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The Essential *tacF* Gene Is Responsible for the Choline-Dependent Growth Phenotype of *Streptococcus pneumoniae*▽

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Streptococcus pneumoniae **has an absolute nutritional requirement for choline, and the choline molecules are known to incorporate exclusively into the cell wall and membrane teichoic acids of the bacterium. We describe here the isolation of a mutant of strain R6 in which a single G→T point mutation in the gene *tacF* (formerly designated *spr1150*) is responsible for generating a choline-independent phenotype. The choline-independent phenotype could be transferred to the laboratory strain R6 and to the encapsulated strain D39 by genetic transformation with a PCR product or with a plasmid carrying the mutated *tacF* gene. The *tacF* gene product belongs to the protein family of polysaccharide transmembrane transporters (flippases). A model is presented in which TacF is required for the transport of the teichoic acid subunits across the cytoplasmic membrane. According to this model, wild-type TacF has a strict specificity for choline-containing subunits, whereas the TacF present in the choline-independent mutant strain is able to transport both choline-containing and choline-free teichoic acid chains. The proposed transport specificity of parental-type TacF for choline-containing subunits would ensure the loading of the cell wall with teichoic acid chains decorated with choline residues, which appear to be essential for the virulence of this pathogen.**

One of the unique properties of the human pathogen *Streptococcus pneumoniae* is the strict nutritional requirement for choline (18), which is taken up from the growth medium and metabolized exclusively to be attached to the wall teichoic acid and the lipoteichoic acid (25). The choline residues of the teichoic acid are involved in a wide variety of physiological functions, including the activation of the murein (peptidoglycan) hydrolases LytA, LytB, and LytC (8, 9, 11, 17), the binding of a class of surface proteins (the choline-binding proteins) (20, 29), and the interaction with bacteriophages (15) and host proteins, for example, with the receptor for the platelet-activating factor (5). The essentiality of choline residues for pneumococcal virulence recently has been demonstrated (13).

Choline is transported into the cytoplasm and is activated to CDP-choline by the products of the *licABC* genes (2, 3). CDP-choline is the substrate of proteins (presumably the products of the *licD1* and *licD2* genes) that attach the phosphoryl choline residues to the teichoic acid precursors (30). These genes are clustered in the *lic* region on the pneumococcal chromosome, which contains three additional genes, *spr1148*, *spr1149*, and *spr1150*, of unknown functions. Based on sequence comparisons, it was suggested that these genes may encode an alcohol dehydrogenase (*spr1148*), a CDP-ribitol pyrophosphorylase (*spr1149*), and a sugar transporter (*spr1150*) (30).

Although the cell wall choline residues are of great importance for the physiology of *S. pneumoniae*, none of the choline-related functions is essential for cell growth, and the molecular mechanism(s) responsible for the choline dependency of pneumococcal growth has remained unknown. Until now, two choline-independent mutant strains of *S. pneumoniae* have been reported. Mutant R6Cho$^-$ was obtained with a background of *S. pneumoniae* strain R6 from a heterologous genetic cross with DNA from *Streptococcus oralis*, which has a choline-containing cell wall but, unlike *S. pneumoniae*, is able to grow in the absence of exogenous choline (22). A second mutant, JY2190, was obtained after successive growth of *S. pneumoniae* strain Rx1 in medium without choline but with decreasing concentrations of ethanolamine (28). It is currently unknown which genetic change(s) is responsible for the choline-independent phenotype of these two mutants.

In this communication, we describe a third choline-independent mutant, R6Chi, which was obtained with a background of *S. pneumoniae* strain R6. The method of isolation was similar to the one used for the isolation of mutant JY2190 of strain Rx1: cultures of strain R6 were grown in chemically defined medium in which the choline component was replaced by gradually decreasing concentrations of ethanolamine and eventually by no aminoalcohol at all. We have identified a single-point mutation residing in the so-far uncharacterized gene *spr1150* (which we rename *tacF*), which appears to be responsible for the choline independence of R6Chi. We present a model for the physiological function of TacF and for the mechanism of the pneumococcal nutritional requirement for choline.

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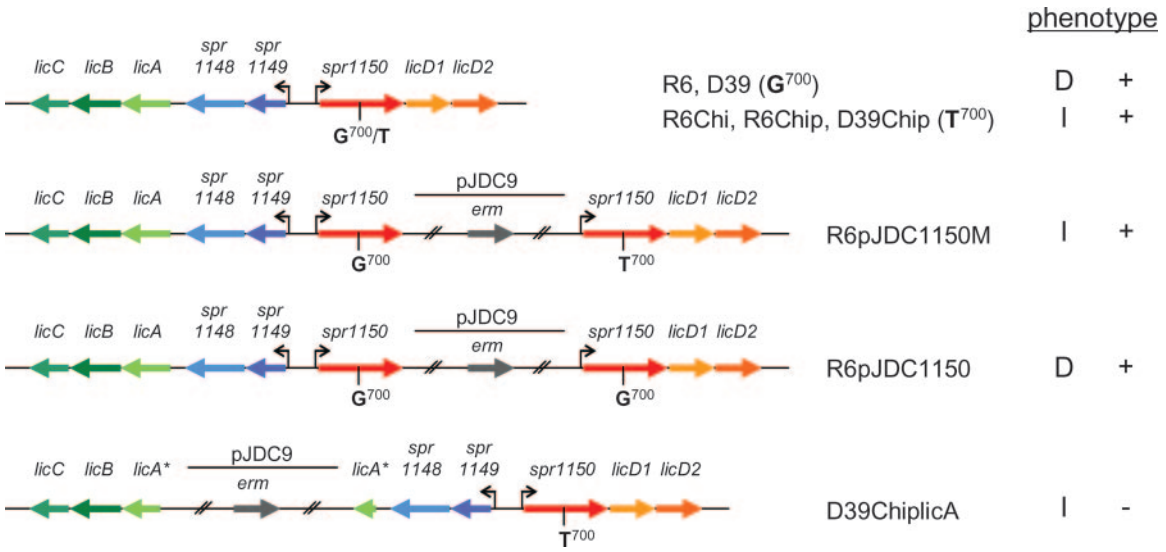


FIG. 1. Organization of the *lic* region of the different strains used in this study. The base at position 700 of the *spr1150* (*tacF*) gene is indicated. An asterisk indicates an inactivated *licA* gene. Divergent transcription of the choline utilization genes driven by promoters is denoted by a perpendicular arrow before *spr1149* and *spr1150*, respectively. erm, erythromycin resistance gene present on the integration plasmid pJDC9. Phenotype abbreviations: D, choline-dependent growth; I, choline-independent growth; +, choline incorporation from the growth medium; −, no choline incorporation from the growth medium.

MATERIALS AND METHODS

Strains and growth conditions. *S. pneumoniae* D39 and R6 (27) were grown at 37°C in Cden medium (26) with 5 μg/ml of choline or in complex C+Y medium containing 1 mg/ml yeast extract (12). Cden agar plates contained 1.5% agar. Growth on Cden agar plates was documented after they were replica plated on nitrocellulose filters, followed by staining with 0.1% amido black solution (45% methanol, 10% acetic acid) and destaining with water. Competent pneumococci were obtained as previously described (14), with the addition of competence peptide (16). *Escherichia coli* DH5α (10) was grown in Luria broth at 37°C with aeration. If necessary, erythromycin was added at a concentration of 1 mg/ml for *E. coli* and 1 μg/ml for *S. pneumoniae*.

Generation of a choline-independent mutant. R6 was grown sequentially at 37°C in Cden medium lacking choline but containing decreasing concentrations of ethanolamine (200, 20, 2, 0.2, and 0 μg/ml) as described previously (28). The culture then was plated on tryptic soy broth containing 3% sheep blood. One clone capable of growing in the absence of choline in the growth medium was named R6Chi. ^{1}H and ^{31}P nuclear magnetic resonance analyses of isolated cell walls confirmed the absence of phosphorylcholine residues in R6Chi grown in the absence of choline.

Generation of R6pJDC1150M, R6pJDC1150, R6Chip, and D39Chip. The plasmids pJDC1150 and pJDC1150M were constructed to transfer the *tacF* gene to pneumococcal strains by insertion duplication mutagenesis. For this, the *tacF* region (positions 1151093 to 1153039) was amplified by PCR with the primers 5′-CCATGCGAGCTCCATAAAAGAAATACTGGTCC-3′ and 5′-TATGTCG GATCCACAGATCAATATCGTCGTCC-3′, using chromosomal DNA from R6Chi (for pJDC1150M) or from R6 (for pJDC1150). The PCR product was restricted with SacI and BamHI and ligated into plasmid pJDC9 (4), which was treated with the same restriction enzymes. pJDC1150M and pJDC1150 were transformed into R6 under selection for erythromycin to generate R6pJDC1150M and R6pJDC1150, respectively. The correct integration of pJDC1150M and pJDC1150 was confirmed by PCR analysis using the primer pairs 5′-ATTACGCCAAGCTTGCATGC-3′ and 5′-AAATAGGTCGATCACC TAGC-3′, 5′-CACGACGTTGTAAAACGACG-3′ and 5′-CGTTGGTAGTCT TCTCTAGG-3′, and 5′-AAAGGGCATTTAACGACGAAACTGG-3′ and 5′-GACAATACTTGCTCATAAGTAACGG-3′. The PCRs with chromosomal DNA from R6pJDC1150M and R6pJDC1150 (but not from R6) yielded products of the expected sizes, covering both boundaries of the plasmid integration site as well as an internal fragment of the erythromycin resistance gene on pJDC9. The choline-independent strains R6Chip and D39Chip were generated by transforming the respective parental strains (R6 and D39) with a PCR product covering the *tacF* region (see above) and selection on choline-free Cden agar plates.

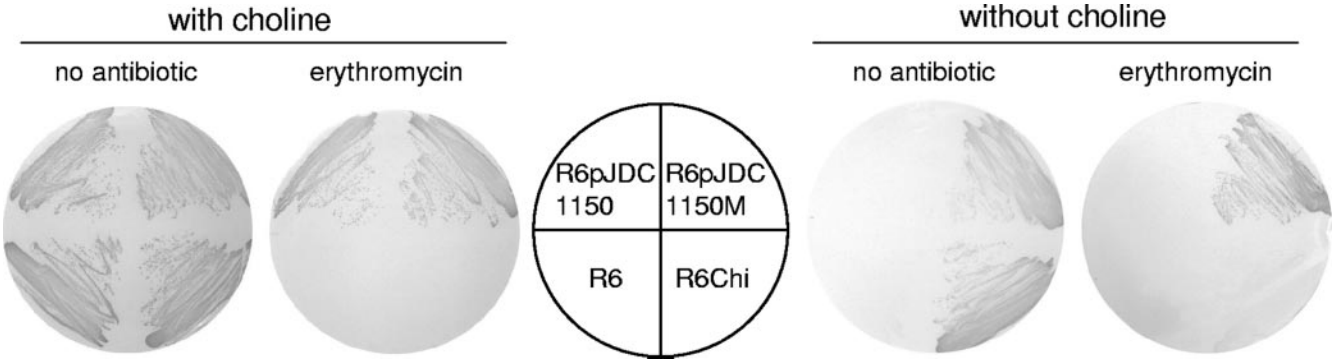


FIG. 2. Growth of the different strains on Cden plates with or without choline and erythromycin. Only the strains with the mutated *tacF* gene, R6Chi and R6pJDC1150M, are capable of growing in the absence of choline. Only the strains transformed with a pJDC9-based plasmid are resistant to erythromycin.

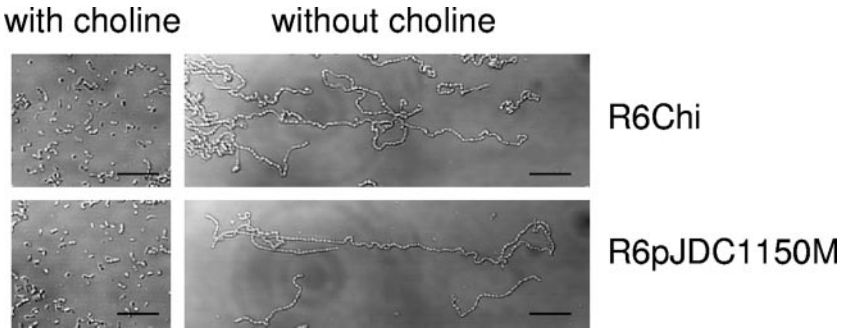


FIG. 3. Cells of R6Chi and R6pJDC1150M visualized by light microscopy. Both strains grew as diplococci, and they grew in short chains in the presence of choline and in long chains in the absence of choline. Bar, 10 μm.

Inactivation of *lic* genes. The *licA*, *licB*, and *licC* genes were inactivated in R6Chi and D39Chi with the help of pJDC9-based plasmids containing internal sequences of the genes to be inactivated, as described before (13). Inactivation of *licA* most likely has polar effects on the expression of *licB* and *licC*. All three *lic* mutants are unable to utilize exogenous choline. Therefore, an inactivation of these *lic* genes is possible only in choline-independent mutant strains.

Intraperitoneal mouse virulence model. Strains D39Chip and D39ChiplicB31 were grown in C+Y medium to an optical density at 590 nm of 0.6, cells were resuspended in pyrogen-free 0.9% sodium chloride solution, and 10-fold serial dilutions were prepared. Groups of 8-week-old female CD1 mice (5 mice per bacterial concentration) were injected (via the peritoneal cavity) with 0.5 ml of the inocula containing between 10^3 to 10^7 CFU. Mouse survival was monitored for 3 to 7 days.

Intranasal mouse colonization model. Strains D39Chip, D39ChiplicA65, D39ChiplicB31, and D39ChiplicC112 were grown to an optical density at 590 nm of 0.6 and were 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 using a xylazine and ketamine mixture (75 μl injected into the peritoneal cavity), as described by Kharat and Tomasz (13). Suspensions of bacteria (10 μl) were inoculated through the nostrils with a 10-μl blunt-end Hamilton syringe. Mice were sacrificed 48 h after inoculation by injection with 100 μl of Nembutal into the peritoneal cavity. Bacteria colonizing the nasopharynx were collected by expelling 50 μl saline solution through the trachea and were enumerated on blood agar plates supplemented with 5 μg/ml gentamicin.

Other methods. DNA sequencing of PCR products and plasmids was done with the help of custom-made oligonucleotides (http://biomers.net/default.aspx) at 4BaseLab, Reutlingen, Germany. The isolation of chromosomal DNA and of plasmids, PCR and purification of PCR products, and separation of DNA in agarose gels were done according to standard procedures described previously (21). Bacterial cells were visualized by light microscopy using a 40× objective.

RESULTS

Identification of a point mutation in the *spr1150* gene of R6Chi. The choline-independent mutant R6Chi was isolated as described in Materials and Methods. Several genes with known or hypothetical functions in pneumococcal cell wall metabolism were sequenced from R6Chi and from its parental strain, R6. The obtained sequences were compared to the corresponding sequences of strains TIGR4 and R6 in the public databases. A single-point mutation, a T replacing a G at position 1152062, was identified in the chromosomal region of bp 1150093 to 1153021 of mutant R6Chi. The mutation was identified to reside at position 700 in the hypothetical *spr1150* gene, and it was absent from the sequence determined from chromosomal DNA of the parental strain, R6 (Fig. 1).

The point mutation in *spr1150* is responsible for the choline-independent phenotype. The chromosomal region from 261 bp upstream to 179 bp downstream of *spr1150* (bp 1151093 to 1153039) from R6Chi or from R6 was cloned into plasmid pJDC9, resulting in the plasmid pJDC1150M or pJDC1150, respectively. Upon transformation into R6 and selection for resistance to erythromycin, the plasmids integrated into the chromosome, leading to strains R6pJDC1150M and R6pJDC1150. R6pJDC1150M carries a wild-type and a mutated copy of *spr1150*, whereas R6pJDC1150 carries two copies of the wild-type *spr1150* (Fig. 1). R6pJDC1150M, but not R6pJDC1150, was able to grow in the absence of exogenous choline in liquid medium and on agar plates (Fig. 2). In another genetic experiment, the product of the PCR amplification of the *spr1150* region of R6Chi was directly transformed into R6 or into the encapsulated D39 strain with selection on choline-free Cden plates. Choline-independent transformants with the mutated *spr1150* gene were obtained, and one clone of each transformation experiment was isolated and named R6Chip and D39Chip, respectively.

These results prove that the $G^{700}\rightarrow T$ mutation in *spr1150* enables pneumococci to grow in the absence of exogenous choline. Furthermore, the mutated *spr1150* gene is dominant

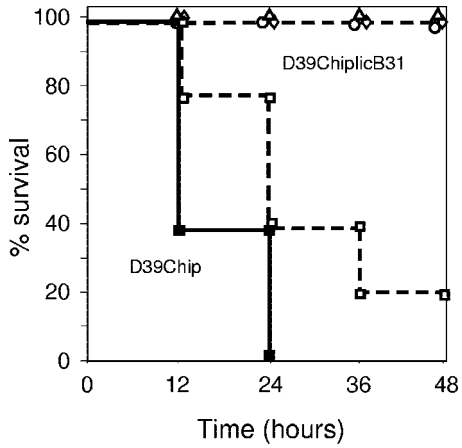
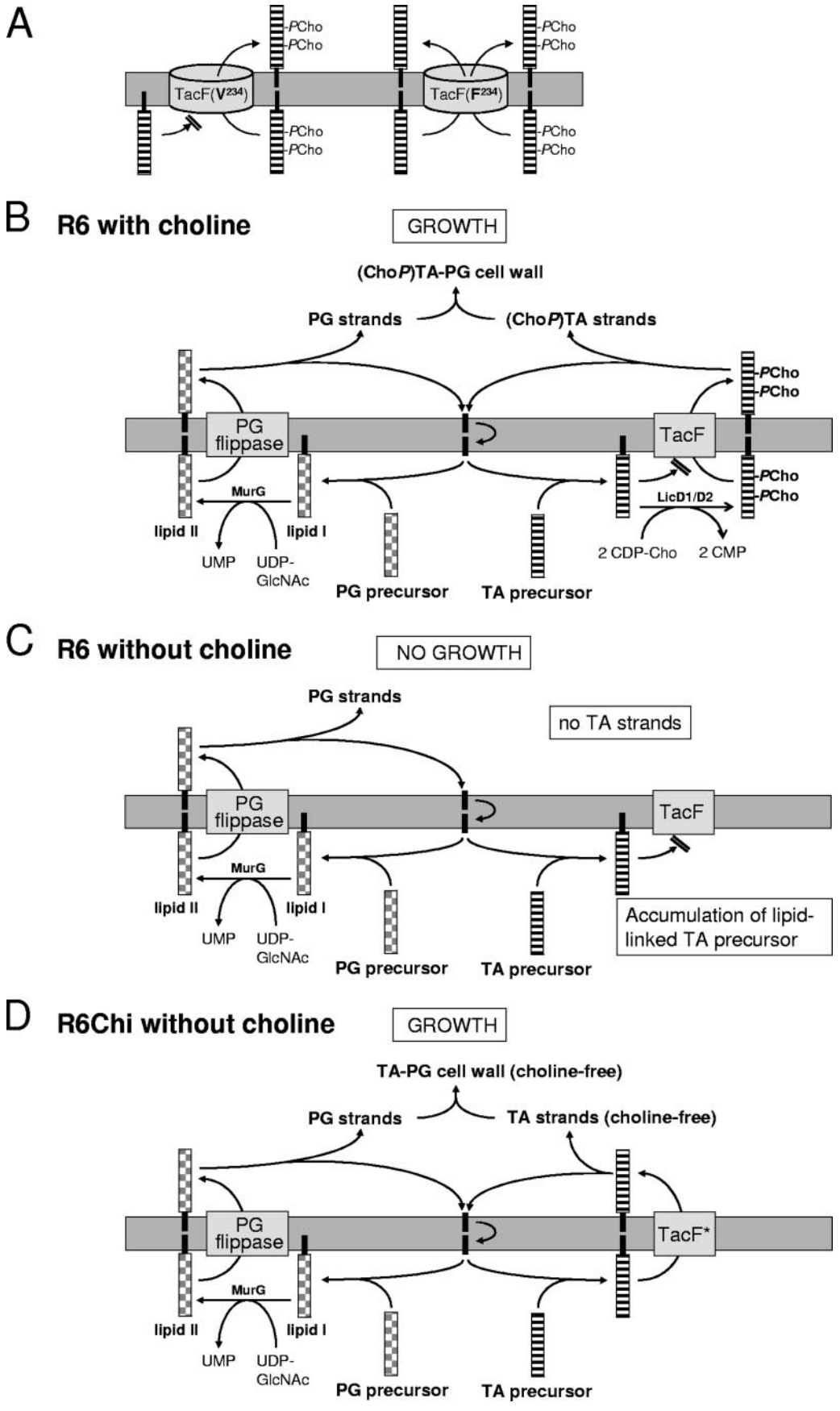


FIG. 4. Virulence of choline-free *S. pneumoniae* in the mouse intraperitoneal model. Bacteria were injected into the intraperitoneal cavity at different cell concentrations, and the survival of mice was monitored for 3 to 7 days. Strain D39Chip was inoculated with 10^3 CFU/mouse (solid lines with solid squares), and strain D39ChiplicB31 (dashed lines and open symbols) was inoculated with the following concentrations (in CFU/mouse): 10^3 (open triangles), 10^5 (open circles), 10^6 (open diamonds), and 10^7 (open squares).


A
PCho
PCho
TacF(V234)
TacF(F234)
B
R6 with choline
GROWTH
(ChoP)TA-PG cell wall
PG strands
(ChoP)TA strands
PG flippase
TacF
MurG
lipid II
lipid I
UMP
UDP-GlcNAc
LicD1/D2
2 CDP-Cho
2 CMP
PG precursor
TA precursor
C
R6 without choline
NO GROWTH
PG strands
no TA strands
Accumulation of lipid-linked TA precursor
D
R6Chi without choline
GROWTH
TA-PG cell wall (choline-free)
TA strands (choline-free)
TacF*

and renders pneumococci choline independent in the presence of the unmutated *spr1150* gene.

Hypothetical function of Spr1150. The *spr1150* gene is located in the *lic* region, which includes three genes for choline uptake and activation (*licA*, *licB*, and *licC*), two genes (*licD1* and *licD2*) presumably involved with the incorporation of choline into teichoic acid precursor, and three genes (*spr1148*, *spr1149*, and *spr1150*) of unknown function. The mutation in the *spr1150* gene of R6Chi results in the replacement of the valine residue at position 234 of the derived amino acid sequence of the wild-type protein with a phenylalanine. Spr1150 has 14 predicted transmembrane helices (http://bp.nuap.nagoya-u.ac.jp/sosui/) and belongs to the protein family PF01943, which currently contains 1,132 integral membrane proteins, many of which are implicated in the production of polysaccharides (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF01943). Furthermore, Spr1150 is one of 113 proteins of the cluster of orthologous groups of proteins 2244 (COG2244) designated "membrane proteins involved in the export of O antigen and teichoic acid" (http://www.ncbi.nlm.nih.gov/COG/grace/wiew.cgi?COG2244) (24). Two members of COG2244 from *E. coli* have a proven transmembrane polysaccharide transport (flippase) function, the O-antigen flippase Wzx (RfbX) (1) and the lipid III flippase WzxE (19). Spr1150 may function as a flippase to transport the lipid-linked, choline-containing teichoic acid precursors across the cytoplasmic membrane in *S. pneumoniae*, and we propose to rename the *spr1150* gene *tacF* (for teichoic acid flippase).

Growth characteristics of choline-independent strains carrying the mutated *tacF* gene. All strains carrying a mutated *tacF* gene, R6Chi, R6Chip, D39Chip, and R6pJDC1150M, grew in long chains and did not autolyze in the stationary phase when grown in the absence of choline (Fig. 3). However, if choline was present in the growth medium, they grew as diplococci or as short chains and lysed in the stationary phase, indicating that the mutants have retained the capacity to incorporate choline into their cell walls. This growth behavior is similar to that described for the two previously reported choline-independent pneumococcal mutants (22, 28).

Effect of cell wall choline on pneumococcal virulence. A recent study has demonstrated the drastic reduction in the virulence of a choline-independent capsular type 2 mutant strain derived from R6Cho$^-$ in several models of pneumococcal disease (13). However, mutant R6Cho$^-$ was obtained from a genetic cross in which *S. oralis* was the source of donor DNA (13). Thus, strain R6Cho$^-$ may contain genetic elements from *S. oralis*, possibly affecting virulence. Therefore, it was important to repeat the virulence studies with a choline-independent strain free of heterologous genetic material. Choline-independent strain D39Chip, expressing a type 2 capsule, was constructed by transforming the PCR product of *tacF* from strain R6Chi. In order to prevent D39Chip from incorporating choline from the in vivo environment, one of the *lic* genes required for choline uptake and/or activation also was inactivated to produce a strain in which the impact of the choline-free cell wall could be tested in vivo for virulence (13). Inactivation of *licA*, *licB*, or *licC* in D39Chip resulted in mutants that grew in chains and did not autolyze in stationary phase, even in choline-containing semisynthetic medium. D39Chip (with intact *licB*) and D39ChiplicB31 (with inactivated *licB*) represent an isogenic pair of strains that differ in their ability to decorate the cell wall with choline. These strains were used in the mouse intraperitoneal model.

Mice injected with as few as 10^3 CFU of D39Chip caused death within 24 h, whereas mice injected with D39ChiplicB31 continued to exhibit 20% survival at the end of experiment, even when injected with a concentration of bacteria as high as 10^7 CFU (Fig. 4).

Mouse intranasal colonization by mutants lacking cell wall choline. Strains D39Chip, D39ChiplicA65, D39ChiplicB31, and D39ChiplicC112 were grown in the choline-containing medium C+Y. Eight-week-old CD1 female mice (10 mice per strain) were anesthetized and inoculated with 10 μl bacterial suspension containing 10^8 CFU/ml in sterile saline. Mice inoculated with choline-deficient mutant bacteria failed to colonize, whereas mice inoculated with D39Chip showed bacterial loads as high as 300 to 500 per mouse.

DISCUSSION

Pneumococci are the only known bacterial species with the same, chemically complex structure in the repeating unit of their wall teichoic acid and lipoteichoic acid, both of which carry one or two choline residues per teichoic acid chain (7). Most genes required for the biosynthesis of the pneumococcal teichoic acids are unknown. The following observations indicate that the *tacF* gene has a role in teichoic biosynthesis. (i) The *tacF* gene is located in the *lic* gene cluster of the chromosome, adjacent to five known genes of choline metabolism. (ii) The mutation $G^{700}\rightarrow T$ in the *tacF* gene described here annulled the nutritional requirement for choline.

The deduced integral membrane protein TacF belongs to the COG2244 group of proteins, members of which are confirmed or putative flippases involved in the transmembrane transport of lipid-linked carbohydrate subunits. Considering its link to the choline-dependent growth phenotype, we propose that TacF is the flippase for the transmembrane transport of the teichoic acid subunits. According to this model (Fig. 5A), the wild-type protein, TacF(V^{234}), would have a strict specific-

FIG. 5. Model for the specificity of the proposed TacF teichoic acid subunit flippase and for the choline-dependent growth phenotype. (A) The wild-type TacF(V^{234}) flippase has a strict specificity for choline-containing teichoic acid subunits and does not transport subunits without choline, ensuring the loading of the cell wall with choline residues (left side). The TacF(F^{234}) flippase, encoded by the mutated *tacF* gene in R6Chi, is able to transport both choline-containing and choline-free teichoic acid subunits. RU, repeating unit; *P*Cho, phosphoryl choline; black bar, lipid anchor. (B) The membrane steps of teichoic acid and peptidoglycan biosynthesis pathways are interconnected by the undecaprenyl phosphate transport lipid (black rectangle), which is released in the diphosphate form during polymerization of the peptidoglycan (PG) and teichoic acid (TA) strands, respectively, and then is recycled for further cycles of subunit transport. (C) In the absence of choline, wild-type cells cannot produce teichoic acids because of the specificity of TacF for choline-loaded teichoic acid subunits. (D) The mutant strain R6Chi is able to grow in the absence of choline and to produce a choline-free cell wall, because the altered flippase (TacF*) has lost its specificity for choline-containing subunits.

ity for transport of choline-containing subunits, whereas the mutant TacF(F^{234}) flippase also would transport subunits without choline, which accumulate when choline-independent mutant cells grow in the absence of exogenous choline. This model predicts a dominant effect of the mutated *tacF* gene in the presence of the wild-type gene, as we have observed in R6pJDC1150M in this work. It is predicted that TacF has 14 transmembrane helices, with V^{234} being located on a loop region between the transmembrane helices 7 and 8. This loop might guard the transport channel to provide substrate specificity. In the absence of another homologous pneumococcal protein with the same role, the predicted teichoic acid transport function of TacF should be essential for pneumococcal cell wall biosynthesis and growth. Indeed, the *tacF* gene was among 133 genes identified as being essential in *S. pneumoniae* (23).

Our data indicate that *tacF* is the only component determining the choline requirement for growth, and it is likely that the choline-independent mutant JY2190, isolated by the same method from strain Rx1 (28), harbors a mutated *tacF* gene. Having a flippase with transport specificity for choline-containing teichoic acid subunits would be an elegant way for the cell to ensure the loading of the cell wall with choline residues, which are essential for many important physiological functions. By this mechanism, the subunits would remain at the inner leaflet of the cytoplasmic membrane until they are loaded with choline, a process that is catalyzed (presumably) by the cytoplasmic LicD1 and/or LicD2 enzyme, using CDP-choline as substrate.

Considering the proposed flippase function of TacF, there are several factors that could account for the choline dependency of growth in wild-type pneumococci. First, teichoic acids may be essential polymers of the cell wall, and the lack of transport of choline-free teichoic acid chains in strains carrying the wild-type *tacF* would be lethal for the cells. Second, the possible accumulation of lipid-linked, choline-free precursors in the inner leaflet of the cytoplasmic membrane could disrupt the membrane integrity. Finally, the accumulation of lipid-linked, choline-free precursors might trap the undecaprenyl phosphate transport lipid, which then is unavailable for the transport of precursor for the essential peptidoglycan (murein) synthesis. The latter possibility has been proposed before, because peptidoglycan synthesis was inhibited upon choline deprivation (6). The interconnection between teichoic acid and peptidoglycan synthesis and the resulting consequences for growth of wild-type and choline-independent mutants are shown in Fig. 5B to D.

The cell wall choline residues have a variety of physiological functions. In this communication, we have demonstrated the essential role of choline for pneumococcal virulence and colonization, fully confirming the findings already reported for the other choline-independent pneumococcal strain, R6cho$^-$, which harbors an unknown DNA sequence obtained from *S. oralis* in the heterologous genetic cross (13).

The proposed transport process of the choline-loaded teichoic acid precursors by the hypothetical flippase TacF may present a novel target for antimicrobial therapy against pneumococci.

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REFERENCES

1. **Alaimo, C., I. Catrein, L. Morf, C. L. Marolda, M. Callewaert, M. A. Valvano, M. F. Feldman, and M. Aebi.** 2006. Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides. EMBO J. **25:**967–976.
2. **Bean, B., and A. Tomasz.** 1977. Choline metabolism in pneumococci. J. Bacteriol. **130:**571–574.
3. **Campbell, H. A., and C. Kent.** 2001. The CTP:phosphocholine cytidylyltransferase encoded by the licC gene of *Streptococcus pneumoniae*: cloning, expression, purification, and characterization. Biochim. Biophys. Acta **1534:** 85–95.
4. **Chen, J. D., and D. A. Morrison.** 1987. Cloning of *Streptococcus pneumoniae* DNA fragments in *Escherichia coli* requires vectors protected by strong transcriptional terminators. Gene **55:**179–187.
5. **Cundell, D. R., N. P. Gerard, C. Gerard, I. Idanpaan-Heikkila, and E. I. Tuomanen.** 1995. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. Nature **377:**435–438.
6. **Fischer, H., and A. Tomasz.** 1985. Peptidoglycan cross-linking and teichoic acid attachment in *Streptococcus pneumoniae*. J. Bacteriol. **163:**46–54.
7. **Fischer, W.** 1997. Pneumococcal lipoteichoic and teichoic acid. Microb. Drug Resist. **3:**309–325.
8. **García, P., M. P. Gonzalez, E. Garcia, R. Lopez, and J. L. Garcia.** 1999. LytB, a novel pneumococcal murein hydrolase essential for cell separation. Mol. Microbiol. **31:**1275–1277.
9. **García, P., M. Paz Gonzalez, E. Garcia, J. L. Garcia, and R. Lopez.** 1999. The molecular characterization of the first autolytic lysozyme of *Streptococcus pneumoniae* reveals evolutionary mobile domains. Mol. Microbiol. **33:** 128–138.
10. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
11. **Höltje, J. V., and A. Tomasz.** 1975. Specific recognition of choline residues in the cell wall teichoic acid by the *N*-acetylmuramyl-L-alanine amidase of *Pneumococcus*. J. Biol. Chem. **250:**6072–6076.
12. **Horne, D. S., and A. Tomasz.** 1993. Possible role of a choline-containing teichoic acid in the maintenance of normal cell shape and physiology in *Streptococcus oralis*. J. Bacteriol. **175:**1717–1722.
13. **Kharat, A. S., and A. Tomasz.** 2006. Drastic reduction in the virulence of *Streptococcus pneumoniae* expressing type 2 capsular polysaccharide but lacking choline residues in the wall. Mol. Microbiol. **60:**93–97.
14. **Lacks, S.** 1966. Integration efficiency and genetic recombination in pneumococcal transformation. Genetics **53:**207–235.
15. **Lopez, R., E. Garcia, P. Garcia, C. Ronda, and A. Tomasz.** 1982. Choline-containing bacteriophage receptors in *Streptococcus pneumoniae*. J. Bacteriol. **151:**1581–1590.
16. **Morrison, D. A.** 1997. Streptococcal competence for genetic transformation: regulation by peptide pheromones. Microb. Drug Resist. **3:**27–37.
17. **Mosser, J. L., and A. Tomasz.** 1970. Choline-containing teichoic acid as a structural component of pneumococcal cell wall and its role in sensitivity to lysis by an autolytic enzyme. J. Biol. Chem. **245:**287–298.
18. **Rane, L., and Y. Subbarow.** 1940. Choline, panthothenic acid and nicotinic acid as essential growth factors for pneumococcus. J. Biol. Chem. **134:**455–456.
19. **Rick, P. D., K. Barr, K. Sankaran, J. Kajimura, J. S. Rush, and C. J. Waechter.** 2003. Evidence that the *wzxE* gene of *Escherichia coli* K12 encodes a protein involved in the transbilayer movement of a trisaccharide-lipid intermediate in the assembly of enterobacterial common antigen. J. Biol. Chem. **278:**16534–16542.
20. **Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortqvist, and H. R. Masure.** 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. Mol. Microbiol. **25:**819–829.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. **Severin, A., D. Horne, and A. Tomasz.** 1997. Autolysis and cell wall degradation in a choline-independent strain of *Streptococcus pneumoniae*. Microb. Drug Resist. **3:**391–400.
23. **Song, J.-H., K. S. Ko, L.-Y. Lee, J. Y. Baek, W. S. Oh, H. S. Yoon, J.-Y. Jeong, and J. Chun.** 2005. Identification of essential genes in *Streptococcus pneumoniae* by allelic replacement mutagenesis. Mol. Cells **19:**365–374.
24. **Tatusov, R. L., E. V. Koonin, and D. J. Lipman.** 1997. A genomic perspective on protein families. Science **278:**631–637.

25. **Tomasz, A.** 1967. Choline in the cell wall of a bacterium: novel type of polymer-linked choline in Pneumococcus. Science **157:**694–697.
26. **Tomasz, A.** 1964. Studies on the competence (for genetic transformation) of *Diplococcus pneumoniae* in a synthetic medium, abstr. G87. Abstr. 64th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC.
27. **Tomasz, A., and R. D. Hotchkiss.** 1964. Regulation of the transformability of the pneumococcal cultures by macromolecular cell products. Proc. Natl. Acad. Sci. USA **51:**480–487.
28. **Yother, J., K. Leopold, J. White, and W. Fischer.** 1998. Generation and properties of a *Streptococcus pneumoniae* mutant which does not require choline or analogs for growth. J. Bacteriol. **180:**2093–2101.
29. **Yother, J., and J. M. White.** 1994. Novel surface attachment mechanism of the *Streptococcus pneumoniae* protein PspA. J. Bacteriol. **176:**2976–2985.
30. **Zhang, J. R., I. Idanpaan-Heikkila, W. Fischer, and E. I. Tuomanen.** 1999. Pneumococcal *licD2* gene is involved in phosphorylcholine metabolism. Mol. Microbiol. **31:**1477–1488.