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Different Pathways of Choline Metabolism in Two Choline-Independent Strains of *Streptococcus pneumoniae* and Their Impact on Virulence▽

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The two recently characterized *Streptococcus pneumoniae* strains—R6Chi and R6Cho$^-$—that have lost the unique auxotrophic requirement of this bacterial species for choline differ in their mechanisms of choline independence. In strain R6Chi the mechanism is caused by a point mutation in *tacF*, a gene that is part of the pneumococcal *lic2* operon, which is essential for growth and survival of the bacteria. Cultures of *lic2* mutants of the encapsulated strain D39Chi growing in choline-containing medium formed long chains, did not autolyze, had no choline in their cell wall, and were completely avirulent in the mouse intraperitoneal model. In contrast, while the Cho$^-$ strain carried a complete pneumococcal *lic2* operon and had no mutations in the *tacF* gene, deletion of the entire *lic2* operon had no effect on the growth or phenotype of strain Cho$^-$. These observations suggest that the biochemical functions normally dependent on determinants of the pneumococcal *lic2* operon may also be carried out in strain Cho$^-$ by a second set of genetic elements imported from *Streptococcus oralis*, the choline-independent streptococcal strain that served as the DNA donor in the heterologous transformation event that produced strain R6Cho$^-$. The identification in R6Cho$^-$ of a large (20-kb) *S. oralis* DNA insert carrying both *tacF* and *licD* genes confirms this prediction and suggests that these heterologous elements may represent a "backup" system capable of catalyzing P-choline incorporation and export of teichoic acid chains under conditions in which the native *lic2* operon is not functional.

The cell wall and membrane teichoic acid of the bacterial pathogen *Streptococcus pneumoniae* is unusual in that it contains in its structure phosphorylcholine residues, which are involved with a wide variety of physiological and ecological functions (5, 6). *S. pneumoniae*—as a species—is also unique in its dependence on an exogenous source of choline for growth (15).

Choline is taken up from the culture medium (or from the in vivo environment) and converted to CDP-choline by the sequential activity of proteins encoded by determinants organized into the *lic1* operon (31). The protein products of *licB*, *licA*, and *licC* catalyze cellular uptake (5) and conversion of intracellular choline to phosphorylcholine in an ATP-dependent reaction (28) followed by the conversion of phosphorylcholine in a CTP-dependent reaction to CDP-choline (2, 14, 17), which is assumed to be the substrate of an additional enzyme(s) that catalyzes the incorporation of P-choline residues (one or two, depending on the particular *S. pneumoniae* strain) into the teichoic acid chains. The genetic determinants involved appear to be part of a second operon (*lic2*) adjacent to *lic1* on the pneumococcal chromosome (11, 12, 31).

While the unique auxotrophic requirement for choline can be fulfilled by other structurally different amino alcohols (24, 27), the normal physiological properties of the bacterium require the trimethylamino group of choline. *S. pneumoniae* strains growing in medium in which choline has been replaced by ethanolamine show numerous abnormalities: they form long chains, they do not autolyze, and they are resistant to bacteriophage and cannot undergo genetic transformation (24). Interestingly, these are exactly the same abnormalities shown by three recently isolated choline-independent strains of *S. pneumoniae* when cultured in choline-free medium (4, 19, 30).

Two of these choline-independent strains were laboratory mutants isolated by an enrichment procedure in which the parental strain was serially passaged in a culture medium, the choline component of which was replaced by gradually decreasing concentrations of ethanolamine. This procedure eventually yielded mutants R6Chi (4) and JY2190 (30), which could grow in medium completely lacking the amino alcohol component. The mechanism of choline independence in JY2190 remains unknown at the present time. On the other hand, genetic and biochemical studies of R6Chi allowed the identification of a single G→T point mutation in spr1150—one of the genes of the *lic2* operon—as the molecular basis of choline independence in this mutant. The gene spr1150 (renamed *tacF*) was proposed to encode a polysaccharide transmembrane transferase ("flippase") that catalyzes transport of teichoic acid chains to the outer surface of the pneumococcal plasma membrane (4).

The mechanism of choline independence in the third extensively studied *S. pneumoniae* strain, R6Cho$^-$ (19), is not

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† We consider the studies of Dalia Denapaite (identification of *S. oralis* DNA sequences) and Florian Gehre (characterization of the in vitro and in vivo phenotypes of mutants) to represent contributions of equal importance for the studies described in this article.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Streptococcus pneumoniae strains		
R6	Penicillin-susceptible laboratory strain derived from R36A	RU[a] collection
R6Cho$^-$	Choline-independent derivative of R6	19
R6Chi	Spontaneous mutant of *S. pneumoniae* strain R6 carrying a G→T point mutation at nucleotide position 700 in spr1150	4
R6Cho$^-$*spr1150ID*	Insertion/duplication mutant of R6Cho$^-$, with inactivated spr1150	This study
R6Cho$^-$Δ*licD1D2*	*licD1-licD2* deletion mutant of R6Cho$^-$	This study
R6Cho$^-$Δ*lic2*	The *lic2* operon deletion mutant of R6Cho$^-$	This study
D39	Type 2 capsular virulent *S. pneumoniae* strain	RU collection
D39Cho$^-$	Choline-independent derivative of D39	12
D39Cho$^-$Δ*licD1D2*	*licD1-licD2* deletion mutant of D39Cho$^-$	This study
D39Cho$^-$Δ*lic2*	The *lic2* operon deletion mutant of D39Cho$^-$	This study
D39Chi	Choline-independent derivative of D39 carrying G700T point mutation in the *tacF* gene	4
D39ChiΔ*licD1D2*	*licD1-licD2* deletion mutant of D39Chi	This study
Plasmids		
pJDC9	*E. coli* plasmid; Erm^r	3
pR410	*E. coli* plasmid; Kan^r	21
pGEM3Z	*E. coli* plasmid; Amp^r	29
pSPR1150ID	Plasmid pJDC9 carrying an internal DNA fragment of *tacF* amplified with spr1150IDF-RI and spr1150IDR-BamHI primers	This study
pΔD1D2DU3	Plasmid pGEM3Z carrying an insert composed of upstream flank of the *licD1* gene and downstream flank of the *licD2* gene	This study
pΔD1D2DU31	Plasmid pGEM3Z carrying an insert composed of upstream flank of the *licD1* gene/kanamycin resistance cassette and downstream flank of the *licD2* gene	This study
pΔLIC2	Plasmid pGEM3Z carrying an insert composed of upstream flank of the spr1150 (*tacF*) gene/kanamycin resistance cassette and downstream flank of the *licD2* gene	This study

[a] RU, Rockefeller University.

known. R6Cho$^-$ shared many of the physiological properties of R6Chi but had a more complex origin: it was isolated as the product of a heterologous genetic cross in which the recipient was *S. pneumoniae* strain R6 and the source of donor DNA was *Streptococcus oralis*, a streptococcal species that contains choline in its teichoic acid but has no auxotrophic requirement for it (19).

The purpose of the studies described here was to compare the status and essentiality of genes in the *lic1* and *lic2* operons in R6Chi and R6Cho$^-$, in order to better understand the mechanism of the physiological abnormalities of these choline-free strains of pneumococci, particularly the dramatic reduction in their virulence-related properties (4, 9, 12).

MATERIALS AND METHODS

Strains and bacterial growth. Bacterial strains and plasmids used in this study are listed in Table 1. Strains of *S. pneumoniae* were grown in a casein-based semisynthetic medium (C+Y) and Cden medium (25) with 5 μg/ml choline or without choline at 37°C without aeration. *Escherichia coli* strains containing the pJDC9 plasmid (3) or its derivatives were grown in Luria-Bertani (LB) medium in the presence of 1 mg/ml erythromycin (Sigma). Strains of *S. pneumoniae* containing pJDC9 were grown in C+Y medium or Cden medium in the presence of 1 μg/ml erythromycin.

DNA manipulations. All routine DNA manipulations were performed by using standard methods (18). PCR primers were synthesized at the custom primer facility of Invitrogen. Chromosomal DNA from strains was isolated as described earlier (13), and PCR products and DNA recovered after restriction endonuclease digestions were purified using the QIAquick PCR purification kit (Qiagen). Long-range PCR was performed using iProof High-Fidelity DNA polymerase (Bio-Rad Laboratories) according to the manufacturer's instructions. DNA sequencing was done at Genewiz (South Plainfield, NJ) and at the Nano + Bio Center, University of Kaiserslautern. Nucleotide sequences were analyzed by using DNAStar and CloneManager software.

Transcription of the *lic2* operon. The R6Cho$^-$ strain was grown in C+Y until the culture reached an optical density at 590 nm (OD_{590}) of 0.4. At this time steady-state RNA was isolated using the Trizol reagent. A sample of 10 μg denatured RNA was loaded on a 1.5% formaldehyde denaturation agarose gel. The RNA from the gel was transferred to a Super Nitran membrane using the Turbo Blot system. Cross-linked membrane was next hybridized to the [α-^{32}P]dCTP-labeled combined probe of *licD1-licD2* genes. Hybridization was followed by washing of the membrane, and the transcript of the *lic2* operon was detected by autoradiography.

Inactivation of *tacF* (spr1150) by insertion duplication in the background of R6Cho$^-$. An insertion duplication mutant of spr1150 was constructed in the genetic background of R6Cho$^-$ (Fig. 1). An internal 640-bp DNA element of *tacF* was PCR amplified from R6 genomic DNA using primers spr1150IDF-RI (Table 2) and spr1150IDR-BamHI (Table 2). The product was digested with EcoRI and BamHI and ligated to pJDC9, digested with the same restriction enzymes. The ligation mixture was transferred into competent *E. coli* cells and plated on LB agar containing 1 mg/ml erythromycin. The resulting plasmid, pSPR1150ID, contained the desired insert (*tacF*) as determined by restriction analysis and DNA sequencing. After transformation of *S. pneumoniae* R6Cho$^-$, transformants were selected on blood agar plates (BAP) supplemented with 1 μg/ml erythromycin. One such erythromycin-resistant transformant was named R6Cho$^-$*spr1150ID*. Genomic DNA from R6Cho$^-$*spr1150ID* was used to confirm the insertion duplication event by PCR amplification and sequencing of the PCR product from both ends.

Inactivation of *licD1-licD2* in R6Cho$^-$, D39Cho$^-$, and D39Chi strains. The *licD1-licD2* genes were inactivated in the R6Cho$^-$ genetic background with the help of plasmid pΔD1D2DU31 containing the kanamycin resistance gene *aphIII* as well as the flanking regions for deletion. The 3′ end of *licD1* and the 5′ end of *licD2* including the translation initiation region were deleted, leading to a complete loss of expression of functional proteins. The genetic organization of *licD1* and *licD2* and the extent of the deletion are shown in Fig. 1.

A 657-bp downstream fragment containing *′licD2* was amplified by PCR from R6 genomic DNA using the primers delD2ForPstI and delD2RevHindIII (Table 2). The purified PCR product and plasmid pGEM3Z (29) were digested with PstI and HindIII, ligated, and transformed into competent *E. coli* cells. Transformants were selected on LB agar plates supplemented with 100 μg/ml ampicillin, 40 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and 8

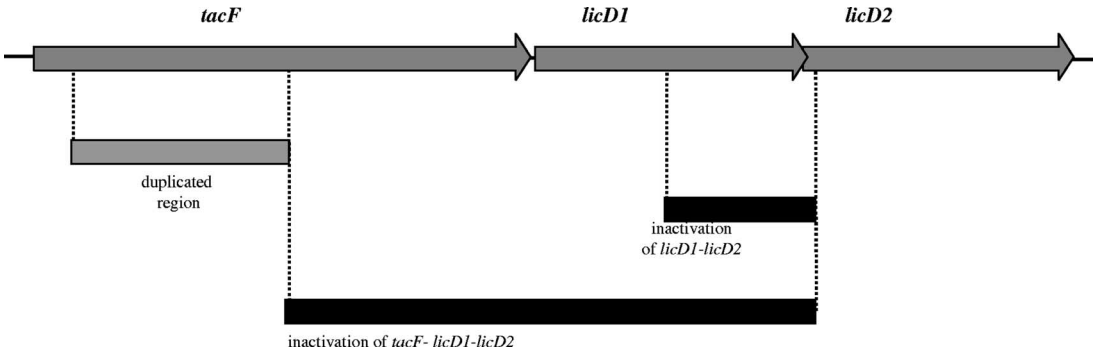

FIG. 1. Schematic representation of the *S. pneumoniae* R6 *lic2* operon and inactivation constructs. The genetic organization of the *tacF*, *licD1*, and *licD2* genes forming the *lic2* operon is presented. Black bars show the extent of deletions in strains R6Cho$^-$Δ*licD1D2* and R6Cho$^-$Δ*lic2*. The indicated parts of the genes were replaced by a kanamycin resistance gene. The internal region of *tacF* used to inactivate the gene by insertion-duplication in strain R6Cho$^-$*spr1150ID* is shown by a gray bar. The same constructs were applied to inactivate genes in a different genetic background.

μg/ml IPTG (isopropyl-β-D-thiogalactopyranoside). White colonies were processed for plasmid isolation and restriction analysis. A recombinant plasmid with the desired insertion was used to clone an upstream fragment harboring *licD1′* for homologous recombination. The upstream fragment was PCR amplified from R6 genomic DNA using primers PRO17 and PRO16 (31). The PCR product and recombinant (*′licD2*) vector were digested with EcoRI-BamHI, purified, ligated, and transformed into *E. coli*. Transformants were selected on LB agar plates supplemented with 100 μg/ml ampicillin. Plasmid pΔD1D2DU3 was confirmed to release a 1.2-kb insert when digested with EcoRI-HindIII corresponding to the combined upstream (*licD1′*) and downstream (*′licD2*) fragments.

Plasmid pΔD1D2DU3 (Table 1) then served to clone the kanamycin resistance gene *aphIII* into the XbaI site located between the upstream and downstream recombination fragments. The resistance gene was PCR amplified from plasmid pR410 (Table 1) using primers DAM301 and DAM347 (Table 2). Plasmid pΔD1D2DU3 was digested with XbaI and end filled with *E. coli* DNA polymerase I (Klenow fragment). Subsequently, the blunt-end kanamycin cassette was ligated to pΔD1D2DU3 and *E. coli* cells were transformed with the ligation mixture. Transformants conferring kanamycin resistance were selected on LB agar supplemented with 50 μg/ml kanamycin. Plasmid pΔD1D2DU31 was confirmed to carry the kanamycin resistance gene *aphIII* between *licD1′* and *′licD2* (Fig. 1). This 2.1-kb insert was PCR amplified with primer pair PRO16 and delD2RevHindIII (Table 2), and the gel-purified amplicon served as donor DNA to transform competent R6Cho$^-$ cells. Kanamycin-resistant transformants were selected on BAP supplemented with 400 μg/ml kanamycin and confirmed to have the constructed insertion/deletion in *licD1-licD2* by both PCR analysis and Southern hybridization experiments. The resulting strain was designated R6Cho$^-$Δ*licD1D2*. In order to inactivate *licD1* and *licD2* in an encapsulated *S. pneumoniae* strain, genomic DNA isolated from R6Cho$^-$Δ*licD1D2* was used to transform competent cells of strain D39Cho$^-$ (expressing capsular polysaccharide 2) to generate transformant D39Cho$^-$Δ*licD1D2*, which was confirmed to carry the insertion/deletion in the *licD1-licD2* genes.

Similarly, D39ChiΔ*licD1D2* was constructed by using genomic DNA isolated from R6Cho$^-$Δ*licD1D2* to transform competent D39Chi cells. Transformants were selected on blood agar-kanamycin plates and were confirmed for deletion of *licD1-licD2*.

Inactivation of the entire *lic2* operon. The *lic2* operon was deleted in the R6Cho$^-$ genetic background using plasmid pΔD1D2DU31 as a vector. In order to achieve this, the upstream recombination fragment (*licD1′*) had to be replaced by a *tacF* fragment (Fig. 1). This fragment was PCR amplified on R6 genomic DNA with the use of spr1150IDF-RI and spr1150IDR-BamHI primer pairs (Table 2) and *Pfu* DNA polymerase. The PCR product was digested with EcoRI-BamHI and ligated to the large fragment of EcoRI-BamHI-digested pΔD1D2DU31. Competent *E. coli* cells were then transformed with the ligation mixture, and transformants were selected on LB agar supplied with the desired amounts of ampicillin and kanamycin. The resulting plasmid was designated pΔLIC2. The insert carried on pΔLIC2 (*tacF′-aphIII-′licD2*) was PCR amplified with *Pfu* DNA polymerase and spr1150IDF-RI and delD2RevHindIII primers. Transformation was performed by using the gel-purified insert as donor and R6Cho$^-$ as recipient. Transformants (R6Cho$^-$Δ*lic2*) were selected on BAP supplemented with 400 μg/ml kanamycin and were confirmed to carry the insertion/deletion by PCR analysis, DNA sequencing, and Southern hybridization experiments. R6Cho$^-$Δ*lic2* genomic DNA was then transferred to D39Cho$^-$, and transformants were selected on BAP containing kanamycin. The transformant D39Cho$^-$Δ*lic2* was confirmed to carry the insertion/deletion by PCR analysis and Southern hybridization experiments.

Detection of choline in purified cell walls. Choline incorporation into the cell wall was determined in R6Cho$^-$, R6Cho$^-$Δ*licD1D2*, R6Cho$^-$Δ*lic2*, D39Chi, and D39ChiΔ*licD1D2*. All strains were grown in Cden medium supplemented with 80 μCi and 5 μg/ml of [^{3}H]choline to an OD_{590} of 0.6. Cell walls were purified (19), and 100 μg purified cell walls was digested with muramidase and processed for quantification of radioactive counts.

Intraperitoneal (i.p.) mouse virulence model. All strains with the D39Cho$^-$ and D39Chi backgrounds were grown in C+Y medium to an OD_{590} of 0.6. Cells were resuspended in pyrogen-free 0.9% sodium chloride solution, 10-fold serial dilutions were prepared, and groups of 8-week-old female CD1 mice (10 mice

TABLE 2. Primers used in this study

Primer	Sequence	Reference
spr1150IDF-RI	CCGAATTCACTATGGTTACTTCAACTCAGTC	This study
spr1150IDR-BamHI	CCGGATCCTCGCTGAGCTATGGTATAGTAAC	This study
delD2ForPstI	CCCTGCAGGACTATATTGATGAGACTTGTAAG	This study
delD2RevHindIII	CGATAAGCTTTAATGCTATGACTATACCACTC	This study
PRO16	CCTGAATTCTTAAAATGAAACAACTAACCGT	21
PRO17	GAAGGGATCCTCAAAGCGATCTATAGGGAAAAT	21
DAM301	CGCGCAAGCTGGGGATCCG	21
DAM347	CCGAATTCTAGGTACTAAAACAATTCATCCAGTAA	21
Uo5_pheA_for	GCCTATTCATCAGCAGTTGATGGTGGTTCC	This study
Uo5_hom_PCR_down	CTTGTCACCCTCTTTGCCATCTTGAAGGATTTGC	This study

per bacterial concentration) were injected in the peritoneal cavity with 0.5 ml of the inoculum containing 10^3 to 10^7 CFU. Mouse survival was monitored.

Intranasal colonization of mice. Strains D39Cho$^-$, D39Cho$^-$Δ*licD1D2*, D39Cho$^-$Δ*lic2*, D39Chi, and D39ChiΔ*licD1D2* were grown to an OD_{590} of 0.6 and centrifuged to pellet bacterial cells. Bacteria were resuspended in pyrogen-free saline (0.9% NaCl) to obtain a bacterial concentration of 10^8 CFU/ml. Groups of 8-week-old CD1 female mice (10 per strain) were anesthetized by i.p. injection of 75 μl of a xylazine and ketamine mixture (12). Suspensions of bacteria (10 μl) were inoculated through the nostrils with a 10-μl blunt-ended Hamilton syringe. Mice were sacrificed 48 h after inoculation by i.p. injection of 100 μl pentobarbital sodium (Nembutal). Bacteria colonizing the nasopharynx were collected by expelling 50 μl saline solution through the trachea. Viable counts were determined on BAP supplemented with 5 μg/ml gentamicin.

RESULTS AND DISCUSSION

The status of the *lic1* operon in R6Chi and R6Cho$^-$. Confirmation of the existence of a *lic1* operon in R6Cho$^-$ (12) and R6Chi (4) was provided by isolation and characterization of steady-state mRNA which showed that the five genes spr1148, spr1149, *licA*, *licB*, and *licC* formed a polycistronic message the molecular size of which corresponded to the combined size of these genes (12).

Genes of the *lic1* operon were shown to be essential for growth and survival of all isolates of *S. pneumoniae* except in strains R6Cho$^-$ and R6Chi. Strain R6Cho$^-$ and R6Chi with inactivated *licA*, *licB*, or *licC* continued to grow in choline-containing growth medium in the form of long autolysis-resistant chains of cells. Formation of long autolysis-resistant chains was also the phenotype of R6Chi and R6Cho$^-$, which carried functional genes in the *lic1* operon but were growing in choline-free medium. In vitro, addition of choline to the medium of such cultures resulted in a rapid breakup of chains and return of the autolysis-prone phenotype. This choline-dependent reversion was blocked in mutants of the *lic1* operon in both R6Cho$^-$ and R6Chi (4, 12).

In vivo, inactivation of the same *lic1* operon genes in derivatives of both R6Cho$^-$ and R6Chi expressing a type 2 capsular polysaccharide caused a drastic reduction in virulence presumably for the same reason, i.e., by preventing utilization of choline and preserving a choline-free cell surface (4, 12).

The status of the *lic2* operon in R6Chi and R6Cho$^-$. CDP-choline, the final product of proteins encoded by genes of the *lic1* operon, was proposed to serve as the substrate of one or two P-choline transferases encoded by the genes *licD1* and *licD2* (31)—hypothetical choline transferases which catalyze the incorporation of P-choline into teichoic acid precursors or teichoic acid polymers. The genetic organization of *licD1*, *licD2*, and a third gene, spr1150, on the chromosomes of strains R6 and TIGR4 suggested that these genes may form a single *lic2* operon. In order to confirm this, we performed steady-state mRNA analysis on extracts prepared from strain R6Cho$^-$ grown in a choline-containing medium. The RNA preparation was hybridized to an [α-^{32}P]dCTP product prepared from the PCR product of *licD1-licD2*. Northern blot analysis demonstrated the presence of a single 3.2-kb polycistronic RNA which corresponds to the combined sizes of the genes spr1150 (renamed *tacF*), *licD1*, and *licD2* (data not shown).

The structure and function of *tacF* in the choline-independent strain R6Cho$^-$. The mechanism of choline independence in mutant R6Chi is a single G→T point mutation at base position 700 in *tacF*, the first gene in the *lic2* operon (4). The protein product of *tacF* was shown to have structural features characteristic of polysaccharide transmembrane transferases (flippase). It was proposed that the flippase of parental-type pneumococci can transport lipid-linked teichoic acid units to the outer surface of the pneumococcal plasma membrane only if the teichoic acid chains carry P-choline residues. This structural requirement appears to be lost in the *tacF* G700T point mutant, which can also transport choline-free teichoic acid chains and thus provides the mutant R6Chi with a choline-independent phenotype (4).

We sought to test whether R6Cho$^-$ also carried the G700T transversion within the *tacF* gene. Three types of experiments were performed.

In the first experiment *tacF* along with its promoter was PCR amplified with *Pfu* DNA polymerase on R6Cho$^-$ genomic DNA and processed to decipher the nucleotide sequence. Results of DNA sequencing of the *tacF* gene from R6 and R6Cho$^-$ revealed that the R6Cho$^-$ sequence was 100% identical to that of the parental strain R6, i.e., strain R6Cho$^-$ did not carry the G700T transversion.

In a second experiment, the *tacF* gene of R6Cho$^-$ along with its promoter was cloned in pMSP3535 (1) and transformed into strain R6. If overexpression of *tacF* of R6Cho$^-$ were responsible for the choline independence of this strain, then R6 transformants carrying copies of plasmid-borne *tacF* from R6Cho$^-$ should become choline independent and grow in a choline-free environment. Erythromycin-resistant transformants were tested for growth in a choline-free environment. None of the transformants obtained could grow in choline-free Cden medium.

Finally, we inactivated *tacF* in strain R6Cho$^-$ by an insertion duplication strategy (Fig. 1) using the set of insertion duplication primers described in Materials and Methods. Transformant R6Cho$^-$*spr1150ID* remained fully viable and continued to exhibit physiology similar to that of parental strain R6Cho$^-$ when grown in choline-containing medium. Attempts to inactivate spr1150 (renamed *tacF*) with the same strategy in the background of R6Chi were unsuccessful, indicating the essentiality of this gene. The essentiality of *tacF* (spr1150) in the parental strain R6 has already been documented (20).

Inactivation of the genes of the *lic2* operon: impact on the growth and phenotype of R6Cho$^-$ and R6Chi. Deletion mutants of *licD1* and *licD2* were constructed in the background of R6Cho$^-$ (see Materials and Methods; also Fig. 1). Cultures of the deletion mutant R6Cho$^-$Δ*licD1D2* grew in choline-containing medium in the form of diplococci or short chains, autolyzed in the stationary phase, and retained sensitivity to deoxycholate-induced lysis (Fig. 2A).

Next we proceeded to delete the entire *lic2* operon—including not only *licD1* and *licD2* but *tacF* (spr1150) as well (Fig. 1)—to yield strain R6Cho$^-$Δ*lic2*. The strain R6Cho$^-$Δ*lic2* has remained fully viable and retained the normal physiology: growth in the form of autolysis-prone pneumococci with diplococcal morphology, indistinguishable from the phenotype of the parental strain R6Cho$^-$ when grown in choline-containing medium (Fig. 2A).

In sharp contrast to the *licD1D2* deletion mutant of R6Cho$^-$, inactivation of the same genes in R6Chi (R6ChiΔ*licD1D2*) produced a striking and abnormal phenotype: the mutant cells grew

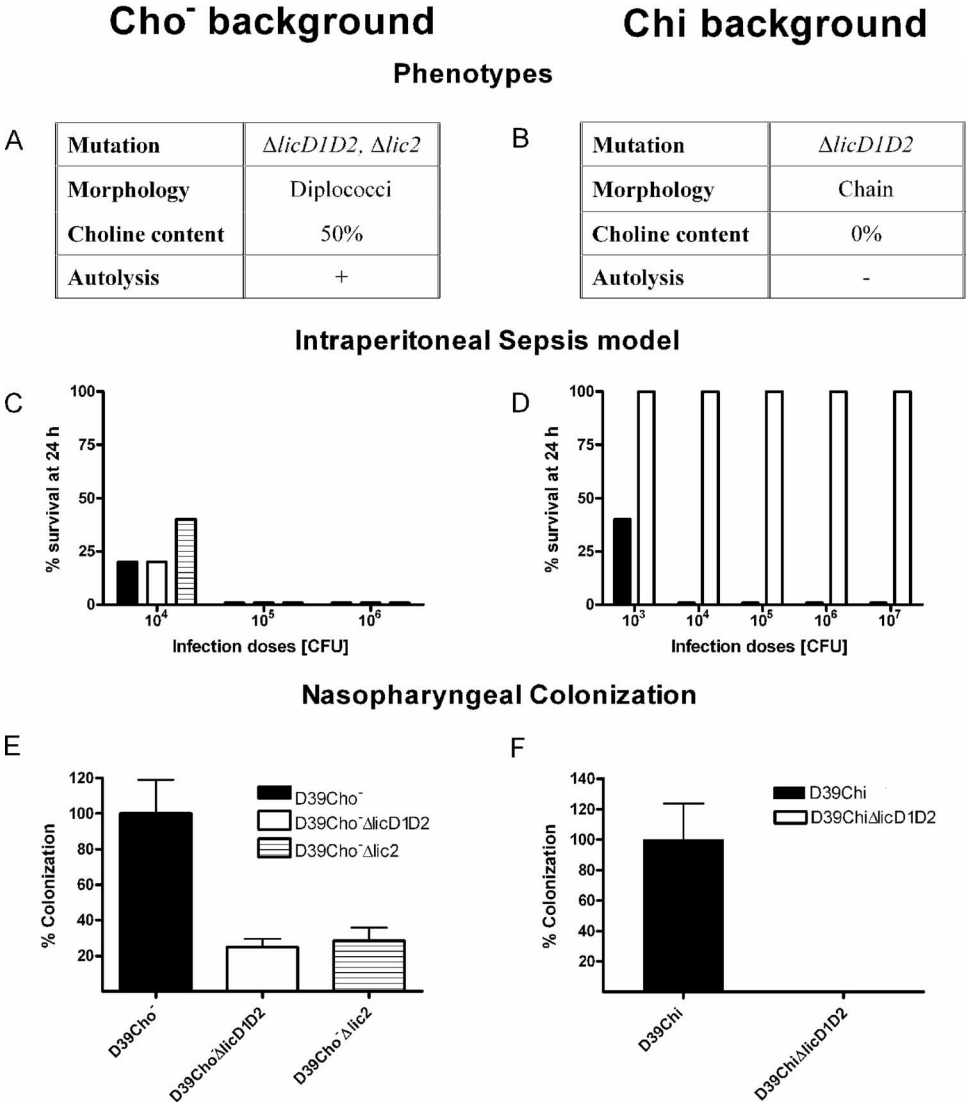

FIG. 2. Effects of mutations in the *lic2* operon on the virulence and phenotypes of the two choline-independent strains D39Chi and D39Cho$^-$. Strains D39Chi, D39ChiΔ*licD1D2*, D39Cho$^-$, D39Cho$^-$Δ*licD1D2*, and D39Cho$^-$Δ*lic2* were tested and compared in the mouse nasopharyngeal colonization assay (E and F) and in the mouse i.p. virulence assay (C and D). Phenotypes of strains R6Chi (B) and R6Cho$^-$ (A) carrying mutations in the *lic2* operon. Strains were grown in choline-containing medium.

in long chains and became completely resistant to autolysis even when grown in choline-containing medium (Fig. 2B). Thus, in R6Chi, the inactivation of *licD1* and *licD2*—putative P-choline transferases—produced the same phenotype as did inactivation of genes in the *lic1* operon (12). These observations strongly suggest that this deletion mutant must produce a choline-free teichoic acid even when grown in medium in which choline is available. Chemical determination of the choline content of such mutants has confirmed this prediction (see below).

In conclusion, inactivation of *licD1D2* caused completely different phenotypes in the two choline-independent strains, strongly suggesting that the mechanism of choline independency should be different in the two strains.

Choline content of the cell walls in *lic2* mutants of R6Cho$^-$ and R6Chi. We determined the choline content of cell walls purified from strains R6Cho$^-$ and R6Cho$^-$Δ*licD1D2* (and R6Cho$^-$Δ*lic2*) and from R6Chi and R6ChiΔ*licD1D2*—each culture growing in choline-containing medium which was also supplemented with [^{3}H]choline. An aliquot of 100 μg purified cell walls was digested with muramidase and processed to determine choline content as described in Materials and Methods. Results shown in Fig. 2A indicate that the cell walls of the R6Cho$^-$Δ*licD1D2* mutants had reduced choline content, corresponding to about 50% of the choline content of the parental strain R6 grown under the same conditions. Apparently, this reduced choline content in the cell walls was sufficient for the cells to maintain a normal (diplococcal and autolysis-prone) phenotype (Fig. 2A).

In contrast, deletion of *licD1-licD2* in the Chi mutant resulted in a completely choline-free cell wall (Fig. 2B), which paralleled the abnormal (chain-forming and autolysis-defective) phenotype of the bacteria.

Impact of inactivation of genes in the *lic2* operon on the virulence of D39Cho$^-$ and D39Chi. The availability of strains D39Cho$^-$Δ*licD1D2* and D39Cho$^-$Δ*lic2*—containing just 50% of the normal amount of surface-bound choline—gave us a tool to determine the impact of cell wall choline content on virulence. Cultures of D39Cho$^-$, D39Cho$^-$Δ*licD1D2*, D39Cho$^-$Δ*lic2*, D39Chi, and D39ChiΔ*licD1D2* were grown in choline-containing medium to an OD_{590} of 0.6. Bacteria were pelleted, washed, and resuspended in pyrogen-free saline; various bacterial concentrations were inoculated into the peritoneal cavity of 8-week-old CD1 female mice; and the survival rate of the animals was determined. As expected, D39Cho$^-$ and D39Chi showed similar high degrees of virulence (Fig. 2C and D).

Interestingly, deletion of *licD1-licD2* as well as of the entire *lic2* operon in D39Cho$^-$, which caused a reduction of the choline content of cell walls to about half of that of the parent strain D39Cho$^-$, had only a minor effect on the virulence potential of this strain (Fig. 2C).

In contrast, D39ChiΔ*licD1D2*, in which the cell wall choline content was reduced to zero, exhibited a dramatic drop in virulence: mice inoculated with 10^7 CFU of D39ChiΔ*licD1D2* showed 100% survival even after 24 h whereas mice inoculated with 10^4 CFU of D39Chi died within 24 h (Fig. 2D).

Impact of inactivation of genes of the *lic2* operon on the colonizing capacity of D39Cho$^-$ and D39Chi. Strains D39Cho$^-$, D39Cho$^-$Δ*licD1D2*, D39Cho$^-$Δ*lic2*, D39Chi, and D39ChiΔ*licD1D2* were grown in choline-containing medium to an OD_{590} of 0.6. Suspensions of bacteria (10 μl) at a concentration of 10^8 CFU/ml were inoculated through the nostrils, and mice were sacrificed after 48 h as described in the work of Kharat and Tomasz (12). Colonizing bacteria were enumerated on BAP supplemented with 5 μg/ml gentamicin.

While the approximately 50% reduction in the amount of surface-bound choline in strains D39Cho$^-$Δ*licD1D2* and D39Cho$^-$Δ*lic2* had little effect on virulence in the i.p. assay (Fig. 2C), the colonizing capacity of these mutants was reduced significantly to 25% and 28% of the control levels, respectively (Fig. 2E).

The completely choline-free D39ChiΔ*licD1D2* strain failed to colonize (Fig. 2F). This complete loss of colonizing capacity was comparable to the suppression of colonizing capacity already described for *lic1* mutants of both D39Cho$^-$ (12) and D39Chi (4), neither one of which had any detectable choline residues in their cell walls.

R6Cho$^-$ has a large deletion in the vicinity of the spr1226 gene. Earlier studies showed that, during the process of the genetic cross that led to the acquisition of choline independence, the transformant R6Cho$^-$ lost a SmaI recognition site (19) and the lost SmaI recognition sequence was mapped within spr1226 (12). Interestingly, spr1225, the gene located upstream of spr1226 in the R6 genome, is annotated as a *licD1* paralog and the same gene in the TIGR4 genome is annotated as *licD3* (11, 23). The spr1225 gene is not an exact copy of *licD1* or *licD2* but bears significant homology to *licD1* of R6 and TIGR4. The function of the spr1225 gene is not known. To distinguish this gene clearly from *licD1* in the *lic2* locus, we adopted the TIGR4 annotation and designated it *licD3*.

We sought to test the status of spr1225 in R6Cho$^-$. Genomic DNA obtained from R6 and R6Cho$^-$ was digested with various restriction endonucleases and subjected to Southern blot analysis using an [α-^{32}P]dCTP-labeled probe. No hybridization signals were detected with R6Cho$^-$ DNA (data not shown), indicating that *licD3* has been deleted in R6Cho$^-$.

Since both spr1226 and *licD3* (spr1225) were deleted in the R6Cho$^-$ strain, we sought to investigate the extent of the deletion in R6Cho$^-$ by designing probes for *pheA* and *aroK* upstream of spr1226 and for spr1224, *hom*, and *msrA* downstream of *licD3* (Fig. 3B). In these Southern blot experiments no signals were obtained from R6Cho$^-$ DNA with spr1224 and *hom* probes and only a weak signal with *pheA* (data not shown). Clear hybridization was detected with *msrA* and *aroK* probes. These results demonstrate that a region of at least 10 kb has been deleted in the *licD3* region of strain R6Cho$^-$. In addition, weak hybridization signals may indicate replacement of R6 genes by similar but not identical *S. oralis* homologues.

Identification of the inserted *S. oralis* DNA in strain R6Cho$^-$. In the course of the Southern blotting experiments, a preliminary genome sequence of strain *S. oralis* Uo5 (16) became available. The genomic region of this strain corresponding to the observed deletion in R6Cho$^-$ suggested that the genes that were not detected in the Southern blot experiments could have been replaced by a large *S. oralis* region, which included *lic* genes. The sequences surrounding this *S. oralis* locus appear to be similar enough to allow its integration into the pneumococcal genome by homologous recombination. In order to document this large replacement in strain R6Cho$^-$, long-range PCR experiments were performed using primers located inside a number of genes in this genomic region. Primers were designed to match genes of both strains, R6 and *S. oralis* Uo5, which should improve their chances of also matching DNA from *S. oralis* ATCC 35037, the strain used to produce strain R6Cho$^-$. Several PCR products were obtained by this approach, all indicating that a large replacement of R6 DNA by *S. oralis* DNA occurred. As an example, the 13-kb product amplified from R6Cho$^-$ DNA with primers located in *hom* and *pheA* is shown in Fig. 3. The corresponding fragment of R6 is only 10 kb in size (Fig. 3A).

The nucleotide sequence of this 13-kb PCR product revealed that genes implicated in choline metabolism were integrated in R6Cho$^-$, replacing endogenous R6 genes (Fig. 3B). A new copy of *licD3* is found right next to the spr1226 homologue followed by a gene encoding a putative glycosyltransferase (*orf2*). A second *licD* gene, tentatively designated *licD4*; genes (*orf4* and *orf5*) for two hypothetical proteins; and the phosphocholine esterase gene *pce* (26) are additionally present in this locus. The LicD4 protein has a large extension of about 450 amino acids at its N terminus, which is not found in other LicD proteins. A *tacF* homologue encoding teichoic acid flippase is found downstream of *pce* and on the opposite strand (Fig. 3B).

The DNA sequence of the *hom-pheA* long-range PCR fragment of R6Cho$^-$ also revealed that the DNA is completely of *S. oralis* origin. Therefore, integration of *S. oralis* DNA occurred beyond the borders of this fragment. DNA sequencing by primer walking eventually identified the site of crossing over within *thrB* and *aroE* (Fig. 3B). Thus, a large 20,249-bp fragment of *S. oralis* DNA replaced the 16,920 bp of the R6 genome during the transformation event leading to the loss of

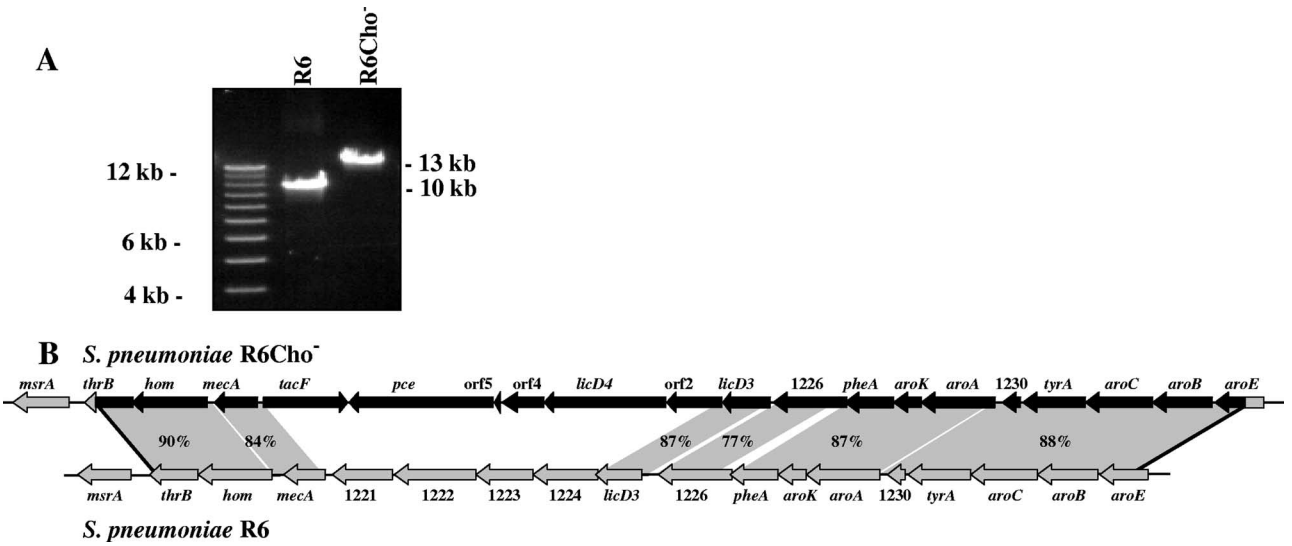

FIG. 3. (A) PCR analysis of the *licD3* locus in *S. pneumoniae* R6 and the choline-independent derivative R6Cho$^-$. Long-range PCR products using primers located in *hom* and *pheA* (see panel B) with genomic DNA of R6 and R6Cho$^-$ are shown on a 1% agarose gel. (B) Schematic representation of the *licD3* locus in *S. pneumoniae* R6 and the choline-independent derivative R6Cho$^-$. The genomic organization of the *licD3* region of *S. pneumoniae* R6 (bottom) is shown along with the same region of *S. pneumoniae* R6Cho$^-$ after integration of *S. oralis* ATCC 35037 DNA (top). Endogenous R6 genes are shown by gray arrows and are indicated by gene names or "spr" numbers (11, 12, 31). Acquired *S. oralis* genes are shown by black arrows. The sites of recombination are indicated by black lines. Replacement of *S. pneumoniae* R6 DNA by *S. oralis* ATCC 35037 DNA occurred between *thrB* (position 1.214.973 in the R6 genome) and *aroE* (position 1.231.893). The degree of identity (percent) between *S. pneumoniae* and *S. oralis* genes is indicated. Some of the gene products, whose genes showed no significant similarity to R6, could be identified by their protein similarity. Designations *orf2*, *orf4*, and *orf5* were given according to their positions in the presumed *licD3* operon of *S. oralis*. Identities of R6 genes spr1221 to spr1224 to *S. oralis* genes could not be assessed because the whole-genome sequence of *S. oralis* ATCC 35037 is not available. *S. oralis* homologues of R6 genes spr1226 and spr1230 were given the same number to highlight similarity. They are not likely to occupy the same position in the *S. oralis* genome. The *S. oralis* nucleotide sequence is available from GenBank (accession number EU675999).

choline auxotrophy—a property unique to the species *S. pneumoniae*.

The mechanisms of choline independence in R6Chi and R6Cho$^-$. The experimental results described in this communication indicate that the mechanisms of choline independence in R6Chi and R6Cho$^-$ are different in several respects.

R6Chi carries a mutated form of the first gene of the *lic2* operon, *tacF*, and this mutation appears to be the molecular basis of the choline-independent phenotype. A functional *lic2* operon remains essential for the survival and the choline-independent phenotype of this strain.

R6Cho$^-$ also appears to have retained the pneumococcal *lic2* operon as indicated by the capacity of the strain to produce a polycistronic message that corresponds precisely to the size of the pneumococcal *lic2* operon. While this pneumococcal operon is no longer essential for the survival of bacteria, it may remain functional under some conditions of growth.

The survival and physiological properties of R6Cho$^-$ derivatives from which the entire *lic2* operon was deleted clearly indicate that genes of the *lic2* operon play no roles in the choline-independent phenotype of this strain. Therefore, one could predict that the acquisition of genetic elements from the heterologous *S. oralis* strain included a functional equivalent of *tacF* and a second gene that could replace the phosphorylcholine transferase function of the pneumococcal *licD1*. The nucleotide sequence of the *S. oralis* insertion in R6Cho$^-$ is fully consistent with this prediction, as it revealed homologues of *tacF* and *licD* (Fig. 3B).

The most likely candidate to carry out the phosphorylcholine transferase reaction in strain Cho$^-$ is the protein encoded by the imported *S. oralis* gene *licD4*. Its C-terminal domain is most similar to the LicD1 and LicD2 proteins of *S. pneumoniae*, showing 35% and 32% identical residues, respectively. Another LicD domain is found in the gene product of *orf4*, but similarity is rather limited and incomplete. The LicD3 protein is also less likely to be involved in phosphorylcholine transfer, since the very similar LicD3 of R6 (93% identity) plays no role in this process. This is confirmed by our finding that strain D39ChiΔ*licD1D2* is free of surface-bound choline despite the fully functional *licD3* gene. Gene inactivation studies will be needed to unambiguously identify the phosphorylcholine transferase(s) encoded in this region.

The second component expected to be present in the heterologous *S. oralis* DNA is *tacF*, encoding the teichoic acid flippase. While the endogenous R6 gene is part of the *lic2* operon located about 60 kb from the *licD3* locus, *tacF* of *S. oralis* is located immediately downstream of the presumptive *licD3* operon (Fig. 3B) and appears to be a single transcriptional unit. In contrast to TacF of R6, *S. oralis* flippase is able to export teichoic acids with or without attached choline phosphoryl groups as indicated by the ability of *S. oralis* to grow in choline-containing and choline-free medium as well (10). Both enzymes have 47% residues in common.

In addition to *tacF* and *licD* genes, a gene (*orf2*) for a glycosyltransferase, a small gene (*orf5*) encoding a protein of unknown function, and *pce* specifying phosphocholine esterase (26) are also contained in the acquired *S. oralis* DNA. Interestingly, the glycosyltransferase of *orf2* is similar (42% identical

amino acids) to that of spr1223, which was lost upon *S. oralis* DNA insertion (Fig. 3B). Since *pce* of R6 is located outside the *licD3* region, R6Cho$^-$ is equipped with two phosphocholine esterases with 45% identical residues.

Both choline-independent mutants, R6Cho$^-$ and R6Chi, share the common physiological abnormalities of chain formation and defective autolysis when grown in choline-free medium. These phenotypes are understandable in terms of the known structural requirements of two pneumococcal enzymes, LytB and LytA, both of which require choline residues in the cell wall for their activity (7, 8, 22). However, we found that 50% surface-bound choline—as seen in the *lic2* deletion mutants of R6Cho$^-$—was still sufficient to guarantee a functioning autolytic system, allow complete daughter cell separation, and ensure the wild-type diplococcal morphology of the bacteria.

Another surprising—and novel—observation described in this report was the drastic and selective reduction in the colonizing capacity of strains D39Cho$^-$Δ*licD1D2* and D39Cho$^-$Δ*lic2* carrying only a single choline residue per teichoic acid chain, which was not accompanied by a comparable reduction in the level of virulence of these strains as measured in the i.p. mouse model of disease. These findings suggest that different stages of pneumococcal pathogenesis may have differential sensitivities to the number of choline residues available at the cell surface.

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