Streptococcus pneumoniae requires choline for growth, and it decorates the teichoic and lipoteichoic acids of the cell wall with this essential nutrient (5). It is established that choline metabolism plays an indispensable role in cell separation, transformation, autolysis, and pathogenicity of S. pneumoniae. For example, the cell surface phosphocholine (P-Cho) participates in the interaction with the host surface and induces attachment and invasion (4, 15). The importance of choline in pathogenesis is not confined to S. pneumoniae but is also found in Haemophilus influenzae (9, 10, 18, 19), Pseudomonas aeruginosa, and Neisseria gonorrhoeae (12, 17). In addition, extracellular P-Cho serves as the scaffold for a group of choline-binding proteins that are secreted from the cells and are subsequently attached to the cell surface by their homologous choline-binding domains (see reference 11 and references therein).

The pathway for choline metabolism in S. pneumoniae and H. influenzae has been hypothesized to consist of a choline transport system, choline kinase, CTP:phosphocholine cytidylyltransferase (CCT), and a choline phosphotransferase that transfers P-Cho to lipoteichoic acid or lipopolysaccharide (18). The existence of this pathway is supported by the detection of choline kinase and CCT activity in crude extracts of S. pneumoniae (1, 20). Genetic elements required for choline incorporation into the lipopolysaccharide of H. influenzae are found in the lic1 locus, which contains four open reading frames. The hypothesis drawn from the bioinformatic analysis of the lic1 locus (19) is that licC encodes a candidate for the CCT due to the resemblance of its amino terminus to the amino-terminal 60 residues of nucleoside triphosphate (NTP) transferase family members, leaving the licD gene as a candidate for the choline phosphotransferase. A homologous licC gene exists in S. pneumoniae (21), and the predicted LicC proteins of H. influenzae and S. pneumoniae are 37% identical and 60% similar. There are no examples of bacterial CCTs, and both of the predicted LicC proteins lack homology to either the prototypical metazoan CCTs (7, 14) or the CTP:glycerol-3-phosphate cytidylyltransferase from Bacillus subtilis (16). Rather, licC is related to members of the NTP transferase superfamily that primarily activate phosphosugars, such as galU of Escherichia coli and rmlA of P. aeruginosa (Fig. 1), opening the possibility that licC is involved in carbohydrate metabolism. The goal of this study was to determine if the licC gene encodes a novel CCT.

Similarity of LicC to NTP transferases. LicC is classified as a member of the NTP transferase (nucleotidylyltransferase) superfamily of proteins based on the presence of the signature sequence (KAXGXGTRXPK) for this group of enzymes at its amino terminus. Figure 1 shows the relationship of the primary sequence of LicC to two representative members of this family, a phosphosugar uridylyltransferase (GalU) and thymidylyltransferase (RmlA) from gram-negative bacteria. LicC possessed a high degree of similarity with these two nucleotidylyltransferases in the amino-terminal domain (Fig. 1). The crystal structures of members of the NTP transferase family verify that this amino-terminal sequence motif is involved in nucleotide binding (2, 3).

Expression, purification, and activity of LicC. The licC gene was amplified using primers designed to introduce an NdeI site at the initiator codon and a BamHI site after the stop codon. The fragment was sequenced and cloned into pET-15b for expression as a His-tagged protein. The construct began at Ile1 and ended at Asn234 (Fig. 1). The construct was sequenced to verify the absence of PCR artifacts.

The protein was purified on Ni-nitrilotriacetic acid agarose followed by gel filtration on Superdex-200 26/60 (Fig. 2). The enzyme was homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The apparent molecular mass calculated from the gel filtration column was 30 kDa, which agreed with the predicted molecular mass of LicC plus the His tag based on the amino acid sequence (Fig. 1). This result indicates that LicC exists as a monomer (Fig. 2). The protein was stored in 50% glycerol–20 mM Tris-HCl, pH 7.5, at −20°C and retained full activity for several months.
LicC catalyzed the formation of CDP-Cho as identified by thin-layer chromatography (Fig. 3). LicC cytidylyltransferase activity was linear with time and protein and exhibited a specific activity of 2.5 μmol/min/mg of protein in the standard assay conditions (Fig. 3, inset).

Biochemical properties. We compared the activity of LicC using either ATP, TTP, UTP, or GTP in place of CTP, all at 2 mM concentration. No activity was detected with TTP, UTP, or GTP. The activity with ATP was 0.25% of that with CTP (data not shown). The high degree of nucleotide selectivity was consistent with the identification of LicC as a cytidylyltransferase rather than another nucleotidyltransferase. The Km for CTP was 133 μM (Fig. 4A) and for P-Cho was 83 μM (Fig. 4B). In both cases, the V_max was 7.7 ± 0.2 μmol/min/mg. There was no indication of cooperativity in the binding of either substrate consistent with LicC existing as a monomer.

We assessed the ability of LicC to use phosphoethanolamine (P-Etn) as a substrate by substituting 1 mM P-[14C]Etn (specific activity of 3.68 mCi/mmol) for P-[14C]Cho in the standard assay described for Fig. 3 and raising the LicC concentration to 10 mg/assay. LicC was capable of utilizing P-Etn as the substrate but was much less efficient. The protein exhibited a Km of 1.43 mM for P-Etn and a V_max with this substrate of 0.06 μmol/min/mg. Thus, LicC was able to convert P-Etn to CDP-Etn, but it used P-Etn much less efficiently as a substrate.

FIG. 1. Similarity of LicC to NTP transferases. The predicted amino acid sequence of the licC gene of S. pneumoniae is aligned with the predicted amino acid sequences of the E. coli galU and P. aeruginosa rmlA genes, two nucleotidyltransferases involved in the activation of phosphosugars for the biosynthesis of cell wall polysaccharides. Identical amino acids between LicC and either of the two NTP transferases are highlighted. Members of the NTP transferase family contain the KAX,GXGTRXᴬ PK amino-terminal motif.
FIG. 2. Purification of LicC. The His-tagged LicC protein was purified by Ni-nitrotriacetic acid affinity chromatography as described previously (6). The protein was immediately applied to a Superdex-200 26/60 column (Pharmacia Biotech) equilibrated with 50 mM Tris-HCl (pH 7.0), 1 mM dithiothreitol, and 1 mM EDTA and was eluted at a flow rate of 2.5 ml/min. LicC was monitored at 280 nm and eluted at 226 ml. The molecular mass was estimated to be 30 kDa by graphic analysis of a standard curve based on the elution volumes (arrows) of protein molecular mass standards (Sigma), βA, β-amylase (200 kDa); ADH, alcohol dehydrogenase (150 kDa); BSA, bovine serum albumin (66 kDa); CA, carbonic anhydrase (29 kDa); CytC, cytochrome c (12.4 kDa). LicC was pure, based on analysis with a sodium dodecyl sulfate–12% polyacrylamide gel followed by Coomassie blue staining (inset).

FIG. 3. LicC is a CCT. The LicC assay contained 2 mM CTP, 10 mM MgCl₂, 1 mM phospho[methyl-¹⁴C]choline (specific activity of 3.68 mCi/mmol), 150 mM bis-Tris-HCl (pH 7.0), and 62.5 ng of LicC in a final volume of 50 μl. LicC was added last to initiate the reaction, and after 10 min at 37°C, the reaction was stopped by the addition of 5 μl of 0.5 M EDTA. A 40-μl aliquot of the reaction mixture was applied to a preabsorbent silica gel G plate that was developed with 95% ethanol–2% ammonium hydroxide (1:1, vol/vol) (8). The single product was visualized with a Bioscan Imaging detector and migrated with authentic CDP-Cho. The formation of CDP-Cho per assay was linear with LicC (inset).
moiety to a phosphorylated acceptor substrate concomitant with the release of pyrophosphate (National Center for Biotechnology Information Conserved Domain Database, protein family no. 00483). The inclusion of LicC in this family is illustrated by its similarity to the amino termini of GalU and RmlA, two nucleotidyltransferases that are involved in the formation of nucleoside diphosphate-hexose derivatives that are used in the biosynthesis of cell wall components (Fig. 1). The less conserved regions in the middle of these proteins represent the substrate-binding regions. Many family members are dimers or tetramers; however, LicC appears to be a monomer, based on the results of gel filtration chromatography (Fig. 2). The structure of RmlA (2) shows that the subunit interaction surface is encoded by the carboxy-terminal domain. Interestingly, LicC is smaller than typical NTP transferases due to the lack of a carboxy-terminal tail (Fig. 1), consistent with its monomeric behavior in solution.

The biochemical properties of LicC are consistent with its proposed role in the pathway for the decoration of teichoic acids with P-Chol. EtN does replace Cho as a nutritional requirement and is incorporated to approximately the same extent into the cell wall (13). However, the replacement of Cho with EtN results in several severe phenotypic changes, including the lack of daughter cell dissociation and autolysis, resistance to deoxycholate-induced lysis, and the inability to undergo genetic transformation. Significantly, the presence of Cho in the medium completely blocks the incorporation of EtN into cell walls, illustrating that the pathway is highly selective for Cho. This physiological observation suggests that the Cho incorporation pathway is highly selective for Cho but is capable of using EtN. This conclusion is reflected in the substrate specificity of LicC, and we suggest that the other enzymes in the pathway will have the same dual specificity and Cho selectivity. The importance of choline metabolism to viability and virulence suggests that LicC would be an excellent target for the development of antibacterial therapies.

Conclusions. Our study establishes that the third gene in the lic1 cluster, licC, encodes a functional CCT responsible for the formation of CDP-Chol. LicC does not have similarity in primary sequence to the known metazoan CCTs and thus represents a unique member of this functional enzyme class. However, LicC is a member of the NTP transferase (nucleotidyltransferase) superfamily of enzymes that transfer the nucleoside monophosphate

FIG. 4. Kinetic constants for LicC. The kinetic constants for the two LicC substrates are shown. Double reciprocal plots were used to calculate the apparent $K_m$ for CTP (A), P-Chol (B), and P-Etn (C) by employing the radiochemical assay described in the legend to Fig. 3. (A) The CTP $K_m$ was 133 $\mu$M measured in the presence of 1 mM P-Chol. (B) The P-Chol $K_m$ was 83 $\mu$M measured in the presence of 2 mM CTP. (C) The $K_m$ for P-Etn of 1.43 mM was determined in the presence of 2 mM CTP. Assays for CDP-Etn formation were the same as the standard assay, except that P-Etn (specific activity of 3.68 mCi/mol) was substituted for P-Chol and that the LicC concentration was 10 $\mu$g assay. The error bars represent the range of data.

REFERENCES