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Decoration of Lipopolysaccharide with Phosphorylcholine: a Phase-Variable Characteristic of *Haemophilus influenzae*

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Choline, although not a nutritional requirement for *Haemophilus influenzae*, is taken up from the growth medium and incorporated into its lipopolysaccharide (LPS). Incorporated choline is in the form of phosphorylcholine (ChoP) based on the reactivity with the monoclonal antibody with specificity for this structure, TEPC-15. Incorporation of [^{3}H]choline from the growth medium and expression of the TEPC-15 epitope undergo high-frequency phase variation, characteristic of other LPS structures in this species. The expression and phase variation of ChoP require a previously identified locus involved in LPS biosynthesis, *lic1*. The first gene in *lic1*, *licA*, contains a translational switch based on variation in the number of intragenic tandem repeats of the sequence 5′-CAAT-3′. The full-length LicA polypeptide resembles choline kinases of eucaryotes, suggesting that the pathway for choline incorporation into the *H. influenzae* glycolipid has similarities to the pathway for choline incorporation in eucaryotic lipid synthesis. The display of ChoP, a host-like structure, renders the organism more rather than less susceptible to the bactericidal activity of human serum. The increased serum sensitivity of variants with ChoP correlates with higher serum immunoglobulin G titers to LPS containing this structure. ChoP appears to be a cell surface feature common to a number of pathogens of the human respiratory tract, including *Streptococcus pneumoniae* and mycoplasmas. In the case of *H. influenzae*, its primary contribution to pathogenesis does not appear to be antigenic variation to evade host humoral clearance mechanisms.

Choline in the form of phosphatidylcholine is rare in procaryotes. In contrast, phosphatidylcholine is the major eucaryotic membrane phospholipid. The pathway for choline incorporation into eucaryotic lipids involves the phosphorylation of choline by choline kinase, coupling to CTP by the enzyme phosphocholine cytidylyl transferase, and, finally, transfer to diacylglycerol (17, 23). In the few bacteria that have phosphatidylcholine, there is de novo synthesis of phosphatidylcholine by N-methylation of phosphatidylethanolamine rather than the pathway involving choline kinase (2). Choline may also be acquired from the environment by some bacteria tolerant to osmotic stress as a precursor of glycine betaine, which functions as an osmolyte (27). In the bacteria that take up environmental choline, however, it is not used in the synthesis of structural molecules. One notable exception is the gram-positive respiratory tract pathogen *Streptococcus pneumoniae*, which takes choline from growth medium and incorporates it in the form of choline phosphate or phosphorylcholine (ChoP) on its cell wall-associated teichoic acid as well as its glycolipid, lipoteichoic acid (6, 32). Choline is a nutritional requirement for the pneumococcus, although the enzymatic steps and genes required for choline incorporation in the pneumococcus have not been identified. Recently, other residents of the human respiratory tract were screened for the presence of ChoP with an antibody that recognizes this structure (10). Of the 15% of isolates with the ChoP antigen, *Haemophilus influenzae* was the only gram-negative species represented. This observation suggests that *H. influenzae*, which occupies the same environmental niche in the human nasopharynx as the pneumococcus, also contains ChoP on its surface.

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The surface glycolipid or lipopolysaccharide (LPS) of *H. influenzae* consists of lipid A, an inner core of a singly phosphorylated 2-keto-3-deoxyoctulosonic acid (KDO) linked to three heptose molecules, and an outer core consisting of a heteropolymer of glucose and galactose (33, 49). The *H. influenzae* LPS lacks the multiple O-linked saccharide units characteristic of the smooth LPS of the family *Enterobacteriaceae*. The complete structure of the glycolipid has not been resolved because of marked intra- and interstrain heterogeneity in the composition and linkage of saccharide units in the outer core (20, 24). There are high-frequency (10^{-2} to 10^{-3}/generation), spontaneous, reversible gain and loss (phase variation) of oligosaccharide epitopes expressed by genes in three chromosomal loci, *lic1*, *lic2*, and *lic3* (46). A common feature of each of the three loci is multiple tandem repeats of the tetramer 5′-CAAT-3′ within the coding region of the first gene (45). Slipped-strand mispairing within this highly repetitive sequence shifts the 5′ end of the gene into or out of frame with the remainder of the open reading frame, creating a translational switch. Complete sequencing of the *H. influenzae* genome has shown that highly repetitive sequences which generate molecular switches are a common feature of many genes, especially those involved in the expression of cell surface components (8). In the case of the *lic* loci, downstream genes are associated with expression of oligosaccharide epitopes identified by monoclonal antibody (MAb) 4C4 for *lic2* and MAb 12D9 for *lic1* (13, 46). Only for *lic2* and *lic3* are specific saccharide structures controlled by these loci known; *lic2* is required for expression of the structure Galα(1-4)βGal, while *lic3* contains the galactose epimerase gene, *galE* (15, 30). In contrast, *lic1*, which is present in all strains of a representative survey of *H. influenzae* isolates, has not been associated with any specific oligosaccharide structure (46). Defined mutations in *lic1*, which comprises four genes (*licA*, *B*, *C*, and *D*), elimi-

TABLE 1. *H. influenzae* strains used

Strain	Genotype or characteristic(s)	TEPC-15 reactivity[a]	Source or reference
Eagan	Serotype b clinical isolate	+, PV	Collection of A. Smith
Fuju	Serotype b clinical isolate	+, PV	42
RM7004	Serotype b clinical isolate	+, PV	44
RM7004-AH1-2	*lic1* Tet^r	−	47
RM7004-EX1	*lic2* Tet^r	−	47
RM7004-RVdel8	*lic3* Kan^r	+, PV	30
I69	Rd$^-$, deep rough LPS mutant	−	48
Rd$^-$	Serotype d clinical isolate, unencapsulated	+, PV	42
Rd$^-$ (*lic1* mutant)	Rd$^-$ × RM7004-AH1-2 DNA, Tet^r	−	This study
Rd$^-$ (*lic2* mutant)	Rd$^-$ × RM7004-EX1 DNA, Tet^r	+, PV	This study
Eagan (*lic1* mutant)	Eagan × RM7004-AH1-2 DNA, Tet^r	−	This study
Eagan (*lic2* mutant)	Eagan × RM7004-EX1 DNA, Tet^r	+, PV	This study
H137	Nontypeable clinical isolate	+, PV	This study
H143	Nontypeable clinical isolate	+, PV	This study
H233	Nontypeable clinical isolate	+, PV	This study
Rd$^-$b$^+$02	Serotype b transformant of Rd$^-$	+, PV	50

[a] Reactivity with MAb TEPC-15 with specificity for phosphorylcholine (+) was determined by Western blotting; reactivity with phase variation of the TEPC-15 epitope (PV) was determined by colony immunoblotting.

nate expression of the 12D9 epitope but have little apparent effect on the LPS size (42).

Several reports have suggested that phase-variable LPS structures contribute to the pathogenesis of *H. influenzae* infection (25, 30, 50). In a virulent, serotype b clinical isolate, a *lic1 lic2* mutant was able to colonize the nasopharynx in an infant rat model of *H. influenzae* carriage but was relatively deficient at causing septicemia following intranasal inoculation (47). This suggests that structures encoded by *lic1* and *lic2* enhance the invasive capacity of *H. influenzae*. It has also been suggested that phase-variable LPS structures associated with differences in colony opacity contribute to resistance to the complement-mediated bactericidal activity of serum (42). Phase variants with an opaque colony morphology were more serum resistant and had a slightly smaller LPS on Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels compared to phase variants with a transparent colony morphology.

This report describes the uptake of choline and its addition as ChoP to the LPS of *H. influenzae*. The substitution of choline onto LPS undergoes phase variation mediated by the *lic1* locus and requires expression of *licA*, which contains the molecular switch $(5'\text{-CAAT-}3')_n$ and is translated into a protein with similarity to eucaryotic choline kinase.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *H. influenzae* strains used for the study are shown in Table 1. *H. influenzae* strains were grown in brain heart infusion broth supplemented with 1.5% Fildes enrichment with or without 1% agar (Difco Laboratories, Detroit, Mich.). When specified, a chemically defined medium was used (31). *S. pneumoniae* Rx1 was grown as previously described (43). Colony morphology was determined with a stereo zoom dissecting microscope with a substage mirror and 100-W halogen illuminator. Lipoteichoic acid was obtained by a published method (7). Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified.

LPS analysis. *H. influenzae* LPS was prepared from mid-log-phase cultures according to a hot phenol-water extraction method (21). Cells used in LPS preparation were >97% of the desired phenotype as confirmed by colony immunoblotting. LPS size was assessed in 16.6% acrylamide gels by Tricine-SDS-PAGE (29). LPS was visualized with the modified silver stain described by Hitchcock and Brown (16).

Choline incorporation and detection. *H. influenzae* was radiolabeled by adding [^{3}H]choline (New England Nuclear Co., Boston, Mass.) to chemically defined medium (final concentration, 0.25 μCi/ml). *H. influenzae* was grown to an optical density at 620 nm of 0.3 and washed three times in an equal volume of phosphate-buffered saline. An aliquot was removed for determination of cell density, and incorporation of the label was determined in whole cells in duplicate.

Thin-layer chromatography. Purified LPS was hydrolyzed in 48% HF at 25°C for 4 h. After evaporation, the material was resuspended in water, spotted, and then dried onto cellulose sheets (EM Science, Cherry Hill, N.J.). Chromatography was carried out in water-saturated phenol-ethanol-water (50:5:6) (22). Choline was detected with Dragendorff's reagent, which gives a transient colorimetric reaction specific to quaternary amine structures such as choline (22).

Transformation of *H. influenzae*. *H. influenzae* was made competent by the method of Herriot et al. (14). Preparation of genomic DNA from *H. influenzae* has been described previously (18). Transformants were selected on medium containing tetracycline (1 μg/ml).

Western transfer and immunoblotting. Electrotransfer onto Immobilon-P (Millipore Corp., Bedford, Mass.) and Western blotting were carried out as described previously (39). Immunoblotting of membranes was carried out in a 1/10,000 dilution of MAb TEPC-15, and bands were visualized following incubation in goat anti-mouse immunoglobulin A antibody conjugated to alkaline phosphatase (36).

Autoradiography. LPS was prepared from mid-log-phase cultures by treatment of whole-cell lysates with proteinase K (Boehringer, Mannheim, Germany) according to the method of Kimura and Hansen (24). LPS was separated on SDS–18% PAGE gels and transferred to Immobilon-P as described above in "Western transfer and immunoblotting." Immobilon-P was used for autoradiography following treatment with En^{3}Hance (Dupont NEN Research Products, Boston, Mass.) according to the manufacturer's instructions.

Colony immunoblotting. Procedures used for immunoblotting colonies lifted onto nitrocellulose have previously been reported (44). Tissue culture supernatant containing MAb 12D9 was used at a dilution of 1:100 and detected with goat anti-rabbit immunoglobulin G antibody conjugated to alkaline phosphatase. ChoP on colonies lifted onto nitrocellulose was detected with MAb TEPC-15 as described above in "Western transfer and immunoblotting."

Serum bactericidal assays. Complement-mediated serum bactericidal activity was determined in normal human serum (NHS) pooled from six random adult donors and stored at −80°C. Assays were performed with 20 μl of mid-log-phase organisms (optical density at 650 nm, 0.3 to 0.4) diluted to 10^7 CFU/ml, 140 μl of Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.), and 20 μl of serum. After incubation for 60 min at 37°C with rotation, the assay was stopped by cooling the mixture to 4°C. To calculate the percent survival, colony counts were compared to controls in which complement activity had been eliminated by prior heating of the serum to 56°C for 30 min.

Enzyme-linked immunosorbent assay. Equal quantities of purified LPS from variants of strain Eagan were fixed to 96-well polystyrene plates in 0.9% NaCl with 20 mM $MgCl_2$ for 16 h at 37°C. After five washes in a buffer containing 10 mM Tris (pH 8.2), 150 mM NaCl, 0.05% Brij, and 0.02% azide, serial dilutions of NHS were applied to the plate for 16 h at 25°C. Following an additional five washes, an anti-human immunoglobulin G antibody conjugated to alkaline phosphatase was added and incubated for 2 h at 25°C. Plates were developed, and the titer was determined as previously described (43).

RESULTS AND DISCUSSION

The LPS core of *H. influenzae* contains phosphorylcholine. The presence of phosphorylcholine (ChoP) in *H. influenzae* was examined by screening whole-cell lysates on Western blots with a MAb, TEPC-15 (Fig. 1). TEPC-15 has high specificity

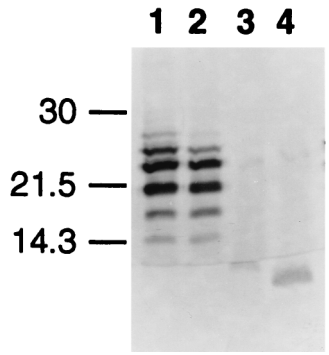

FIG. 1. Western blot of *H. influenzae* and *S. pneumoniae*. Phosphorylcholine-containing molecules were detected with MAb TEPC-15 on whole-cell lysates separated by SDS–15% PAGE. Lanes: 1, *S. pneumoniae*; 2, *S. pneumoniae* (lipoteichoic acid); 3, *H. influenzae* Eagan; 4, *H. influenzae* Rd$^-$. Size markers are protein standards in kilodaltons.

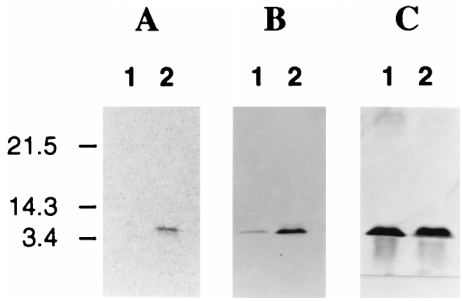

FIG. 3. Autoradiography of TEPC-15-reactive and -nonreactive variants grown in the presence of [^{3}H]choline. Whole-cell lysates were treated with proteinase K and separated in SDS–18% PAGE gels, and the LPS was detected by autoradiography (A). Controls in which LPS was detected by Western analysis with TEPC-15 (B) or silver staining (C) confirmed the phenotype and equivalent loading of samples. Lanes: 1, Rd$^-$ (TEPC-15-nonreactive variant); 2, Rd$^-$ (TEPC-15-reactive variant). Size markers are protein standards in kilodaltons.

for ChoP and does not cross-react with related compounds such as choline, ethanolamine, or phosphorylethanolamine (28). This MAb cross-reacts with the ChoP on the lipoteichoic acid of *S. pneumoniae* in a ladder-like pattern caused by different numbers of repeating units (6). TEPC-15 reacted with low-molecular-weight material from whole-cell lysates of the two unrelated *H. influenzae* strains initially tested, Eagan and Rd$^-$. The low molecular weight and interstrain size differences in the TEPC-15-reactive molecule in *H. influenzae* suggested that the MAb was binding to LPS. This was confirmed by Western analysis demonstrating the TEPC-15 epitope on purified LPS from strains Eagan and Rd$^-$ obtained by a hot phenol-water extraction method (Fig. 2A). Silver staining of the LPS was used to show that there was no significant protein contamination (Fig. 2B). The presence of choline in LPS has not previously been described in any gram-negative organism, although in *Escherichia coli*, ethanolamine, which is structurally related to choline, is linked to both KDO and heptose in the LPS inner core region (35).

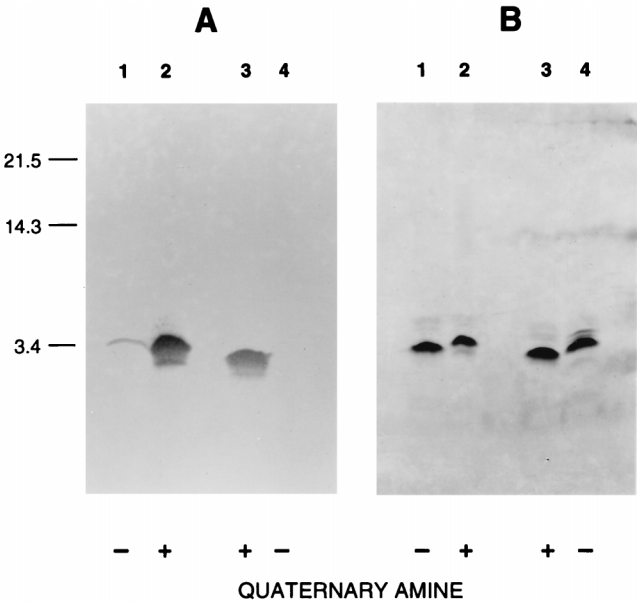

FIG. 2. Western blot and silver staining of purified LPS from phosphorylcholine phase variants. TEPC-15-reactive and -nonreactive colonies were separately grown to mid-log phase. The LPS was extracted, and equal quantities were separated on Tricine-SDS–16.6% PAGE gels and immunoblotted with TEPC-15 (A) or silver stained (B). Lanes: 1, Eagan (TEPC-15 nonreactive); 2, Eagan (TEPC-15 reactive); 3, Rd$^-$ (TEPC-15 reactive); 4, Rd$^-$ (TEPC-15 nonreactive). The presence (+) or absence (−) of a quaternary amine structure in the purified LPS as determined by reactivity with Dragendorff's reagent is indicated below. Size markers are protein standards in kilodaltons.

Further confirmation that the *H. influenzae* LPS contains ChoP was provided by analysis of hydrolysis products by thin-layer chromatography. The LPS hydrolysis products contained material that reacted with a reagent which specifically detects quaternary amines (Dragendorff's reagent). Dragendorff's reagent reacts with quaternary amines such as choline but not with nonquaternary amines that are structural analogs of choline, such as ethanolamine (22). The migration of the Dragendorff's reagent-reactive band from the LPS corresponded to that of the phosphorylcholine control (R_f = 89) and was distinct from that of choline (R_f = 93) (22). Based on this observation and the reactivity with TEPC-15, which has high affinity for ChoP but not for choline, the substituent on the LPS appeared to be in the form of choline phosphate (ChoP) (28).

It was possible to determine whether ChoP is on the lipid or carbohydrate portion of the LPS by analysis of strain I69. This deep rough mutant has a truncated LPS consisting of lipid A linked to a singly phosphorylated KDO (48). The absence of the TEPC-15 antigen in I69 suggests that ChoP is on the heptose and/or hexose portion of LPS (Table 1) (38, 49). The precise LPS structure(s) to which ChoP is linked was not determined in this study.

Choline is obtained from growth medium. Choline is not necessary for the growth of *H. influenzae*, since a chemically defined growth medium lacking choline has been described for this organism (31). It was possible to demonstrate, however, that like the pneumococcus, *H. influenzae* acquires choline from its growth medium. [^{3}H]choline was added to chemically defined growth medium which otherwise lacks choline. The incorporation of [^{3}H]choline from the medium into LPS was demonstrated by autoradiography. The radiolabel comigrated with the LPS bands reactive with TEPC-15 and was detected by silver staining in whole-cell lysates treated with proteinase K and separated by SDS-PAGE (Fig. 3). The incorporation of [^{3}H]choline into LPS provided further evidence that the *H. influenzae* glycolipid contains choline.

Incorporation of choline undergoes phase variation. In addition to the serotype b (Eagan) and d (Rd$^-$) strains, a limited survey of other *H. influenzae* strains was screened for the presence of the ChoP substituent on the LPS. All strains examined,

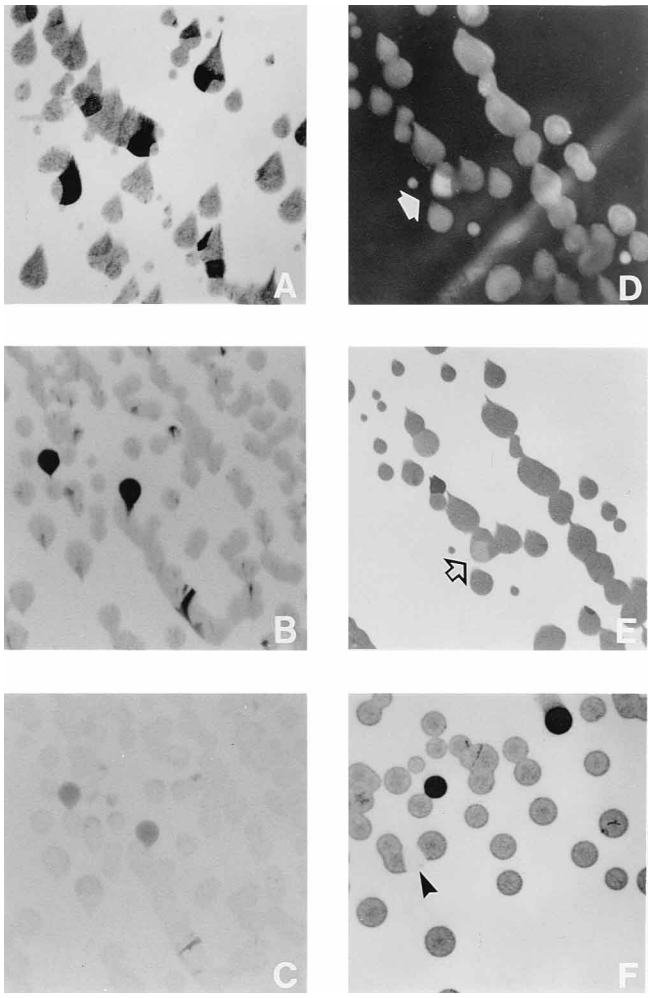

FIG. 4. (A) Immunoblot of colonies of strain RM7004 with TEPC-15 showing sectoring. (B) Colonies of strain Eagan immunoblotted with MAb TEPC-15. (C) The same colonies as shown in panel B were regrown and immunoblotted with MAb 12D9, whose epitope undergoes phase variation controlled by the *lic1* locus. (D) Photograph of variant colony of strain Eagan with the opaque phenotype (white arrow) shows diminished reactivity when immunoblotted (E) with TEPC-15 (open arrow). (F) Immunoblot of strain H233 showing three distinct levels of reactivity with TEPC-15; high reactivity, low reactivity, and nonreactivity (arrowhead).

including two additional serotype b isolates (RM7004 and Fuju) and three unrelated nontypeable isolates (H137, H143, and H233), had the TEPC-15 epitope on Western analysis (Table 1). It was concluded that the ChoP structure is a common and possibly ubiquitous feature of this organism.

In examining these *H. influenzae* strains, considerable strain-to-strain variation was noted in the intensity of reactivity to TEPC-15. Since the LPS of this organism displays marked phase variation in structure, intrastrain differences in content of ChoP were assessed with TEPC-15 on colony immunoblots. Each of the seven strains shown to have ChoP-LPS displayed colony-to-colony variation in expression of the TEPC-15 epitope (Table 1). The observation of sectored colonies and reversion of reactive to nonreactive colonies as well as nonreactive to reactive colonies indicated that intrastrain differences were caused by phase variation of the TEPC-15 antigen (Fig. 4A).

The observation of phase variation allowed the separation of organisms into two relatively uniform populations based on reactivity with the MAb. Purified LPSs from reactive and nonreactive phase variants of strains Eagan and Rd$^-$ were compared following separation on Tricine-SDS-PAGE gels. Variation in expression of the TEPC-15 epitope in colonies correlated with variation in expression of the TEPC-15-reactive structure in LPS on Western analysis (Fig. 2A). Silver staining of LPS from reactive and nonreactive phase variants demonstrated equivalent loading and showed that there were slight differences in LPS migration between variants. The relationship between the presence of the TEPC-15 epitope and LPS size, however, was not consistent between the two strains (Fig. 2B). In addition, [^{3}H]choline incorporation into whole cells was compared in TEPC-15-reactive and -nonreactive variants of two unrelated strains (Rd$^-$ and H137). In both cases, only those variants with the ChoP structure were able to incorporate [^{3}H]choline (Fig. 5). In contrast, the variants lacking the TEPC-15 epitope incorporated approximately 100-fold less of the radiolabeled choline. (The minimal choline incorporation noted in the TEPC-15-nonreactive variants appeared to be caused by a small population of phenotypic revertants.) This result demonstrated that the ability to incorporate choline acquired from the nutrient medium correlated with expression of the ChoP structure on the LPS. Dragendorff's reagent re-

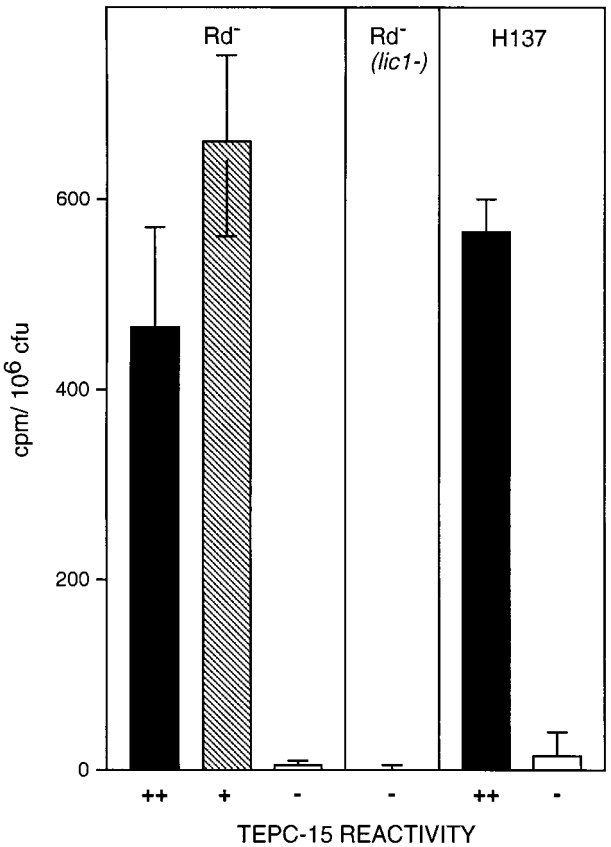

FIG. 5. Comparison of the incorporation of [^{3}H]choline in phase variants that differ in reactivity with MAb TEPC-15. A single colony that was either reactive (striped or solid bars) or nonreactive (open bars) with TEPC-15 on colony immunoblots was grown in a chemically defined medium in the presence of radiolabeled choline to mid-log phase. Incorporation of [^{3}H]choline into whole cells was determined and expressed as the geometric mean number of counts per minute in 10^6 CFU in three separate determinations ± standard deviation. For strain Rd$^-$, variants with three levels of reactivity with TEPC-15 were compared.

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LicA (H.influenzae)              222 CHNDLVPENMLL-------QDDRLFFIDWEYSGLNDPLFDIATIIEEA 262
                                     ||||    | |         ||  |  || || | |    | |    |
Choline kinase (S.cerevisiae)    327 CHNDAQYGNLLFTA*QEQSQDSKLVVIDFEYAGANPAAYDLANHLSEW 414

LicA (H.influenzae)              222 CHNDLVPENMLL-------QDDRLFFIDWEYSGLNDPLFDIATIIEEA 262
                                     |||||   | |            | ||||||    |    | |    ||
Choline kinase (C.elegans)       158 CHNDLTSSNILQ-----LNSTGELVFIDWENASYNWRGYDLAMHLSEA 200

LicA (H.influenzae)              222 CHNDLVPENMLL-------QDDRLFFIDWEYSGLNDPLFDIATIIEEA 262
                                     ||||    | ||           |  || |||  |   |||    |
Choline kinase (R.norvegicus)    394 CHNDCQEGNILLLEGQENSEKQKLMLIDFEYSSYNYRGFDIGNHFCEW 352

LicA (H.influenzae)              222 CHNDLVPENMLL-------QDDRLFFIDWEYSGLNDPLFDIATIIEEA 262
                                      ||||   ||           |   | ||||    |  || |  ||
LicA (M.hominis)                 142 VHNDLWLFNMIK-------VNDKIYFTDWEYATMGDVHFDLAYFIESS 182
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FIG. 6. Sequence similarity of LicA with eucaryotic choline kinases. The region of *H. influenzae* LicA from amino acids 222 through 262 was compared to choline kinases from *S. cerevisiae*, *C. elegans*, and liver of the rat, *Rattus norvegicus*. For the choline kinase of *S. cerevisiae* the asterisk denotes 41 amino acids that were deleted in order to optimize the alignment. Amino acids conforming to the consensus sequence for phosphotransferases and protein kinases described by Brenner (3) are indicated in boldface. Amino acids identical to those of LicA of *H. influenzae* are indicated by vertical lines. Also shown is the sequence comparison of the same region of LicA from *H. influenzae* and the corresponding region of a gene of unknown function identified in *Mycoplasma hominis.*

acted with purified LPS from TEPC-15-reactive variants of strains Eagan and Rd$^-$ but not with an equal quantity of purified LPS from TEPC-15-nonreactive variants of these strains (Fig. 2). These results provided physical evidence that *H. influenzae* LPS undergoes phase variation in expression of a quaternary amine structure, which, based on incorporation of choline and reactivity with the MAb TEPC-15, is ChoP.

***lic1* is necessary for choline incorporation.** The relationship between phase variation in ChoP and phase variation in LPS structures determined by the three *lic* loci was examined in strain RM7004, from which these loci were initially characterized. Mutants with deletion or insertion mutations encompassing all open reading frames in each locus were compared to the parent strain for reactivity with TEPC-15 on colony immunoblots (Table 1). Mutants with changes in *lic1* (RM7004-AH1-2) or *lic2* (RM7004-EX1) were constitutively nonreactive, whereas the mutation in *lic3* (RM7004-RVdel8) had no effect on this epitope. In order to confirm the role of these loci in the expression of ChoP, chromosomal DNA from the *lic1* and *lic2* mutants in RM7004 was transformed into strains Eagan and Rd$^-$. For both strains Eagan and Rd$^-$ acquisition of tetracycline resistance from the *lic1* mutant, but not the *lic2* mutant, correlated with constitutive loss of reactivity with TEPC-15 (Table 1). Additional evidence that *lic1* is necessary for decorating the LPS with ChoP was provided by colony immunoblots. The pattern of variation in reactivity with TEPC-15 correlated precisely with the expression of the LPS epitopes defined by MAb 12D9 and expressed by *lic1* (Fig. 4B and C). All the 12D9-reactive colonies also reacted with TEPC-15, and no 12D9-nonreactive colonies reacted with TEPC-15. Since 12D9 does not recognize ChoP in pneumococcal lipoteichoic acid, its epitope appears to require structures in addition to ChoP. There was no correlation between expression of the 4C4 epitope encoded by *lic2* and reactivity with TEPC-15. A defined mutant in *lic1* in strain Rd$^-$, furthermore, was unable to incorporate significant amounts of [^{3}H]choline from the medium (Fig. 5). These results suggested that the *lic1* locus is necessary for incorporation of choline, whereas LPS structures expressed by the *lic2* locus is required only in some strains.

The contribution of *lic1* genes to choline incorporation. Two possible functions for the *lic1* locus were considered. The first was that *lic1* is not directly involved in choline incorporation but rather is responsible for expression of LPS structures that are required to add ChoP and that phase variation in the presence of ChoP is a result of on-off switching of these LPS structures. An alternative hypothesis is that *lic1* functions directly in the incorporation of ChoP onto the LPS. Evidence supporting the latter hypothesis was provided by comparison of the four gene products of the *lic1* locus to current entries in sequence databases by using the BLAST program through the National Center for Biotechnology Information (1).

The first gene in *lic1*, *licA*, encodes a polypeptide with sequence similarity to choline kinases in *Saccharomyces cerevisiae* (30.8% identity and 54.4% similarity over 68 amino acids), *Caenorhabditis elegans* (50.0% identity and 63.9% similarity over 36 amino acids), and rat liver (Fig. 6) (40). The region of greatest similarity is within the consensus sequence, H*X*D*hXXX*N*hhh* (where *h* is a large hydrophobic amino acid, FLIMVMY), which contains the catalytic domain for phosphotransferases and protein kinases reported by Brenner (3). The similarity to other choline kinases, including the active site of the enzyme in a protein whose function is linked to choline incorporation, indicates that LicA may function as a bacterial choline kinase.

The second gene in *lic1*, *licB*, which encodes a protein comprising nine hydrophobic domains, showed similarity in sequence to several proteins, including a membrane protein from *Clostridium kluyveri* (26.2% identity and 41.8% similarity over 194 amino acids). Although the function of this protein is unknown, it is notable that some species of the genus *Clostridium* have been found to have ChoP-containing cell walls (34). There is also similarity between LicB and the chloroplast triose phosphate transporter from plants (24% identity and 47% similarity over 183 amino acids for the triose phosphate transporter of *Spinacia oleracea*). This is an anion-selective channel protein of the chloroplast envelope that transports fixed carbon as triose phosphate into the cytoplasm (9, 37). In addition, a separate region of LicB resembles the high-affinity choline permease, BetT (41.2% identity and 66.7% similarity over 51 amino acids), identified in *H. influenzae* Rd$^-$ by its similarity to the *E. coli* gene product (8, 27).

The third gene in *lic1*, *licC*, encodes a protein with an N terminus that resembles the allosteric site of a family of pyrophosphorylases that catalyze reactions between nucleoside triphosphates and sugar 1-phosphates in the biosynthesis of polysaccharide. Of the five groups of pyrophosphorylases, the similarity in LicC is most significant to the uridylyltransferases, such as α-glucose-1-phosphate uridylyltransferase involved in

spore coat polysaccharide biosynthesis in *Bacillus subtilis* (42% identical and 63% similar over 52 amino acids) (11).

The last gene in *lic1*, *licD*, encodes a protein with similarity to the product of a gene, *cpsG*, that has been identified in *S. pneumoniae* within a locus for synthesis of the capsular polysaccharide (38% identity and 64% similarity over 65 amino acids) (12).

Based on these similarities to enzymes of known function, the pathway for choline incorporation into LPS may have features in common with the pathway for choline incorporation into eucaryotic lipids. The specific function and cellular location of these enzymes, however, have not been demonstrated. A possible pathway based on these similarities suggests that following uptake of choline into cells, possibly through a transport mechanism involving LicB, there is phosphorylation by choline kinase (LicA). A pyrophosphorylase (LicC) then catalyzes the formation of nucleoside diphosphocholine from choline phosphate and a nucleoside triphosphate (UTP?). The final step in the pathway would be the transfer of the activated form of choline onto the LPS. While there is no specific evidence suggesting that LicD transfers ChoP onto the LPS, similarity to a gene that functions in polysaccharide synthesis provides a link between other *lic* genes that appear to interact with choline and the oligosaccharide of *H. influenzae.*

Relationship between phase variation in phosphorylcholine and other LPS structures. In the strains analyzed, three distinct levels of reactivity (negative, low, and high) with TEPC-15 were noted on colony immunoblots, suggesting that phase variation of the ChoP structure is more complicated than on-off switching (Fig. 4F). Similar observations were made with the *lic1*-associated epitope defined by MAb 12D9 (45). It was observed that two of the three reading frames of *licA* have possible initiation codons, which if translated into full-length polypeptides could lead to two forms of LicA differing at the N terminus. The three observed phenotypes could result from variation between the three reading frames of *licA*. An alternative hypothesis for phase variation between three phenotypes is that the ability of TEPC-15 to bind to ChoP on the LPS is affected by the presence or absence of other variably expressed LPS structures rather than differences in the amount of incorporated choline. Results showing no difference in the incorporation of radiolabeled choline from phase variants of strain Rd^- with high (++) and low (+) levels of reactivity with TEPC-15 support this possibility (Fig. 5).

Another factor in the expression of the TEPC-15 epitope was colony opacity. Although both opaque and transparent phenotypes were capable of expressing the TEPC-15 epitope, the opaque colony morphology had diminished reactivity with the MAb compared to variants with a transparent phenotype (Fig. 4D and E). This indicates that undefined LPS structures associated with opaque organisms may affect the ability of TEPC-15 to bind to its epitope on the LPS. A complete understanding of the relationships of LPS substituents that undergo phase variation will require a more detailed understanding of the specific structures involved.

Contribution of phosphorylcholine to serum sensitivity. The hypothesis that expression of ChoP on the cell surface contributes to antigenic variation was examined. The effect of ChoP on the ability of *H. influenzae* to evade the bactericidal action of complement and antibody in human serum was determined by using the encapsulated strain Eagan with the transparent colony phenotype. Phase variants with the ChoP structure were more than 200-fold more serum sensitive than phase variants without ChoP on their LPS (Fig. 7). In fact, of the $ChoP^+$ population incubated in NHS, the majority of the survivors

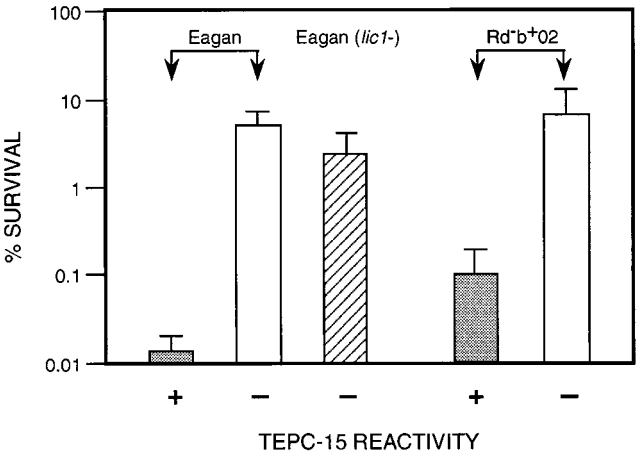

FIG. 7. Resistance to the bactericidal activity of human serum in *H. influenzae* phase variants. A single TEPC-15-reactive (stippled bars) or -nonreactive (open bars) colony of the variant or mutant indicated was grown to mid-log phase and treated for 60 min in 10% pooled NHS. The percent survival is the number of CFU remaining compared to controls in which complement was inactivated. Values are the geometric means of four determinations ± standard deviations.

were from the small number of spontaneous revertants with the $ChoP^-$ phenotype. ChoP-containing variants were also significantly more serum sensitive than a constitutive $ChoP^-$ mutant of strain Eagan in which the *lic1* locus had been interrupted. The increased serum sensitivity of ChoP-containing variants was also confirmed by using an unrelated encapsulated strain, Rd^-b^+02. Expression of ChoP on the LPS by *lic1* therefore renders the organism more rather than less serum sensitive. The immunoglobulin G titer of the pooled NHS to the LPS of strain Eagan containing ChoP was eightfold higher than that to the LPS of the variant of strain Eagan without ChoP, as determined by enzyme-linked immunosorbent assay. This suggests that the increased serum killing was a result of higher levels of antibody to the LPS containing the ChoP structure.

The observation that expression of ChoP is not constitutive suggests that it is a decoration on the LPS rather than a requirement for its structural integrity. Why should a pathogen decorate its surface with ChoP, a structure normally associated with host molecules? The increased serum sensitivity of ChoP-containing phase variants makes it unlikely that this structure functions to camouflage the organism from the host immune response. Results demonstrating that the presence of ChoP renders the organism more susceptible to killing by serum are not entirely surprising, as serum contains natural antibody against ChoP (4). In addition, a serum protein, known as C-reactive protein, binds to ChoP and may contribute to clearance of bacterial pathogens (19, 41). It is of particular interest that a protein with homology to LicA, including the active site for choline kinases, has been identified in several species of the genus *Mycoplasma*, which includes one of the most common human respiratory tract pathogens (Fig. 6) (26). A choline-containing glycolipid has recently been identified in *Mycoplasma fermentans* (5). Results also demonstrate that the glycolipid of *S. pneumoniae*, another major human respiratory tract pathogen, contains the same ChoP epitope as the LPS of *H. influenzae*. We propose that there may be a common mechanism involving ChoP on the bacterial cell surface that contributes to the ability of these pathogens to thrive in the human respiratory tract. In the case of *H. influenzae* infection, results using an infant rat model show that *lic1* mutants colonize the

mucosal surface efficiently but are less likely to invade the bloodstream from the nasopharynx (47). This suggests that ChoP-containing structures expressed by *lic1* are critical to the invasion process itself.

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