing bands would be expected for strain Rd. For isolates 1158 and 1159, four hybridizing bands were seen, confirming that the *sppA* gene is duplicated in these isolates. The next gene downstream of *sppA* in Rd, HI1542, was also used as a hybridization probe, but the results were inconclusive. Hybridization with the HI1543 gene defined the 3′ end of the duplication, giving a single hybridizing band for the isolates. The gene immediately upstream of *lic1* in the Rd genome sequence, HI1536, was found to be duplicated in NTHI isolates 1158 and 1159; however, the HI1535 gene was not duplicated, thus defining the 5′ end of the duplication. To locate *lic1** within the genome, the 2.9-kb band from the MfeI digest of isolate 1158 DNA, predicted to contain the second copy of *lic1A** (HI1537), was cloned and sequenced, and the sequence was used to search the *H. influenzae* strain Rd genome database. The sequence of the insert contained DNA with homology to *sppA* (HI1541) at one end and to *lic1B* (HI1538) at the other end, indicating that there is a tandem duplication of the *lic1* locus in isolate 1158 (Fig. 4B). Further confirmation of the presence of a tandem duplication of the *lic1* locus came from a PCR analysis using a *lic1D* forward primer (1158lic1DA) and a *lic1B* reverse primer (L1B1) (Fig. 4). The results revealed a 4-kb amplification product in isolates 1158 and 1159.

Duplicated *lic1* sequence is different from the sequence of the original *lic1* locus. The two Lic1D sequences from NTHI isolate 1158 were compared (Fig. 5) and found to be 78.9% similar and 74.1% identical. The sequence of *lic1D** is divergent from any *lic1D* sequence described in a survey of the same 25 NTHI isolates by multilocus sequence typing of a number of LPS biosynthetic genes (2). When 20 Lic1D sequences were aligned, three major subgroups which correlated with the hexose extension to which PCho addition is directed were identified (i.e., off HepI, HepII, and HepIII) (data not shown). Preliminary structural analysis of NTHI isolate 1158 LPS indicated the presence of two PCho residues, one linked directly to the hexose off HepIII and the other in a unique location linked to the Hep (designated HepIV) as part of the oligosaccharide extension from HepI. The sequence of Lic1D* likely constitutes a fourth subgroup of sequences in which the addition of PCho is addition to a HepIV residue. The Lic1D* sequence contains two amino acid residues that differ from the residues in any of the other Lic1D sequences analyzed. Both of these residues are located in the EcoRV fragment of DNA used by Lysenko and colleagues (17) in transformation experiments that resulted in a change in the *lic1D* sequence and an altered location of PCho in the LPS. The Thr-95 and His-144 residues in Lic1D* are different from the conserved Ala-95 and Arg-144 residues at the equivalent positions in all other Lic1D sequences analyzed. The sequence of Lic1D from isolate 1158 aligns with the HepIII-like sequences (data not shown), consistent with the hypothesis that Lic1D is responsible for addition of PCho to the hexose attached to HepIII.

The second copy of *lic1* was also investigated in NTHI isolates 176 and 1008. PCR amplification with primers 1158lic1DA and L1B1 resulted in a 1.8-kb product with isolate 1008 (data not shown). HI1541 (*sppA*) was found by Southern analysis not to be duplicated in this isolate, and the size of the PCR product likely indicates that the *lic1** operon is also truncated. This would be consistent with the fact that two

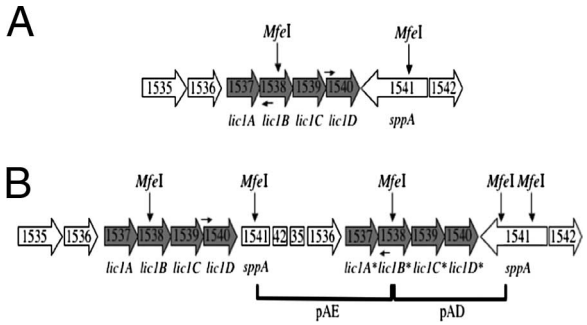

FIG. 4. Model of the organization of the *lic1* tandem duplication in NTHI isolate 1158. (A) Schematic representation of the *lic1* operon and surrounding genes in the strain Rd genome. (B) Organization of the *lic1* duplication in NTHI isolate 1158. Block arrows represent ORFs, and the direction of an arrow indicates the relative direction of transcription. The *lic1* operon is represented by shaded block arrows. TIGR accession numbers are indicated in the arrows, and gene designations are indicated below the arrows. Rectangles in panel B represent partial open reading frames compared to the strain Rd genome sequence (*sppA* had a 147-bp truncation at the 5′ end, only 37 bp at the 5′ end of HI1542 was present, and only 85 bp at the 3′ end of HI1535 was present in the region between the two *lic1* operons in the genome of NTHI isolate 1158). The small horizontal arrows indicate the positions and directions of primers L1B1 (within *lic1B*) and 1158lic1DA (within *lic1D*). The positions of MfeI restriction sites are indicated by vertical arrows. In panel B the region of DNA corresponding to the cloned insert within plasmids pAD and pAE is indicated.

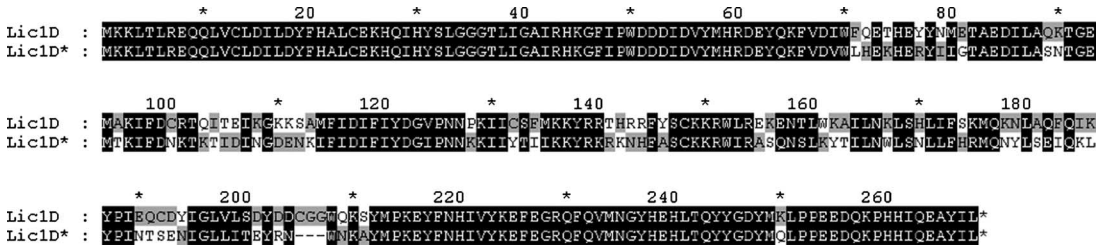

FIG. 5. Alignment of amino acid sequences of Lic1D (A. Cody, personal communication) and Lic1D* in NTHI isolate 1158. Sequence homology is indicated by a black background.

PCho residues were not observed in a structural analysis of the LPS from this isolate (unpublished results). In NTHI isolate 176, in which two PCho residues have been identified in the LPS (E. Schweda, unpublished data), no PCR amplification product was obtained with primers 1158lic1DA and L1B1. This may indicate that the *lic1* duplication is not in a tandem arrangement in the genome of this isolate, that the duplication is incomplete, or that the sequence of the second copy of *lic1D* is different from that of isolate 1158. This heterogeneity is interesting as it suggests that the duplication event may have occurred independently in different isolates or may reflect the instability of this region of DNA once the duplication has occurred.

Analyses of the phenotype and antigenicity of *lic1* mutants show that the duplicated sequences have different properties. Mutant strains of NTHI isolates 1158, 1159, and 176 were constructed using DNA in which the whole *lic1* operon was disrupted by deletion of part or all of the *lic1A*, *lic1B*, *lic1C*, and *lic1D* open reading frames and replacement with a tetracycline resistance cassette (40) (Fig. 2). These mutant strains were investigated by PCR amplification using primers designed specifically to amplify one of the *lic1D* genes (primers lic1DA and lic1Dr to amplify *lic1D* and primers 1158lic1DA and 1158lic1Dr to amplify *lic1D**). The copy of *lic1* mutated and containing the antibiotic resistance cassette was indicated by the absence of a PCR product with the corresponding specific primer set, since the cassette replaced a fragment of the operon including the lic1DA or 1158lic1DA primer binding sites. For each mutant strain only one copy of the *lic1* locus was disrupted. In strains 176*lic1* and 1159*lic1*, *lic1* was disrupted, whereas in strain 1158*lic1*, *lic1** was disrupted (Table 2). Specific targeted disruption of the *lic1D** gene was achieved by replacement of a 663-bp EcoRV restriction fragment in *lic1D** in plasmid pAD with a chloramphenicol resistance cassette (Fig. 1). Construct pAD1cat1 was used to transform NTHI isolate 1158 to obtain transformant 1158*lic1D**. This mutant strain was also confirmed by PCR amplification using the primer sets described above. Each primer set amplified an 800-bp internal fragment of the corresponding *lic1D* gene. With transformant 1158*lic1D** a ~300-bp increase, due to loss of an internal fragment and insertion of the antibiotic resistance cassette, was observed with *lic1**-specific primers, indicating that *lic1D** was disrupted (Table 2).

Bacterial lysates prepared from parental NTHI isolates and the corresponding *lic1* mutants were separated by Tricine-SDS-PAGE, and representative LPS profiles are shown in Fig. 6A. A change in the LPS glycoforms, indicated by the number of bands observed, was evident for wild-type isolates 1158 and 1159, each of which produced three major bands, for 1158*lic1** and 1158*lic1D**, which produced four bands, and for 1159*lic1*, which produced five distinct bands.

The set of *lic1* mutants and their parent strains were compared using immunoblot assays with LPS-specific MAbs 12D9 and TEPC15. MAb TEPC15 specifically recognizes PCho irrespective of its linkage and position, whereas MAb 12D9 discriminates based on the location of PCho within the LPS molecule (17). NTHI isolates 1158, 1159, and 1158*lic1* all reacted with MAbs TEPC15 and 12D9, whereas mutant strain 1159*lic1* did not react with either MAb (data not shown). The loss of PCho expression in mutant strain 1159*lic1* was not due to phase variation since the phenotype was irreversible (data not shown). Instead, it was found to be due to deletion of 222 bp in the *lic1C** gene of NTHI isolate 1159 compared to the Rd *lic1* sequence. The *lic1C* open reading frame is 699 bp long, and the deletion in *lic1C** spans the sequence from bp 455 to 677. Thus, the apparent irreversible loss of PCho expression observed in mutant strain 1159*lic1* was likely due to the lack of a functional Lic1C gene product in this strain.

Western blot analysis of the same set of *lic1* mutants indicated that MAb TEPC15 reacted with all LPS bands in isolates 1158 and 1159 and in mutant strains 1158*lic1** and 1158*lic1D**, indicating that PCho is expressed on the majority of glycoforms. The mobility differences in the banding patterns observed for these strains is consistent with those expected due to differences in the extent of modification with PCho. MAb TEPC15 did not react with strain Rd*lic1* or 1159*lic1* (Fig. 6B), confirming the findings of the immunoblot analysis.

Structural analysis of NTHI 1158 and 1159 LPS. The structures of LPS glycoforms from NTHI strains 1158 and 1159 were determined by nuclear magnetic resonance and MS, and full details will be reported separately. LPS from NTHI isolate 1158 was subjected to mild acid hydrolysis to obtain oligosaccharide material. Part of this oligosaccharide material was dephosphorylated, permethylated, and analyzed by LC-ESI-MS^n (7). The dominant ion in the LC-ESI-MS^n spectrum at *m/z* 1716 corresponded to the sodiated adduct of a glycoform with the composition $Hex_2 \cdot Hep_4 \cdot$ anhydro-Kdo-ol (AnKdo-ol). The sequence of this glycoform was established by performing MS-MS (MS^2) and MS^3 experiments, and the results confirmed the carbohydrate backbone of oligosaccharide shown in Fig. 7. CE-ESI-MS of the oligosaccharide sample revealed predominant $Hex_2 \cdot Hep_4$ glycoforms in which the heterogeneity was due to the degree of acetylation, glycylation, and/or phosphorylation. All glycoforms comprised an AnKdo-ol formed during delipidation by β-elimi-

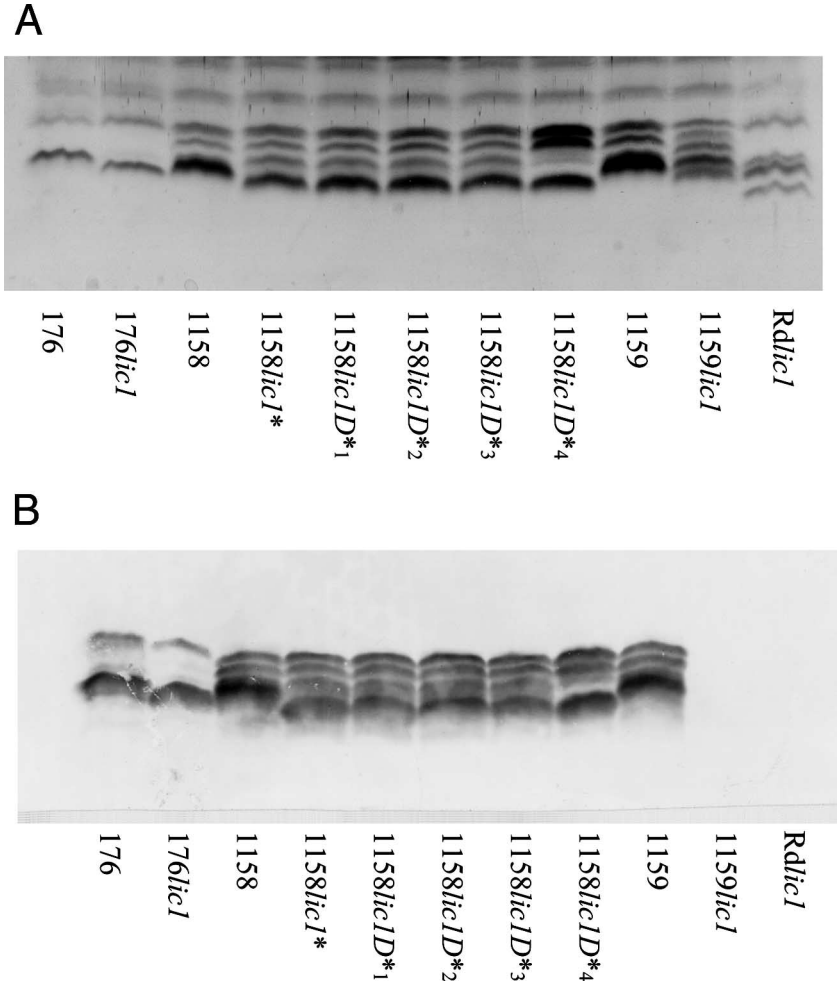

FIG. 6. Western blot analysis using MAb TEPC15 with LPS from NTHI isolates 176, 1158, and 1159 and the corresponding *lic1* mutant strains, 176*lic1*, 1158*lic1**, 1158*lic1D** (four independent mutants [1158*lic1D**$_1$ to 1158*lic1D**$_4$]), 1159*lic1*, and *H. influenzae* mutant strain Rd*lic1*. (A) LPS profile prior to blotting. (B) Western blot with MAb TEPC15.

nation of a pyrophosphoethanolamine (PPEtn) group from C-4 of Kdo as observed in previous studies (41). Of particular interest were major ions at *m/z* 954.4, 975.5, and 997.5 (data not shown) corresponding to glycoforms which each contained two PCho substituents. Information concerning the location of PCho in the oligosaccharide sample was provided by ESI-MS-MS experiments in the positive-ion mode following online separation by capillary electrophoresis. The product ion spectrum obtained from the doubly charged ion at *m/z* 844 (composition, PCho · Ac_2 · Hex_2 · Hep_4 · PEtn · AnKdo-ol) (Fig. 7A) contained marker ions at *m/z* 316, 328, and 370 corresponding to PEtn · Hep, PCho · Hex, and PCho · Ac · Hex. Ions at *m/z* 1358 and 1124 corresponded to loss of PCho · Hex and PCho · Ac · Hex · Hep, respectively, consistent with the hypothesis that PCho is a substituent of the terminal hexose. The product ion spectrum obtained from the doubly charged ion at *m/z* 954 (composition, $PCho_2$ · Ac_2 · Hex_2 · Hep_4 · PEtn · AnKdo-ol) (Fig. 7B) contained, inter alia, the marker ions at *m/z* 358 and 400 corresponding to PCho · Hep and PCho · Ac · Hep, respectively, to which additions of Hex (*m/z* 562), Hep (*m/z* 754), and AnKdo-ol (*m/z* 976) could be seen. This provided evidence that PCho replaced the terminal hexose as well the heptose in the outer core.

Strains 1159*lic1* and 1158*lic1D** were analyzed by CE-ESI-MS. In the CE-ESI-MS spectrum of 1158*lic1D** (positive mode) (Fig. 8A and Table 4), predominant ions corresponding to PCho · Hex_2 · Hep_4 glycoforms were observed, in which the heterogeneity was due to the degree of acetylation (Ac_{0-6}) and glycylation (Gly_{0-1}). Major ions corresponding to glycoforms having two PCho substituents were not observed. All ions corresponded to glycoforms having one PCho (Table 4). The position of the PCho substituent still expressed in this strain was established by CE-ESI-MS-MS for ions at *m/z* 823 and 844 corresponding to the composition PCho · $Ac_{1,2}$ · Hex_2 · Hep_4 · PEtn · AnKdo-ol. The product ion spectrum for *m/z* 823 is shown in Fig. 8B. It contains the marker ions at *m/z* 328 and 370 corresponding to PCho · Hex and PCho · Ac · Hex. The product ion spectrum for *m/z* 843 was virtually identical to that shown in Fig. 7A. None of the product ion spectra contained *m/z* 358 or 400 corresponding to PCho · Hep and PCho · Ac · Hep, respectively. It was therefore evident that LPS purified from strain 1158*lic1D**, containing a disrupted copy of *lic1D**, lacked PCho off HepIV but retained

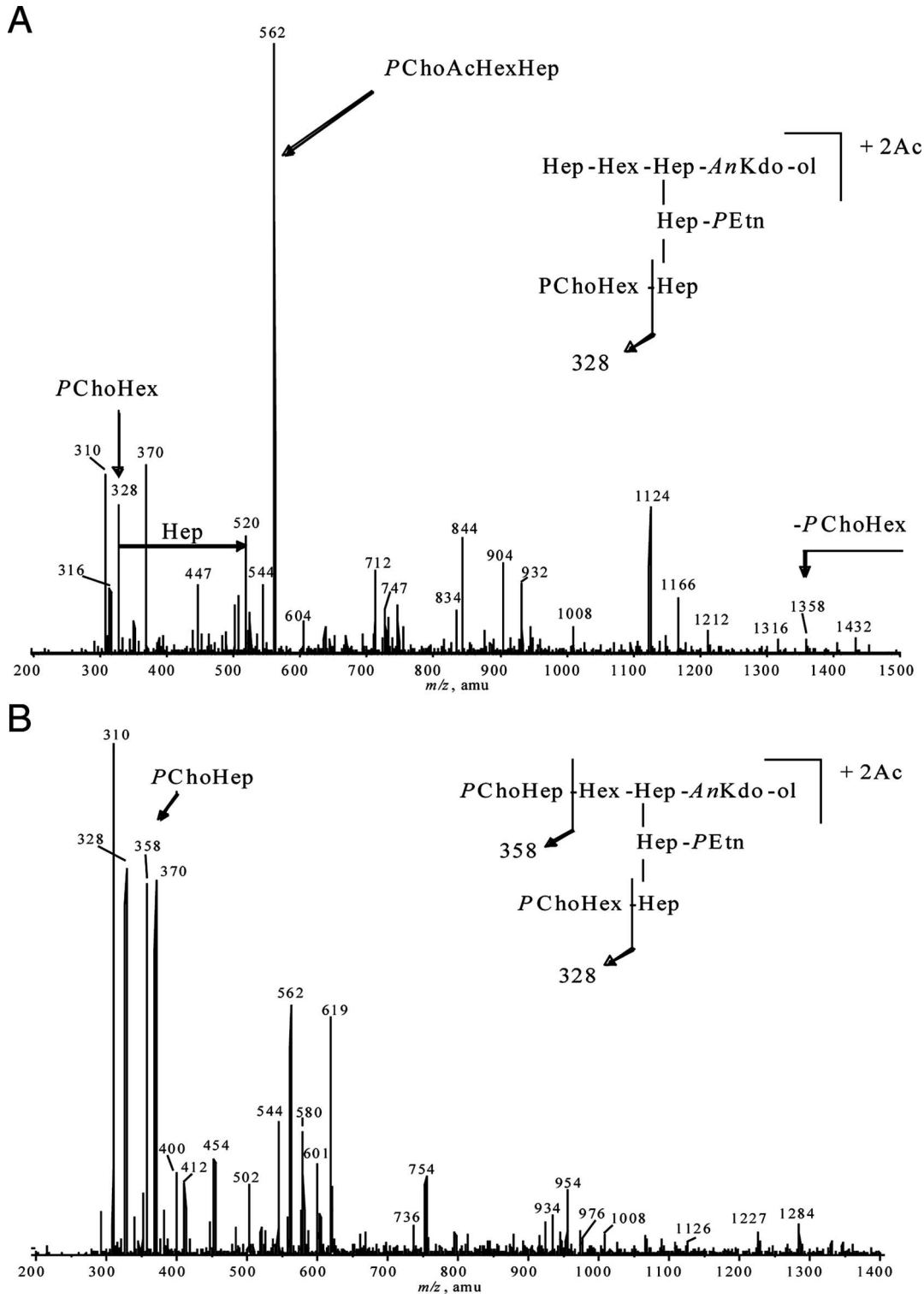

FIG. 7. CE-ESI-MS^2 (positive mode) analyses of the oligosaccharide from NTHI isolate 1158. (A) Product ion spectrum of $[M+2H]^{2+}$ *m/z* 843 corresponding to *P*Cho · Ac_2 · Hex_2 · Hep_4 · *P*Etn · AnKdo-ol. (B) Product ion spectrum of $[M+2H]^{2+}$ *m/z* 954 corresponding to *P*Cho_2 · Ac_2 · Hex_2 · Hep_4 · *P*Etn · AnKdo-ol. The proposed carbohydrate backbone and selected fragments are indicated.

PCho off the HepIII extension. It is noteworthy, however, that a precursor ion-monitoring tandem MS experiment (positive-ion mode) (data not shown) scanning for loss of *m/z* 358 (PCho · Hep) on O-deacylated 1158*lic1D** showed ions at *m/z* 803 and 885.5 corresponding to $PCho_{1,2}$ · Hex_2 · Hep_4 · PEtn · AnKdo-ol. From the abundance of these ions it could be concluded that these glycoforms accounted for about 1 and 0.4% of the total glycoforms. Thus, an *lic1D** mutation certainly effects

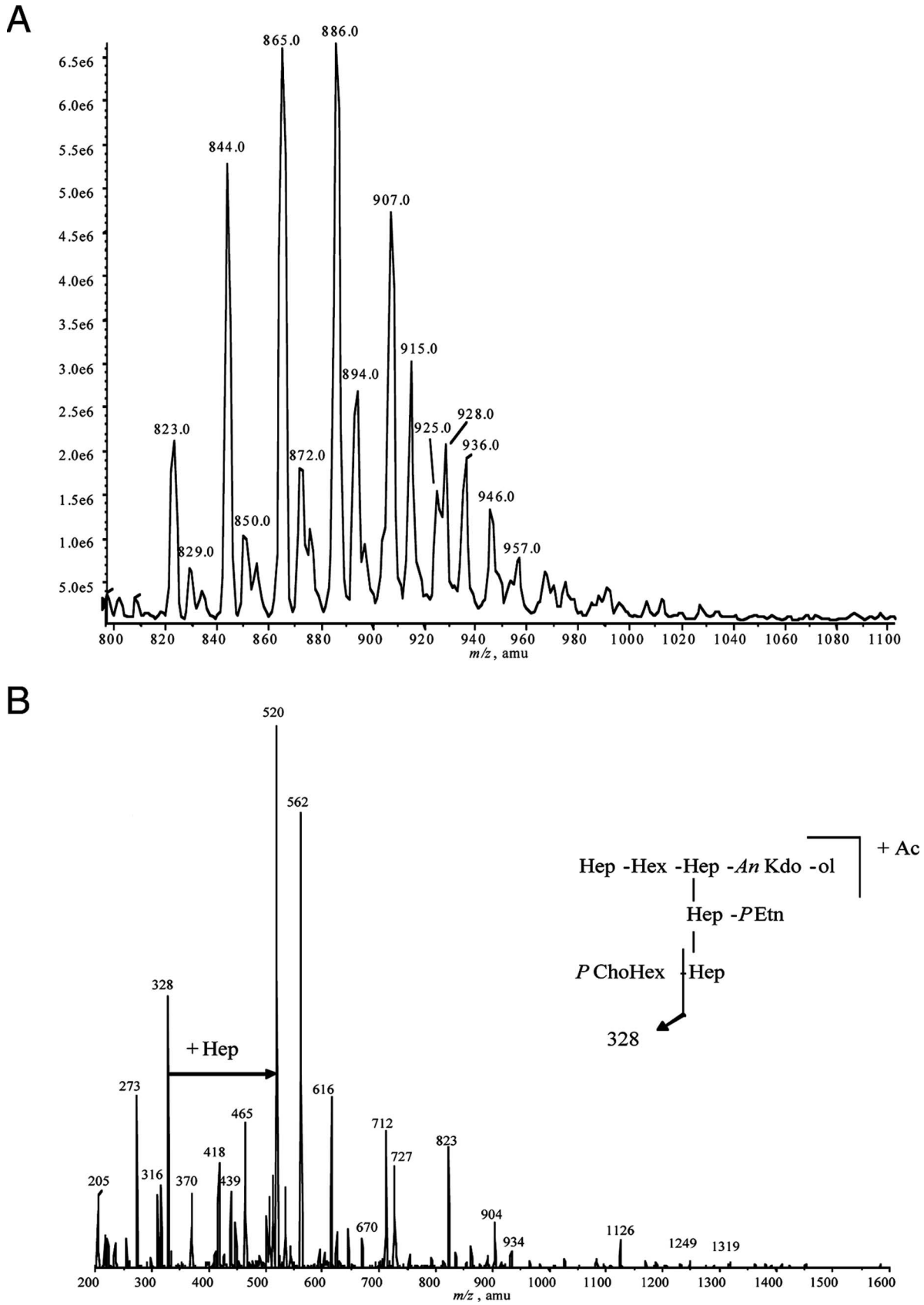

FIG. 8. CE-ESI-MS^2 (positive mode) analyses of the oligosaccharide from the NTHI isolate 1158*lic1D** mutant strain. (A) Part of the CE-ESI-MS spectrum (positive mode) of the oligosaccharide from strain 1158*lic1D**. (B) Product ion spectrum of $[M+2H]^{2+}$ *m/z* 823 corresponding to *P*Cho · Ac · Hex_2 · Hep_4 · *P*Etn · AnKdo-ol. The proposed carbohydrate backbone and selected fragments are indicated.

TABLE 4. Positive-ion ESI-MS data and proposed compositions for oligosaccharide material derived from LPS of NTHI strain 1158*lic1D**

Observed $[M+2H]^{2+}$ ion (*m/z*)	Molecular mass (Da)[a]		Proposed composition
	Observed	Calculated	
823.0	1,644.0	1,645.38	PCho · Ac · Hex_2 · Hep_4 · PEtn · AnKdo-ol[b]
844.0	1,686.0	1,687.42	PCho · Ac_2 · Hex_2 · Hep_4 · PEtn · AnKdo-ol
850.0	1,698.0	1,699.44	Ac · Gly · Hex_3 · Hep_4 · PEtn · AnKdo-ol
865.0	1,728.0	1,729.46	PCho · Ac_3 · Hex_2 · Hep_4 · PEtn · AnKdo-ol
872.0	1,742.0	1,744.47	PCho · Ac_2 · Gly · Hex_2 · Hep_4 · PEtn · AnKdo-ol
886.0	1,770.0	1,771.51	PCho · Ac_4 · Hex_2 · Hep_4 · PEtn · AnKdo-ol
894.0	1,786.0	1,786.52	PCho · Ac_3 · Gly · Hex_2 · Hep_4 · PEtn · AnKdo-ol
907.0	1,812.0	1,813.56	PCho · Ac_5 · Hex_2 · Hep_4 · PEtn · AnKdo-ol
915.0	1,828.0	1,828.57	PCho · Ac_4 · Gly · Hex_2 · Hep_4 · PEtn · AnKdo-ol
925.0	1,848.0	1,849.56	PCho · Ac_2 · Hex_3 · Hep_4 · PEtn · AnKdo-ol
928.0	1,854.0	1,855.61	PCho · Ac_6 · Hex_2 · Hep_4 · PEtn · AnKdo-ol
936.0	1,870.0	1,870.62	PCho · Ac_5 · Gly · Hex_2 · Hep_4 · PEtn · AnKdo-ol
946.0	1,890.0	1,891.61	PCho · Ac_3 · Hex_3 · Hep_4 · PEtn · AnKdo-ol

[a] Average mass units were used to calculate molecular mass values based on proposed compositions, as follows: Hex, 162.14; Hep, 192.17; AnKdo-ol, 222.20; P, 79.98; PEtn, 123.05; PCho, 165.13; Ac, 42.04; and Gly, 57.05.

[b] After O deacylation of the oligosaccharide material with aqueous ammonia, the resulting CE-ESI-MS spectrum revealed a major ion at *m/z* 802.8 corresponding to a glycoform with the composition PCho · Hex_2 · Hep_4 · PEtn · AnKdo-ol. Minor ions at *m/z* 787.8 and 883.9 corresponded to PCho · Hex_3 · Hep_3 · PEtn · AnKdo-ol and PCho · Hex_3 · Hep_4 · PEtn · AnKdo-ol, respectively.

PCho substitution at HepIV but may also show the promiscuity of *lic1D*. Mutant strain 1159*lic1*, shown by PCR amplification to contain a disrupted copy of the complete *lic1* locus, displayed no detectable PCho on its LPS. Structural analysis was also carried out for the LPS of the *lic1* mutant strain of NTHI isolate 176. Like isolate 1158 LPS, isolate 176 LPS displays PCho linked to the first hexose in the chain extension from HepIII and linked directly to HepIV in the oligosaccharide chain extension from HepI. In mutant strain 176*lic1*, shown to contain a disrupted copy of *lic1*, no PCho was present on the HepIII extension, and PCho remained on HepIV (E. Schweda, unpublished results). This finding confirms that *lic1* is responsible for the addition of PCho to the extension off HepIII in NTHI isolate 176 LPS, implying that similar to NTHI 1158 LPS, *lic1** is responsible for addition of PCho to HepIV.

Functional analysis of *lic1* mutant strains. PCho expressed on the bacterial surface is a target for binding of CRP, an acute-phase reactant in serum that plays a role in serum killing of the bacteria. Binding of CRP to the *lic1* mutant strains was investigated by Western analysis using an MAb which specifically recognizes human CRP (Fig. 9). Larger amounts of bound CRP were detected with the wild-type strain expressing two PCho residues on the LPS than with mutant strain 1158*lic1D**, which expresses one PCho residue. No bound CRP was detected with mutant strain 1159*lic1*, which lacks PCho in its LPS.

The resistance of *lic1* mutant strains and the parental isolates to the killing effect of pooled human serum and of pooled human serum with CRP removed was assayed and compared (Fig. 10). Wild-type strain 1158 and mutant strain 1158*lic1D**, expressing two and one PCho residues, respectively (Fig. 10A and B), and wild-type strain 1159 and mutant strain 1159*lic1*, expressing two and no PCho residues, respectively (Fig. 10C and D), had relatively similar serum resistance profiles. However, for strains expressing two or one PCho residue, an increase in serum resistance was evident when CRP was removed from the serum, whereas no change in serum resistance was evident with mutant strain 1159*lic1*, which lacks PCho in its LPS, when CRP was removed.

Expression of PCho has been reported to play a role in the persistence of *H. influenzae* on the mucosal surface of the human respiratory tract (38), but CRP, also present in the human respiratory tract (6), might be able to block the attachment of PCho-expressing bacteria to host cells. The association of *lic1* mutant strains and the parental isolates with human airway epithelial cells ($16HBE14_0^-$) was assayed. Experiments, carried out in triplicate, compared the levels of association of NTHI isolates and their *lic1* mutants with $16HBE14_0^-$ cells in the presence and in the absence of human serum. NTHI isolate 375 and mutant strain 375*lic1* adhered better than strains derived from isolates 1158 and 1159, but no reproducible trends were observed for parental and mutant strains in these experiments. This indicates that alteration of the number of PCho molecules attached to the LPS in the NTHI isolates studied had no significant effect on association of the bacteria with epithelial cells under our in vitro assay conditions (data not shown).

FIG. 9. Western blot analysis using isogenic strains of *H. influenzae* expressing differing amounts of *P*Cho and an MAb against human CRP. Lane 1, wild-type strain 1158; lane 2, 1158*lic1D**; lane 3, 1159*lic1*.

DISCUSSION

PCho is a frequent noncarbohydrate substituent of *H. influenzae* LPS, and its expression is highly variable. This variation is generated by the *lic1D* gene sequence, which determines the site of attachment of PCho within the LPS molecule (17), and phase variation, which is mediated by a 5′CAAT tetranucleotide repeat tract located within the *lic1A* gene (34). PCho can be linked to a hexose or heptose as part of the oligosaccharide

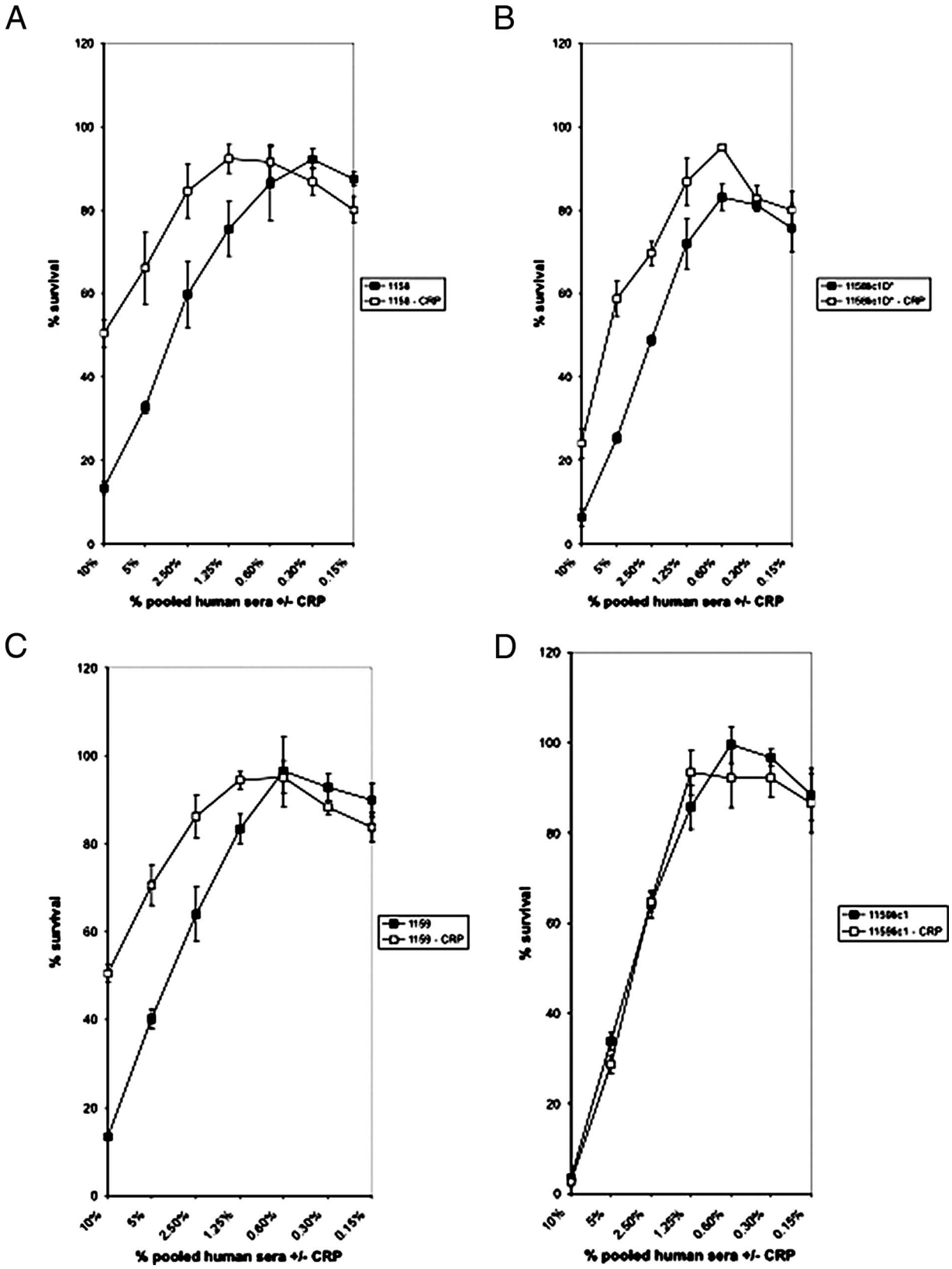

FIG. 10. Resistance of *lic1* mutant strains and the parental strains to the killing effect of pooled human serum compared to the effect of pooled human serum with CRP removed. (A) NTHI isolate 1158; (B) mutant strain 1158*lic1D**; (C) NTHI isolate 1159; (D) mutant strain 1159*lic1*. The results are expressed as a plot of the percentage of survival of inoculating bacteria versus serum concentration. The symbols indicate average values from a single experiment done in triplicate; the error bars indicate standard deviations. The data are representative of multiple independent experiments.

extension from any of the three inner core heptoses. PCho addition to LPS is nonstoichiometric; consequently, not all LPS glycoforms expressed on the surface of the bacterium are identical with respect to PCho expression, adding a further level of heterogeneity. In this study we showed that within a single LPS molecule the addition of multiple PCho residues is due to duplication of the genes of the *lic1* operon. Phenotypic, antigenic, and structural analyses of the LPS of *lic1* mutant strains of NTHI provided evidence that each copy of *lic1D* specifically directs the addition of PCho to one of two distinct positions on the LPS molecule. One of these sites of PCho addition was novel, and addition was directed to a heptose as part of the oligosaccharide extension from the proximal heptose of the backbone of the LPS inner core. Expression of multiple PCho residues on a single LPS molecule of nondisease isolates of NTHI has been reported previously (15), potentially indicating a role for this epitope in both the commensal and pathogenic states of the bacterium.

In this study we report that the *lic1* operon is duplicated in 4 of the 25 NTHI isolates tested. In three of these four isolates, the duplication has been shown to be a tandem duplication of the *lic1* operon. In two of the isolates, the genes adjacent to *lic1* are also duplicated. Duplicated sequences of HI1535, HI1536, HI1541 (*sppA*), and HI1542 were all identified in NTHI isolate 1158. However, with the exception of HI1536 the second copies of gene sequences were incomplete and therefore would not be expected to encode additional functional gene products. The *lic1* tandem duplication may have arisen in a single strain, followed by divergence of the sequence of the distinct copies. Alternatively, the duplication may have occurred through acquisition of DNA by horizontal transfer from another strain, where the sequence was already divergent. The organization of the region of DNA encompassing the *lic1* locus, with approximately 6 kb of DNA duplicated in a tandem arrangement, is potentially unstable and would be expected to undergo high-frequency loss of the duplication through homologous recombination, analogous to the *cap* locus in *H. influenzae* type b strains. The instability of this region may be reflected by the fact that the extent of the duplication is distinct in at least three of the four NTHI isolates possessing two copies of *lic1*. Examination of the recently published NTHI genome of isolate R2846 also revealed a duplication of the *lic1* locus, with a partial *lic1B* gene and full-length *lic1C* and *lic1D* genes, in a tandem duplication adjacent to a complete *lic1* locus. Preliminary data from an LPS structural analysis indicated that two PCho residues are expressed on the LPS of this isolate (E. Schweda, unpublished data), likely directed by the two distinct copies of *lic1D*, which are homologous to the *lic1D* alleles of isolate 1158.

The maintenance of this region of duplicated DNA in the genome of NTHI suggests that there is positive selection; i.e., the phenotype confers increased fitness. In this study, we investigated *lic1* mutant strains to assess the effect of expressing zero, one, or two PCho residues on the LPS on their association with cultured human epithelial cells, binding of CRP, and resistance to the killing effect of normal human sera. PCho expression on LPS facilitates colonization and persistence of NTHI in the human respiratory tract by mediating bacterial adherence to and invasion of the host epithelia. Swords and colleagues (30) have demonstrated that a particular subset of PCho-expressing LPS glycoforms, those highly reactive with MAb TEPC15, preferentially mediate the interaction of NTHI with the receptor for platelet-activating factor found on bronchial epithelial cells. Conversely, the level and location of PCho expression can be detrimental to fitness as PCho is a target for the binding of CRP and subsequent complement-mediated killing (37, 38). CRP is an acute-phase reactant in serum which is also expressed on the epithelial surface of the human respiratory tract (6). In this study, no detectable effect on association with cultured human epithelial cells was observed when wild-type strains were compared to *lic1* mutant strains. In these types of experiments the findings are often strain dependent due to the indirect effects of other LPS epitopes; therefore, similar studies with a range of strain backgrounds are required to evaluate the effect of various degrees of PCho decoration on interactions with host cells. A difference was observed, however, in the binding of CRP to strains expressing various amounts of PCho, with larger quantities of CRP bound to strains expressing two PCho residues than to strains expressing one PCho residue. No detectable CRP was bound to cells not expressing PCho on their LPS. This finding is consistent with the work of Weiser and colleagues, who proposed that PCho is a target for CRP binding and that bound CRP elicits complement-mediated killing of *H. influenzae* (37, 38). To test whether the levels of bound CRP observed in strains in this study were related to serum resistance, serum resistance assays using pooled human serum and pooled human serum with CRP depleted were performed with wild-type and *lic1* mutant strains. For strains expressing one or two PCho residues in their LPS, increased serum resistance was observed when CRP was depleted from the sera, and no corresponding increase was observed for a strain expressing no PCho. These findings confirm previous findings suggesting that CRP elicits bactericidal killing of *H. influenzae* strains expressing PCho on their LPS. The expression of one or more PCho residues would be predicted to increase the susceptibility of *H. influenzae* to host clearance during invasive disease. The ability to vary expression of PCho, therefore, is likely to play an important role in both the commensal and pathogenic lifestyles of this organism.

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