NOTES

Choline Starvation Induces the Gene *licD2* in *Streptococcus pneumoniae*

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Received 13 June 2002/Accepted 8 October 2002

Mutant strains of *Streptococcus pneumoniae* were constructed to monitor the regulation of three dispersed genes known or predicted to act in choline metabolism. One gene (*licD2*) was regulated in response to choline deprivation over a 30-fold range. The other two (SP1860 and *licC*) responded little if at all to the same challenge.

Choline residues play important roles in the structure and function of the *Streptococcus pneumoniae* cell surface. Long known to be an essential nutrient for pneumococcus (1, 9), choline was found by Tomasz to be an unusual and important component of the pneumococcal cell wall (13, 14). In this species, choline is incorporated almost exclusively in the cell wall, where it occurs in teichoic acids (TAs) and lipoteichoic acids (LTAs), linked to sugars of the teichoic acid carbohydrate via phosphate ester linkages (3, 4). Choline is required for growth of the wild type, although it can be partially replaced by ethanolamine. Amounts of choline sufficient for unrestricted growth of pneumococcus are in the range of 5 to 50 $\mu\text{g/ml}$ (14, 18), while a concentration of 1 $\mu\text{g/ml}$ or less leads to premature growth cessation at levels proportional to the amount of available choline. In media with limiting levels of choline, wild-type cells grow until choline is exhausted and then stop replicating but do not lyse (13, 18). Autolysis is also blocked in choline-deficient cells (18) and in cells in which ethanolamine has been substituted for choline (14). During inhibition by penicillin, LTA and peptidoglycan are made and released from the cell, but this synthesis is halted if the cells are also starved for choline. Thus, it appears that these external macromolecule synthesis pathways are regulated in response to the choline supply (5). Potential steps in a pathway for choline utilization including a choline kinase and a CTP:phosphocholine cytidylyl transferase have been reported (10, 16, 17), and the gene *licD2* was shown to be required for phosphocholine incorporation into LTA (19). While research over the past 43 years has developed a picture of choline fates in pneumococcus, no studies that explored the regulation of any of these genes have yet been reported. Three operons implicated in choline metabolism in the literature were selected for study. SP1860 and SP1861 were described as possible choline transport genes (12). The gene *licD2* (SP1274), required for insertion of one-half of the choline residues into TAs, appears to form one

operon with *licD1* (SP1273) (19). The genes *licA* and *licB* (SP1269 and SP1268) are thought to provide more functions in choline utilization (19), while *licC*, apparently in the same operon, encodes CTP:phosphocholine cytidylyl transferase (10).

To investigate whether the regulation of any of these operons depends on choline, we created a set of nondisruptive reporter fusions and characterized their responses to choline limitation.

Bacterial strains, media, and plasmids. The insertion vector pEVP3 (2) was used for cloning pneumococcal targeting fragments in *Escherichia coli* ER2566. The pneumococcal strain CP1250 (Mal[−] Str^r Nov^s Cm^s Com⁺) (8) was the recipient strain for mutagenic plasmids. Luria-Bertani medium was used for culturing *E. coli*, and a casein hydrolysate broth was used for culturing pneumococcus (7). Nutritional studies were done in a chemically defined medium (CDM) (15).

Genetic strategy: nondisruptive promoter tagging. For each operon chosen for evaluation, the targeting fragment of DNA was selected as approximately the downstream half of the 3' (i.e., last) gene in the operon, including its native stop codon (Fig. 1). After insertion in pEVP3, the targeting fragment was adjacent to, in order, stop codons in all three reading frames, a ribosomal binding site, and the *lacZ* gene. After insertion in pneumococcus, the resulting structure was expected to be, in effect, an intact resident (target) operon with one additional gene, *lacZ*, and, in order, the pEVP3 insertion, a duplicate copy of the target, and the downstream sequence beyond the target operon. Thus, we expected to observe transcriptional regulation of the target operon without creating a disruptive mutant phenotype.

Strain construction and transformation. Targeting fragments were amplified by PCR from CP1250 template DNA, digested with *Bgl*II and *Nsi*I, purified with a Qiagen PCR purification kit, quantified, and inserted into the vector pEVP3 between unique *Bgl*II and *Nsi*I sites. Transformation of *E. coli* with plasmid DNA was carried out according to the standard calcium chloride-heat shock method with selection on medium containing 10 μg of chloramphenicol (Cm)/ml. The structures of plasmids prepared from transformed ER2566 (New England Biolabs) by use of the Wizard Plus Maxiprep Kit (Pro-

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FIG. 1. Gene tagging strategy. Genes are designated as in Tettelin et al. (12) or by previously assigned names. (A and B) Loci selected for mutagenesis; (C) organization of the vector pEVP3. The 3' region of each target gene, indicated by an open arrow, was inserted into pEVP3 at its multiple cloning site (MCS) to target insertion of the chimeric plasmid in *S. pneumoniae* by homologous recombination.

mega) were confirmed by restriction digestion and by amplifying junction fragments. A single confirmed plasmid of each type was used as donor DNA for transformation of CP1250, as described earlier (6). Cm^r colonies were selected, and the structure of each pEVP3 insertion was verified by PCR amplification of a junction fragment using a primer complementary to pEVP3 and a second primer matching a sequence upstream of the integrated vector. Three strains, CP1377, CP1378, and CP1379, having insertions in *licD2*, *licC*, and SP1860 (referred to as clone 1), respectively, as well as a second independent transformant of each type (referred to as clone 2), were stored at -84°C until further use. Choline responses of the resulting strains were evaluated by growth of the parental strain CP1250 and the tagged strains in CDM (11). The tagged strains were as susceptible to choline deprivation as the wild-type parent, ceasing growth at reduced cell densities in choline levels of 2 $\mu\text{g}/\text{ml}$ or less (data not shown).

Effects of choline limitation on LacZ activity of the tagged strains. Inocula were prepared as cultures grown overnight in CDM with 1 μg of choline/ml and then diluted into fresh CDM containing 50, 1.8, or 1 μg of choline/ml. During the subsequent growth at 37°C , samples were removed at optical densities (550 nm) of 0.05 to 0.1 and lysed directly to produce

FIG. 2. Effects of choline limitation on the activity of *lacZ* reporters inserted at three chromosomal sites. Two independent transformants with a *lacZ* reporter inserted at each target site were cultured in CDM with the specified amounts of choline until optical densities at 550 nm of 0.05 to 0.1 were reached, and then they were lysed and assayed for beta-galactosidase activity.

extracts for the beta-galactosidase assay at 28°C as described previously (8). Initial experiments revealed that expression of the *licD2::lacZ* fusion was induced by choline limitation but that the reporter fusions at *licC* and SP1860 were induced to a much smaller extent or not at all (Fig. 2). To evaluate the growth kinetics and gene expression profiles of both *licD2*-tagged strains in more detail, the experiment was repeated by using choline concentrations of 50, 2, 1, 0.5, and 0.1 $\mu\text{g}/\text{ml}$ and harvesting samples at intervals of 45 min for the beta-galactosidase assay at 20°C . Under conditions of high choline concentrations (50 $\mu\text{g}/\text{ml}$), the tagged strains exhibited the stationary-phase lysis that is typical of this species (Fig. 3B and C). At lower levels of choline for both parental and tagged strains, such stationary-phase lysis was absent and the total cell number ultimately achieved was constrained by the available choline. The beta-galactosidase background activity of the parental strain CP1250 (panel D) remained low under all culture conditions. In contrast, *licD2::lacZ* expression varied widely in response to limiting choline (Fig. 3E and F). Expression of the *licD2::lacZ* reporters remained at a basal level of approximately 1 Miller unit throughout growth in high choline concentration (50 $\mu\text{g}/\text{ml}$). In cultures provided with lower levels of choline, however, the same expression reporter genes were induced up to 30-fold, apparently as the choline was exhausted during growth—an exhaustion that occurred earlier for lower levels of choline. Thus, *licD2* expression was regulated in a choline-dependent way and increased as cells began to experience choline deprivation.

In this work, three genes implicated in choline metabolism were investigated. The expression of SP1860 and *licC* proved to change little, if at all, in response to choline deprivation, but *licD2*, thought to act in transferring choline phosphate to TA and LTA sugar residues, was strongly induced as cells encountered choline deficits. It may seem surprising that *licD2* is strongly upregulated in response to choline exhaustion while *licC* is not, but the detailed enzymology of this system is not yet com-

FIG. 3. Effects of choline limitation on growth and on gene expression of *licD2* tagged strains. During culture of CP1250 and two *licD2::lacZ* reporter transformants in CDM with the indicated amounts of choline, optical density was monitored at 550 nm and samples were harvested at indicated times for beta-galactosidase assay. (A and D) Beta-galactosidase negative parental strain CP1250; (B and E) *licD2::lacZ* clone 1; (C and F) *licD2::lacZ* clone 2. Upper panels (A, B, and C) show growth curves of the CDM cultures; lower panels (D, E, and F) show their LacZ activity.

pletely defined, and the possibility of one or more types of posttranscriptional regulation has not been investigated. However, the discovery of a gene strongly responsive to choline reveals at least one regulatory circuit that ensures a response to choline deprivation, suggests the possibility that additional genes are similarly regulated, and suggests that pneumococcus does encounter periods of choline limitation in its natural host environment.

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