Mutations in the *tacF* Gene of Clinical Strains and Laboratory Transformants of *Streptococcus pneumoniae*: Impact on Choline Auxotrophy and Growth Rate^{∇†}

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The nutritional requirement that *Streptococcus pneumoniae* has for the aminoalcohol choline as a component of teichoic and lipoteichoic acids appears to be exclusive to this prokaryote. A mutation in the *tacF* gene, which putatively encodes an integral membrane protein (possibly, a teichoic acid repeat unit transporter), has been recently identified as responsible for generating a choline-independent phenotype of *S. pneumoniae* (M. Damjanovic, A. S. Kharat, A. Eberhardt, A. Tomasz, and W. Vollmer, J. Bacteriol. 189:7105–7111, 2007). We now report that *Streptococcus mitis* can grow in choline-free medium, as previously illustrated for *Streptococcus oralis*. While we confirmed the finding by Damjanovic et al. of the involvement of TacF in the choline dependence of the pneumococcus, the genetic transformation of *S. pneumoniae* R6 by using *S. mitis* SK598 DNA and several PCR-amplified *tacF* fragments suggested that a minimum of two mutations were required to confer improved fitness to choline-independent *S. pneumoniae* mutants. This conclusion is supported by sequencing results also reported here that indicate that a spontaneous mutant of *S. pneumoniae* (strain JY2190) able to proliferate in the absence of choline (or analogs) is also a double mutant for the *tacF* gene. Microscopic observations and competition experiments during the cocultivation of choline-independent strains confirmed that a minimum of two amino acid changes were required to confer improved fitness to choline-independent pneumococcal strains when growing in medium lacking any aminoalcohol. Our results suggest complex relationships among the different regions of the TacF teichoic acid repeat unit transporter.

Streptococcus pneumoniae is unique among prokaryotes in that it exhibits an absolute nutritional requirement for choline (26). When choline is withdrawn from the growth medium, peptidoglycan synthesis ceases; it recommences when choline is added (11). As an aminoalcohol, choline becomes incorporated as phosphocholine (PCho) in the cell wall teichoic acid (TA) and membrane lipoteichoic acid of the pneumococcus (36). Depending on the pneumococcal strain, each repeat unit of TA contains one or two PCho residues (18, 39). Following the identification of a genetic locus (*lic*) required for PCho metabolism in *S. pneumoniae*, it has been proposed that choline is transported into the cytoplasm by LicB (40), converted to PCho by LicA (38), and activated to CDP-choline by LicC (4, 27). In addition, LicD2 is thought to mediate the incorporation of choline (presumably from CDP-choline) into lipoteichoic acid and perhaps also into TA (40). Interestingly, the results of recent studies have shown that pneumococcal mutants engineered to lack choline residues on their cell surface exhibit intensely diminished virulence in animal models of infection (20).

Apart from *S. pneumoniae*, PCho-containing TAs have been found in other gram-positive bacteria, including *Streptococcus*

mitis, *Streptococcus oralis*, and *Clostridium beijerinckii* (13). Unlike the pneumococcus, however, *S. oralis* has no nutritional requirement for choline and when grown in its absence, PCho-free TA seems to be incorporated into the cell wall, although the cells show a grossly abnormal shape and size (16). That is, in contrast to the uniform size of normal cells, choline-free bacteria showed a wide distribution of sizes. Moreover, choline-deprived *S. oralis* frequently exhibited septa inserted at oblique angles. Through transformation with *S. oralis* DNA, Severin et al. isolated and partly characterized a pneumococcal transformant (R6Cho[−]) able to grow in the absence of choline (29). Remarkably, however, the R6Cho[−] strain did not show the morphological alterations characteristic of *S. oralis* cells grown in choline-deprived medium. In an independent study, a pneumococcal mutant (JY2190) was isolated that had acquired the ability to grow in the absence of choline and its analogs after serial passage of strain Rx1 in a chemically defined medium containing decreasing concentrations of ethanolamine in each passage (39). When grown in the absence of choline, R6Cho[−] and JY2190 behave similarly to *S. pneumoniae* cells incubated with the choline analog ethanolamine (34) in terms of impaired daughter cell separation at the end of cell division, failure to undergo stationary phase autolysis, and resistance to penicillin- or deoxycholate-induced lysis. All these phenomena can be reversed by the addition of choline to the growth medium. Given that peptidoglycan and TA synthesis both depend on the undecaprenyl phosphate transport lipid, it has been proposed that *S. pneumoniae* has a control mechanism that only allows choline-containing TA subunits to be transferred across the membrane to the growing TA chains (12). In the

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absence of choline (or choline analogs such as ethanolamine), there is a buildup of aminoalcohol-free TA linked to polyprenol phosphate, rendering polyprenol phosphate unavailable to peptidoglycan synthesis, the basis of cell growth. It is thus conceivable that this control is missing in choline-independent (Cho-ind) *S. pneumoniae* strains, allowing the transfer of choline-free TA subunits.

Cell wall PCho residues act as anchors for the so-called choline-binding proteins (23), a family of surface-located proteins, some of which have been reported to play important role(s) in the pathogenicity of *S. pneumoniae* (15, 25). Recently, it has been shown that encapsulated *S. pneumoniae* mutants that have lost their auxotrophic requirement for choline (Cho-ind; see above) and are also blocked from utilizing choline from the growth medium (*licA*, *licB*, or *licC* mutants) show an extensive loss of virulence which must be directly or indirectly related to the loss of choline residues from the pneumococcal surface (14, 20).

Since the Cho-ind *S. pneumoniae* mutants reported so far show TAs completely lacking PCho (29, 39), we decided to try to construct a novel pneumococcal mutant that would preferentially incorporate ethanolamine in its TAs even when grown in a choline-containing environment, such as in vivo. To engineer this mutant, we used DNA prepared from an *S. mitis* strain (SK598) reported to possess ethanolamine-containing TAs irrespective of the incubation medium (3). During the course of our investigation and having isolated the novel Cho-ind pneumococcal strain P072, a report was published ascribing to spr1150, an essential gene (31) located in the *lic* region, a role in TA metabolism and the Cho-ind phenotype of *S. pneumoniae* (5). In this report, Damjanovic et al. (5) noted that a single-point mutation in the spr1150 gene of the spontaneous mutant strain R6Chi was sufficient to generate a Cho-ind phenotype. These authors suggest that the spr1150 gene product is a polysaccharide transmembrane transporter (flippase) required for the transport of TA subunits across the membrane and propose the term *tacF* (standing for TA flippase) to rename the pneumococcal spr1150 gene.

Here we provide experimental evidence confirming the key role of the *tacF* gene in the choline-dependent phenotype characteristic of the pneumococcus, but in addition to the findings of Damjanovic et al. (5), we demonstrate that at least two *tacF* mutations are required to confer an improved fitness to the Cho-ind pneumococcal strains when growing in medium lacking any aminoalcohol.

MATERIALS AND METHODS

Bacterial strains and growth and transformation conditions. The streptococcal strains included in this study are described in Table 1. The R6 strain used here was purchased from the American Type Culture Collection (ATCC BAA-255). Streptococci were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), in C medium (21) supplemented with yeast extract (0.8 mg ml⁻¹; Difco Laboratories) (C+Y medium), or in a chemically defined medium (CDM) (37) supplemented with asparagine (50 µg ml⁻¹) and sodium pyruvate (250 µg ml⁻¹). The CDM was purchased from JRH Biosciences (Lenexa, KS). Where indicated, CDM was supplemented with choline chloride (5 to 50 µg ml⁻¹) or ethanolamine (40 µg ml⁻¹). In some experiments, we observed that Cho-ind pneumococci grew faster in a modified C medium lacking choline (C-Cho medium) than in CDM. Cells were incubated at 37°C without shaking, and growth was monitored by measuring A₅₅₀.

Unless otherwise stated, *S. pneumoniae* was transformed to choline independence by incubating 0.5 ml of competent R6 cells in C medium supplemented

with bovine serum albumin (0.8 mg ml⁻¹) (35) with 1 µg chromosomal DNA prepared from strain SK598 or P072 (see below) for 30 min at 30°C. Afterwards, 1 ml of prewarmed C+Y medium was added and the mixture was incubated at 37°C for 90 min. The culture was then centrifuged and washed with 3 ml of CDM, and the pellet was resuspended in 10 ml of the same medium. After ca. 72 h of incubation at 37°C, cells growing as chains at the bottom of the tube could be observed. A portion of these cells was diluted in fresh C-Cho medium and incubated overnight at 37°C. This procedure was repeated at least twice, and single colonies were recovered by plating appropriate dilutions in tryptic soy agar (Difco Laboratories) plates supplemented with 5% defibrinated sheep blood. The Cho-ind phenotype of the transformants was retested by using C-Cho medium.

PCR amplification, cloning, and nucleotide sequencing. Routine DNA procedures were performed essentially as described elsewhere (28). *S. pneumoniae* chromosomal DNA was prepared as previously described (10). The DNA extraction procedure described by Ezaki et al. (9) was used for all other streptococci. DNA fragments were purified by using a High Pure PCR product purification kit (Roche). For PCR amplification and sequencing of the *tacF* gene, we used the oligonucleotide primers atg (1151360), 5'-TGAATGAAAGTATAA AATTAATGCTCT-3'; b (1151863), 5'-CGATATTGTTGCTATACACTTG TGATG-3'; c (1151890/c), 5'-CATCACAAGTGTATAGACACAATATCG-3'; d (1152264), 5'-GTTTGGACTCATGGTTTATAGGA-3'; e (1152358/c), 5'-AAAAGCGAAGAGAGAGGTCAGATGC-3'; and stop (1152886/c), 5'-GG CATCTTCAACGGTTAGTTGTTTC-3'. The numbers indicate the position of the first nucleotide of the primer in the genomic sequence of strain R6 (17), and /c means that the sequence corresponds to the complementary strand. The ATG initiation codon of *tacF* is shown underlined. For PCR amplification of the *tacF* gene (or gene fragments), high-fidelity *Pfu* polymerase (Biotools) was used.

The nucleotide sequence was determined by following a PCR cycle sequencing method (BigDye terminator version 3.1 cycle sequencing kit) using an automated ABI Prism 3700 DNA sequencer (Applied Biosystems). All primers for PCR amplification and nucleotide sequencing were purchased from Sigma.

The sequence data for the *S. pneumoniae* strains Spain^{23F}-1, INV104B, INV200, and OXC141 were produced by the *S. pneumoniae* Sequencing Group at the Sanger Institute and can be obtained from http://www.sanger.ac.uk/Projects/S_pneumoniae.

Data analysis. Sequence comparisons were undertaken by running the BLAST program (1) using EMBL/UniProtKB databases (<http://www.ncbi.nlm.nih.gov/BLAST>) (last data accessed, 10 August 2007), as well as available preliminary genomic data for *S. pneumoniae* 670 and *S. mitis*^T (The J. Craig Venter Institute website at <http://tigrblast.tigr.org/cmr-blast/>) and for the pneumococcal strains Spain^{23F}-1, INV104B, INV200, and OXC141 (The Sanger Institute; http://www.Sanger.ac.uk/Projects/S_pneumoniae) and G54 (7) (<http://bioinfo.cnio.es/old/data/Spneumo>). Since the published genomic sequence of the *tacF*_{G54} allele has two indeterminate nucleotides, the gene was resequenced. Multiple gene or protein sequence alignments were conducted by using the Clustal W program (33) available at the website of the EMBL-European Bioinformatics Institute (<http://www.ebi.ac.uk/clustalw>). A consensus prediction of transmembrane helices was obtained by comparing the results rendered by 10 different computer programs (see Table S1 in the supplemental material). DNA and protein sequences were also analyzed by using the Genetics Computer Group (GCG) software package (version 10.0) (6). Pairwise evolutionary distances (PEDs) were determined by using the Distances program.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were deposited in the EMBL/GenBank/DBJ databases under the accession numbers AM901296 to AM901310.

RESULTS

Generating and characterizing the Cho-ind pneumococcal strains. We first observed that, as reported for *S. oralis* (16), *S. mitis* strains exhibited a Cho-ind phenotype; that is, they were able to grow in CDM lacking choline or any other choline analog (Fig. 1). However, whereas the type strain of *S. mitis* grew slowly, forming small chains with some cells being abnormal in shape and size (Fig. 1E) as described for *S. oralis*, *S. mitis* strain SK598 formed long chains of cells when incubated in any medium tested, regardless of the presence or absence of choline or choline analogs (Fig. 1C and unpublished observations). Whether SK598 is capable of synthesizing ethanol-

TABLE 1. Streptococcal strains used in this work

Species	Strain (serotype)	Phenotype ^a	Allele ^b	Accession no.	Description/reference(s)
<i>S. pneumoniae</i>	R6 (NT ^c)	+	1	AE008487	17
	D39 (2)	+	1	CP000410	22
	R6x (NT)	+	1	AF106539	40
	TIGR4 (4)	+	1	AE005672	32
	G54 (19F)	+	2	AL449932; AM901297	7 and this work
	670 (6B)	+	3		http://www.tigr.org
	Rx1 (NT)	+	1	AM901296	24 and this work
	JY2190 (NT)	–	4	AM901298	39 and this work
	JY2190 Opt	–			As JY2190 but optochin resistant (5 µg ml ⁻¹); this work
	Spain ^{23F} -1 (23F)	+	5		http://www.Sanger.ac.uk/Projects/S_pneumoniae
	INV104B (1)	+	6		http://www.Sanger.ac.uk/Projects/S_pneumoniae
	INV200 (14)	+	7		http://www.Sanger.ac.uk/Projects/S_pneumoniae
	OXC141 (3)	+	8		http://www.Sanger.ac.uk/Projects/S_pneumoniae
	SP9-BS68 (9V)	+	1	NZ_ABAB01000019	30
	SP14-BS69 (14)	+	9	NZ_ABAD01000005	30
	SP11-BS70 (11)	+	10	NZ_ABAC01000014	30
	SP3-BS71 (3)	+	8	NZ_AAZZ01000007	30
	SP23-BS72 (23F)	+	11	NZ_ABAG01000002	30
	SP6-BS73 (6A)	+	12	NZ_ABAA01000007	30
	SP18-BS74 (18C)	+	3	NZ_ABAA01000007	30
	SP19-BS75 (19F)	+	5	NZ_ABAA01000007	30
	R6Chi (NT)	–	13		5
	R6Chi Cm	–			As R6Chi but chloramphenicol resistant (5 µg ml ⁻¹); this work
	P023 (NT)	–	14	AM901299	Cho-ind R6 transformant using SK598 DNA; this work
	P072 (NT)	–	14	AM901310	Cho-ind R6 transformant using P023 DNA; this work
	P500 (NT)	–	15	AM901300	Cho-ind R6 transformant using spr1150 _{P072} (oligonucleotides atg and stop); this work
	P501 (NT)	–	16	AM901301	As P500 but isolated in an independent exp't; this work
	P501 Str	–			As P501 but streptomycin resistant (150 µg ml ⁻¹); this work
	P502 (NT)	–	17	AM901302	Cho-ind R6 transformant using a PCR fragment of spr1150 _{P072} (oligonucleotides a and b); this work
	P511 (NT)	–	18	AM901303	Cho-ind R6 transformant using a PCR fragment of spr1150 _{P501} (oligonucleotides a and b); this work
	P511 Str	–			As P511 but streptomycin resistant (150 µg ml ⁻¹); this work
	P550 (NT)	–	19	AM901304	Cho-ind R6 transformant using spr1150 _{R6} (oligonucleotides atg and stop); this work
	P551 (NT)	–	20	AM901305	Cho-ind transformant using a PCR fragment of spr1150 _{P550} (oligonucleotides dis, atg, and e); this work
	P575 (NT)	–	21	AM901306	Cho-ind R6 transformant using a PCR fragment of spr1150 _{JY2190} (oligonucleotides atg and e); this work
	P600	–	22	AM901307	Cho-ind R6 transformant using a PCR fragment of spr1150 _{SK598} (oligonucleotides a and b); this work
	P700	–	13		Cho-ind R6 transformant using spr1150 _{R6Chi} (oligonucleotides atg and stop); this work
	M222	+	ND		A multiresistant strain; 10
<i>S. pseudopneumoniae</i>	CCUG 49455 ^T	+	23	AM901308	2 and this work
<i>S. mitis</i>	SK598	–	24	AM901309	3 and this work
	NCTC 12261 ^T	–	25		http://www.tigr.org

^a + and – indicate choline-dependent and Cho-ind strains, respectively.

^b Only the last 1,462 nucleotides (out of 1,488 nucleotides) of the spr1150 gene were compared. ND, not determined.

^c NT, nontypeable.

amine-containing TAs when grown in choline-deprived medium, as it does when grown in choline-containing medium (3), has not been investigated. In contrast, the type strain of *Streptococcus pseudopneumoniae* (the closest relative of *S. pneumoniae* known to date) was unable to multiply in choline-free (or any choline analog-free) medium (data not shown). Based on these observations, we then went on to prepare chromo-

somal DNA from *S. mitis* SK598 to transform *S. pneumoniae* R6 and isolated one clone able to grow in choline-free medium (designated strain P023) for further study. Strain P023 showed all the characteristics previously reported for the Cho-ind isolates of *S. pneumoniae* characterized so far (5, 29, 39). In brief, when incubated in the absence of choline, P023 formed long chains of cells (Fig. 1B) and failed to lyse either in the station-

FIG. 1. Phase-contrast micrographs of Cho-ind strains grown in CDM. Cultures of *S. pneumoniae* P023 (A, B), *S. mitis* SK598 (C), or *S. mitis* NCTC 12261^T (D, E) were grown in the presence of 50 µg ml⁻¹ choline chloride (A, C, and D) or in the absence of choline or its analogs (B, E). Cultures of R6Chi (F, G) or P501 (H, I) were grown in the presence of 40 µg ml⁻¹ ethanolamine (F, H) or in the absence of choline or its analogs (G, I). Arrows indicate cells of abnormal shape and size. Bar, 10 µm.

ary phase of growth or after the addition of deoxycholate. However, P023 was seen to generate deoxycholate-sensitive diplococci (or short chains) several hours after the addition of at least 5 µg ml⁻¹ choline chloride to the choline-free growth medium. The Cho-ind phenotype of strain P023 could be re-transformed to the parental R6 strain (choline dependent) by another round of transformation using P023 DNA as donor material. One of the new Cho-ind R6 transformants, strain P072, was further examined.

Nucleotide sequences of *tacF* alleles in choline-dependent and Cho-ind strains. As already mentioned, it has been reported that a *tacF* gene product containing a single Val²³⁴ to Phe mutation can confer a Cho-ind phenotype to *S. pneumoniae* (5). To confirm this observation, we PCR amplified the entire *tacF* gene of strain P072 using the oligonucleotides atg and stop and used this amplicon to transform the R6 strain. Cho-ind transformants could be isolated, confirming that *tacF*_{P072} was indeed required for choline independence. To gain further insight into the molecular characteristic(s) differentiating the pneumococcal *tacF* gene from that of *S. mitis*, the *tacF* genes of strains Rx1, JY2190, P023, P072, *S. pseudopneumoniae* CCUG 49455^T, and *S. mitis* SK598 were PCR amplified with oligonucleotides atg and stop and sequenced, and the almost-complete nucleotide sequence of the gene (1,462 out of 1,488 bp, not including the oligonucleotide primers) was compared with those deposited in the EMBL database and preliminary nucleotide sequences available from other sources (Table 1). The *S. pneumoniae* strains R6, D39, R6x, TIGR4, SP9-BS68, and Rx1 (present study) share the same *tacF* allele (designated allele 1) (Table 1). The results shown in Fig. 2A and B indicate that the *tacF* alleles of all the *S. pneumoniae* strains analyzed were closely related (PEDs, that is, estimated number of substitutions per 100 bases, <0.9), whereas *tacF*_{P072} (allele 14) was clearly divergent (PED ≈ 5.0) (not shown). As expected, the *tacF* allele of *S. pseudopneumoniae* CCUG

49455^T (choline dependent) proved to be evolutionarily more related to *S. pneumoniae* (PED < 2.0) than to *S. mitis* (PED > 4.7) (Fig. 2B). Naturally, *tacF*_{P072} was very similar (PED = 0.07) to *tacF*_{SK598}, differing only in terms of 1 nucleotide at position 700 (codon 234; position 1) (Fig. 2A). This G-to-T transversion was also found in the *tacF*_{P023} allele, which in turn was observed to be identical to *tacF*_{P072}, as expected (unpublished results). Thus, the product of this last allele, TacF_{P072}, would presumably show the substitution of Val²³⁴ by a Phe residue; quite surprisingly, this point mutation was the one described by Damjanovic et al. for *tacF*_{R6Chi} (5) (Fig. 3). The divergence between the *tacF* alleles of the type strain of *S. mitis* and strain SK598A was estimated at 3.49% (Fig. 2B), possibly reflecting the greater evolutionary divergence among the *S. mitis* isolates than among the *S. pneumoniae* strains (19). Interestingly, Cho-ind strain JY2190 (allele 4) has 2 nucleotides that differ from those of *tacF* allele 1 (Fig. 2A) and both mutations led to the appearance of a new amino acid residue (Pro¹⁰⁴ to Leu and Ala²¹⁴ to Thr) (Fig. 3).

Identifying the amino acid residues of TacF that confer the Cho-ind phenotype. To establish whether one or more of the amino acid changes observed in the Cho-ind strain P072 were responsible for the Cho-ind phenotype, the entire *tacF*_{P072} allele (or parts of it) was PCR amplified using combinations of different oligonucleotide primers, and the resulting DNA fragments were used to transform the pneumococcal R6 strain (Table 1). First, the entire *tacF*_{P072} allele was amplified with the oligonucleotides atg and stop and used as donor DNA. Then, the *tacF* genes in two Cho-ind transformants isolated in independent transformation experiments (designated strains P500 and P501, respectively) were sequenced so that the recombination boundaries between donor and recipient (*tacF*_{R6}) alleles could be determined (Fig. 2A and 4). The recombination crossover points in transformant P500 (allele 15) were located between nucleotide positions 85 to 93 and 1273 to 1294 (taking as 1 the first nucleotide of the ATG initiation codon). In P501, recombination took place at positions 1 to 69 and 701 to 740 (allele 16). Unexpectedly, two additional mutations, present neither in the donor nor in the recipient allele, were found in P500, i.e., A⁶³⁷- and A¹²³¹-to-G transitions (corresponding to the first position of codons 213 and 411, respectively; Fig. 2A). Whether these mutations were introduced during PCR amplification or might have arisen spontaneously after transformation is not known. Interestingly, the spontaneous Rx1 mutant Cho-ind strain JY2190 also contained two amino acid changes (allele 4) (Fig. 3 and 4). To gain further information, an internal fragment of *tacF*_{P072} was PCR amplified using oligonucleotides a and b and the resulting DNA (496 bp) used to transform the R6 strain. The nucleotide sequence of the *tacF* gene of a Cho-ind transformant (named strain P502) was then determined, and two mutations not present in the donor *tacF*_{P072} allele were found (two A to G transitions at positions 660 and 706) that caused a change from Asn²²⁰ to Ser and Thr²³⁶ to Ala, respectively (allele 17) (Fig. 3 and 4).

In sharp contrast with the above results, the transformation of the R6 strain with a 623-bp fragment of *tacF*_{P072} generated by using oligonucleotides c and stop did not give rise to Cho-ind transformants, at least after 96 h of incubation. Identical results were obtained when the PCR-amplified *tacF*_{R6} gene was used as donor DNA. Nevertheless, when the culture trans-

FIG. 2. Sequence variation in the *tacF* gene of *S. pneumoniae* and *S. mitis* strains. (A) Multiple alignment of a 1,462-bp sequence. Only the polymorphic sites (numbered vertically) are depicted. Sites 1, 2, and 3 indicate the first, second, and third nucleotides of the codon, respectively. Black boxes indicate codons in which nucleotide changes cause amino acid substitutions. Colons represent nucleotides identical to those of allele 1. Black and open bars at the top indicate the *tacF* gene fragments that were PCR amplified by using oligonucleotide mixtures atg and c and a and b, respectively. The overlapping region between those DNA fragments is shadowed in gray. (B) A matrix of PEDs between aligned sequences is shown. Values represent the estimated number of substitutions per 100 bases with no distance correction.

formed with *tacF*_{R6} was incubated in C—Cho medium for up to 8 days (instead of the usual 72-h incubation), a Cho-ind pneumococcal strain (designated P550; allele 19) could be isolated. Surprisingly, two previously undescribed mutations (Phe¹⁰⁷ to Ser and Phe²⁰⁹ to Leu) were detected in TacF_{P550} (Fig. 3 and 4). Additional transformation experiments were performed using R6 as the recipient strain. *tacF*_{P501} allele 16 was PCR amplified by using oligonucleotides a and b (496 bp) to generate the P511 transformant. Thus, the PCR-generated gene fragment should contain one nonsilent mutation present in the *tacF*_{P501} allele; that is, G⁷⁰⁰ to T (coding for Phe²³⁴) (Fig. 4). Table 1 describes the results of further experiments (carried out by using different PCR-amplified fragments of *tacF* from the DNA of strains P550, JY2190, or SK598 as donor material) in which we were able to isolate several new Cho-ind pneumococcal transformants, namely, strains P551, P575, and P600, respectively. Comparison of the different deduced amino acid sequences (Fig. 2A, 3, and 4) along with microscopic observations (see below) might suggest that a minimum of two amino acid changes was required to confer improved fitness on a pneumococcal Cho-ind strain when growing in choline-free medium.

Improved growth of Cho-ind strains in aminoalcohol-free medium requires at least two *tacF* mutations. When strain R6Chi or P501 was incubated at 37°C in CDM containing ethanolamine, the cells were always very homogeneous in shape and size (Fig. 1F and H, respectively). However, R6Chi cells grown in CDM lacking choline (or any other choline analog) were of abnormal shape and size (Fig. 1G), which contrasted with the normal morphology exhibited by the P501 strain (Fig. 1I). The growth characteristics exhibited by strain JY2190 were indistinguishable from those of P501. When R6Chi, JY2190, and P501 were incubated in CDM containing ethanolamine, all of them showed a similar growth rate (Fig. 5A). In contrast, when incubated in CDM lacking any aminoalcohol, it could be observed that P501 and JY2190 grew more rapidly and with a shorter lag period than R6Chi (Fig. 1B). This observation was fully confirmed by the results of cocultivation experiments (Fig. 5C and D). Strains JY2190 Opt, R6Chi Cm, and P501 Str were constructed by transformation with DNA prepared from the multiresistant strain M222 (Table 1), mixed in different proportions, and incubated at 37°C in CDM lacking choline (or any choline analog). The viability of the different strains was monitored by plating appropriate di-

FIG. 3. Sequence variation among the different TacF alleles. Only polymorphic residues are depicted (numbered vertically). Colons represent amino acid residues identical to those of allele 1. Black and open bars at the top indicate the locations of *tacF* fragments amplified by PCR using oligonucleotide mixtures atg and e and a and b, respectively. + and - indicate TacF alleles present in choline-dependent and Cho-ind strains, respectively. The positions of amino acid residues predicted to be located in the transmembrane loop structure are shadowed in gray.

lutions in tryptic soy blood agar plates supplemented with the corresponding antibiotic. In agreement with previous observations (see above) the strains harboring two *tacF* mutations, i.e., JY2190 Opt and P501 Str, showed similar growth rates (Fig. 5C). R6Chi Cm displayed a very long lag period such that, even when this strain was cocultivated with P501 Str in a 200:1 proportion, the latter strain was capable of prevailing after 36 h of incubation (Fig. 5D).

To completely discard any unexpected influence of some (unknown) difference(s) between the genome of our R6 recipient strain and that of the R6Chi strain used by Damjanovic et al. (5), we constructed strain P700 (Table 1). To do that, the *tacF*_{R6Chi} allele (allele 13) was PCR amplified using oligonucleotides atg and stop, and this amplicon was used to transform the R6 strain. The transformed culture was diluted and plated in C+Y medium. Up to 50 clones were tested for choline independence by incubation in CDM lacking choline or choline analogs. One Cho-ind R6 transformant containing the *tacF*_{R6Chi} allele was designated strain P700 (Table 1), and its morphology and growth characteristics were studied. As reported above for strain R6Chi, P700 also showed abnormal cell morphology and a long lag period when incubated in CDM lacking any aminoalcohol (data not shown).

Molecular modeling of the TacF flippase. TacF is predicted to be an integral α -helix bundle membrane protein with 14 transmembrane helices (5). Despite the existence of many computer programs to predict the secondary structure of α -helix bundle proteins, a correct topology prediction does not mean that the predicted starts and ends of the transmembrane α -helices can be trusted; only the number of transmembrane helices and their approximate positions are reliable (8). In spite of this limitation, we undertook a comparative prediction

of the structure of TacF_{R6} using 10 different programs (see Table S1 in the supplemental material) and obtained a consensus structure (Fig. 6A). Figure 6B shows the proposed localization of the mutated amino acid residues in selected Cho-ind pneumococcal strains. With the exception of Phe¹⁰⁶, the other amino acid positions could be predicted with reasonable agreement ($\geq 60\%$) among the different programs. It can be seen that with the exception of TacF_{JY2190} (allele 4), in which both mutations reside in different α -helices, and of TacF_{P501} (allele 16), which possesses two mutations apparently located outside the membrane, the mutations appear to occur at various combinations of helices 3, 7, and 9 and amino acid loops 2 and 4 outside the membrane. Moreover, it could be observed in a three-dimensional model of TacF_{R6} (see Fig. S1 in the supplemental material) that the 11 amino acid residues involved in the choline independence of *S. pneumoniae* formed three separate clusters: (i) Ile¹⁰⁰, Pro¹⁰⁴, Phe¹⁰⁶, Phe¹⁰⁷, Phe²⁹⁶, and Ile²⁹⁸; (ii) Val³², Phe²⁰⁹, and Ala²¹⁴; and (iii) Val²³⁴ and Ile²⁴⁷. When this model was compared with the mutations found in the different TacF alleles (Fig. 6B), it emerged that with the exception of alleles 20 and 21 that contain residues belonging only to cluster 1, the amino acid changes observed involved different combinations of residues from two different clusters.

DISCUSSION

The results presented confirm those recently reported by Damjanovic et al. (5) in that the *tacF* gene product (previously designated Spr1150 and SP1272 in the R6 and TIGR4 genome sequences, respectively) is the only factor that determines a requirement for choline for the characteristic growth of *S.*

FIG. 4. Schematic representation of recombinant *tacF* genes generated by genetic transformation. The genomic coordinates of the R6 allele appear at the top. The locations of the different oligonucleotides used for PCR amplification and sequencing are also shown. Stars indicate the location of amino acid residues that are different from those of the *tacF*_{R6} allele. Amino acid changes that appeared de novo after one round of transformation (in the one-letter code) are underlined and depicted as open stars. The shading indicates the approximate positions where recombination took place. Since the *tacF* alleles of strains R6 and JY2190 differ by only two nucleotides, both gene schematics have been equally shadowed.

pneumoniae. However, our observations suggest that at least two amino acid changes with respect to those of the recipient R6 strain are required to confer improved fitness to Cho-ind pneumococcal mutants when growing in medium lacking any aminoalcohol. This conclusion is mainly based on three lines of evidence obtained by analyzing the nucleotide sequences of Cho-ind R6-derivative strains that were generated by using as donor material in transformation experiments (i) *S. mitis* SK598 DNA (strains P023/P072 and P600) or DNA fragments amplified from some of these transformants (strains P500, P501, P502, and P511), (ii) *S. pneumoniae* R6 DNA (strain P550) or PCR fragments of *tacF*_{P550} (strain P551), and (iii) PCR fragments prepared from the Cho-ind spontaneous mutant JY2190 (strain 575). In addition, we demonstrate here that JY2190, which was generated by following a procedure identical to that employed by Damjanovic and coworkers for the R6Chi strain (5), also contains a TacF protein with two amino

FIG. 5. Growth and viability of pneumococcal cultures. R6Chi (●), P501 (◆), or JY2190 (★) cells were grown in C+Y medium until mid-exponential phase and diluted into CDM with ethanolamine (A) or without any aminoalcohol (B, C, and D). In the experiments whose results are shown in panels A and B, about 100 CFU ml⁻¹ of each bacterial strain was initially added. (C) Coculture of P501 (◇) and JY2190 (☆) cells, at an initial ratio of 1:1. (D) Coculture of P501 (◇) and R6Chi (○) cells, at an initial ratio of 1:200. Cultures were monitored for growth, and at different times aliquots were removed for determination of viability.

acid substitutions with respect to its choline-dependent parental strain (Rx1; allele 1) (Fig. 3). Since two clinical pneumococcal isolates (strains INV104B and INV200) that naturally require choline for growth also showed two or even three different amino acid residues, respectively, with respect to the TacF_{R6} protein (Fig. 3), it may be concluded that it is likely that only specific amino acid changes (not random mutations) will render a Cho-ind phenotype. Besides, molecular modeling of the TacF protein suggested that the amino acid residues involved in choline independence (see Fig. S1 in the supplemental material) occupy more-or-less precise positions in TacF and that their mutual interactions play an essential role in transporting choline-containing TA units across the membrane of *S. pneumoniae*. Nevertheless, full confirmation of this prediction will require determining the real three-dimensional folding of the TacF flippase.

The difference between our results and those of Damjanovic et al. (5), who described a single Val²³⁴ to Phe change in the Cho-ind spontaneous mutant R6Chi, was intriguing. Although the mutation (G⁷⁰⁰ to T) reported for strain R6Chi (5) was identical to that detected here in P023 and its derivatives (despite being absent in the progenitor *S. mitis* SK598 strain), our Cho-ind transformants featured additional mutations (Fig. 3 and 4), with the exception of strain P700. It should be emphasized that none of the novel *tacF* mutations (whether in the donor or recipient DNAs) observed in the different Cho-ind

FIG. 6. Localization of mutated amino acid residues in the putative TacF flippase of selected Cho-ind pneumococcus strain alleles. (A) A consensus secondary structure model of TacF_{R6} (495 amino acid residues) produced by comparison of the results obtained using 10 different computer programs (see Table S1 in the supplemental material) is shown. The positions of several residues are indicated to the left of the corresponding amino acid both on the cytoplasm side (inside) and outside the membrane. Transmembrane α -helices are represented as green rectangles. White, yellow, red, and blue circles correspond, respectively, to hydrophobic, polar, negatively charged, and positively charged residues. (B) Proposed localization of mutated residues in several TacF alleles conferring a Cho-ind phenotype. These are shown enlarged in panel A.

transformants generated here (or in the JY2190 strain) were silent. This suggests that, out of all the spontaneous mutations that may appear in *tacF* during PCR amplification and/or during selective growth—that is, during the incubation of the transformed culture in aminoalcohol-free medium—only those conferring some adaptive advantage(s) are preserved. Conversely, our finding could also indicate that all of these mutations are required to confer an improved fitness to Cho-ind mutants of *S. pneumoniae* when growing in a medium lacking any aminoalcohol.

The exact nature of the Cho-ind phenotype is a further question that needs to be addressed. As already mentioned, the type strain of *S. oralis* can grow (albeit abnormally) in

medium lacking exogenously added choline or choline analogs and may thus be designated as a Cho-ind strain. Here we confirm these observations and extend them to the type strain of *S. mitis* (Fig. 1E). Notwithstanding, this was not the case for the *S. mitis* biovar 1 strain SK598, a strain that contains ethanolamine in its cell wall TA regardless of whether it is grown in a choline- or ethanolamine-containing medium (3) and that was also able to form long chains of cells when incubated in a medium lacking choline or an analog of choline (Fig. 1C). Although it was not investigated whether this strain was also able to synthesize TAs containing ethanolamine when grown in these conditions, it is of interest that the R6 transformant strain P023 and its descendants carried the Val²³⁴-to-Phe mu-

tation that was absent in both their parental SK598 strain and *S. mitis* NCTC 12261 (Fig. 2A). Notwithstanding the peculiar case of strain SK598, it could be that to grow as chains of cells, this Phe residue cooperates with other mutations present in the *S. mitis* strains. If this were true, then we could predict that pneumococcal R6 mutants exclusively featuring the Val²³⁴-to-Phe mutation would grow by forming abnormal cells rather than chains of cells. This hypothesis could also apply to other individual mutations found in the different Cho-ind strains (Fig. 3). This hypothesis was confirmed, as R6Chi and P700 (but not P501) cells incubated in a medium lacking any aminoalcohol were of abnormal shape and size (Fig. 1G and I) and showed an extended lag period. Moreover, the results of competition experiments (Fig. 5C and D) clearly indicated that, for improved fitness when growing in aminoalcohol-deprived medium, *S. pneumoniae* Cho-ind mutants must contain at least two specific *tacF* mutations, presumably because of an improved capacity of the TA flippase to transfer aminoalcohol-free TA subunits across the membrane to the growing TA chains.

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