JOURNAL OF BACTERIOLOGY, Nov. 2007, p. 8139–8144 Vol. 189, No. 22
0021-9193/07/$08.00+0 doi:10.1128/JB.01275-07

Isolation and Characterization of Unsaturated Fatty Acid Auxotrophs of *Streptococcus pneumoniae* and *Streptococcus mutans*▽

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Received 7 August 2007/Accepted 31 August 2007

Unsaturated fatty acid (UFA) biosynthesis is essential for the maintenance of membrane structure and function in many groups of anaerobic bacteria. Like *Escherichia coli*, the human pathogen *Streptococcus pneumoniae* produces straight-chain saturated fatty acids (SFA) and monounsaturated fatty acids. In *E. coli* UFA synthesis requires the action of two gene products, the essential isomerase/dehydratase encoded by *fabA* and an elongation condensing enzyme encoded by *fabB*. *S. pneumoniae* lacks both genes and instead employs a single enzyme with only an isomerase function encoded by the *fabM* gene. In this paper we report the construction and characterization of an *S. pneumoniae* 708 *fabM* mutant. This mutant failed to grow in complex medium, and the defect was overcome by addition of UFAs to the growth medium. *S. pneumoniae fabM* mutants did not produce detectable levels of monounsaturated fatty acids as determined by gas chromatography-mass spectrometry and thin-layer chromatography analysis of the radiolabeled phospholipids. We also demonstrate that a *fabM* null mutant of the cariogenic organism *Streptococcus mutants* is a UFA auxotroph, indicating that FabM is the only enzyme involved in the control of membrane fluidity in streptococci. Finally we report that the *fabN* gene of *Enterococcus faecalis*, coding for a dehydratase/isomerase, complements the growth of *S. pneumoniae fabM* mutants. Taken together, these results suggest that FabM is a potential target for chemotherapeutic agents against streptococci and that *S. pneumoniae* UFA auxotrophs could help identify novel genes encoding enzymes involved in UFA biosynthesis.

cis unsaturated fatty acids (UFAs) have crucial roles in membrane biology in organisms ranging from bacteria to humans. The relative abundance of UFAs in cellular phospholipids has a major influence on the physical properties of most biological membranes. UFAs have a much lower transition temperature than saturated fatty acids because the steric hindrance imparted by the rigid kink of the *cis* double bond results in much poorer packing of the acyl chains Thus, UFAs are key molecules in the regulation of cellular membrane fluidity (2). There are two major mechanisms by which living organisms synthesize UFAs; most organisms use an oxygen-dependent pathway, whereas many prokaryotes, including *Escherichia coli*, synthesize UFAs anaerobically. The anaerobic UFA synthesis in *E. coli* occurs through introduction of a double bond into the growing acyl chain by two specialized proteins, FabA and FabB, in a bacterial type II biosynthetic pathway (9). These proteins were first defined by isolation and characterization of UFA auxotrophs (8, 13). FabA is a bifunctional dehydratase/isomerase enzyme that introduces the double bond in the 10-carbon intermediate. This enzyme catalyzes both the dehydration of β-hydroxyacyl acyl carrier protein (β-hydroxyacyl-ACP) to *trans*-2-unsaturated acyl-ACP and also the isomerization of *trans*-2-decenoyl-ACP to *cis*-3-decenoyl-ACP, the key step of the classical anaerobic UFA biosynthetic pathway. FabB is a β-ketoacyl-ACP synthase I that elongates the unsaturated biosynthetic intermediates made by FabA. In *fabA* and *fabB* mutants saturated fatty acid (SFA) synthesis persists due to the presence of another dehydratase, FabZ, and another condensing enzyme, FabF. However, other bacteria lacking *fabA* synthesize UFAs under anoxic conditions. Like *E. coli*, the human pathogen *Streptococcus pneumoniae* produces straight-chain SFAs and monounsaturated fatty acids. This organism does not utilize a FabA-like mechanism for introducing a double bond into the growing acyl chain; instead, it employs a single enzyme with only an isomerase function encoded by the *fabM* gene (16). Although the function of *S. pneumoniae* FabM was inferred from biochemical analysis, this pathway has not yet been confirmed in vivo, and there is no information concerning whether FabM is the only enzyme involved in UFA synthesis in this organism. On the other hand, recent studies have shown that a *fabM* mutant of the cariogenic organism *Streptococcus mutans* is viable, although it exhibits an increased doubling time compared to the parental strain (10). This mutant does not produce UFAs, but it contains a much larger amount of nonidentified fatty acids than the wild-type parental strain (10). Since UFAs are essential in bacteria producing straight-chain fatty acids, these data raise the possibility that *S. mutans fabM* mutants could switch the product distribution of the pathway to generate significant amounts of other fatty acids, i.e., low-melting-point fatty acids, which partially compensate for the absence of UFAs in their membranes.

For these reasons and in order to more definitely establish the role of *fabM* in type II fatty acid biosynthesis in streptococci, we isolated and characterized null *fabM* mutants of *S. pneumoniae* and *S. mutans*. Our data indicate that these mu-

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▽ Published ahead of print on 7 September 2007.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
S. pneumoniae strains		
708	Unencapsulated, avirulent, highly competent *trt*-1 derivative of R6	12
SH9	*fabM*::Cm	This study
SH11	SH9/pDL278*fabM*	This study
SH13	SH9/pDL278*fabN*	This study
S. mutans strains		
UA159	Wild-type strain	4
SA20	*fabM*::Km	This study
SA21	SA20/pDL278*fabM*	This study
Plasmids		
pDL278	*E. coli-S. pneumoniae* shuttle vector; expression plasmid; Sp^r	13
pDL278*fabM*	His-tagged *fabM* coding sequence in pDL278 expressed from the pneumococcal *polA* promoter	16
pDL278*fabN*	His-tagged *fabN* coding sequence in pDL278 expressed from the pneumococcal *polA* promoter	Y. J. Lu
pUCSCATS	pUC18 harboring the syn Cm cassette, pC194 *cat* expressed from the *amiA* promoter[a]	Laboratory collection
pLB1	pGEMT harboring *S. pneumonie fabM*::Cm cassette	This study
pLB2	pGEMT harboring *S. mutans fabM*::Km cassette	This study

[a] See reference 7.

tants are UFA auxotrophs, demonstrating that FabM is an essential enzyme in streptococci. In addition, we found that the *fabN* gene of *Enterococcus faecalis*, which does not complement the growth of *E. coli fabA* mutants (18), is able to functionally complement *S. pneumoniae fabM* mutants. Therefore, *S. pneumoniae* UFA auxotrophic strains could help identify novel genes encoding enzymes involved in UFA biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used in this study are listed in Table 1. *S. pneumoniae* 708 and its derivatives were grown in AGCHYE medium (11) or on brain heart infusion (BHI) agar with 5% sheep blood. AGCHYE medium was prepared with fatty acid-free bovine serum albumin for all experiments unless indicated otherwise. Antibiotics were added at the following concentrations: chloramphenicol (Cm), 3 $\mu g\ ml^{-1}$; and spectinomycin (Sp), 500 $\mu g\ ml^{-1}$. *S. mutans* UA159 and its derivatives were maintained on BHI (Difco) agar plates or in Todd-Hewitt broth with 0.3% yeast extract (THYE) (Difco) at 37°C. The antibiotic concentrations used for *S. mutans* strains were 50 $\mu g\ ml^{-1}$ Sp and 1 $mg\ ml^{-1}$ kanamycin (Km). Overnight cultures were inoculated from −70°C glycerol stocks. The following day fresh cultures were started by washing and diluting the overnight cultures 1:10. *E. coli* strains were grown in Luria-Bertani medium supplemented when needed with 50 $\mu g\ ml^{-1}$ Sp and 100 $\mu g\ ml^{-1}$ ampicillin. UFAs neutralized with KOH were added to the medium at the concentrations indicated below. All chemicals were purchased from Sigma Chemical Co.

Construction of mutant strains. Plasmid pLB1, used to disrupt the *fabM* gene in *S. pneumoniae* (Table 1), was constructed as follows. Two sets of primers were designed to amplify the predicted FabM coding sequence plus additional flanking regions from the *S. pneumoniae* genome. The 710-bp upstream fragment containing the 5′ region of the *fabM* gene was amplified by PCR with primers L1 (5′-TGCATCCGATACCATTTTGGTTAAAGTAGGCAGCA-3′) and L2 (5′-TTGAAGAGATTTCGAAGAGGATCCACTGCTTAT-3′; BamHI site underlined). The 870-bp downstream fragment containing the 3′ region of the *fabM* gene was amplified by PCR with primers 5′-ACTGAACCTACAGAATCGCATGCTCAAACAGAGGATTTCA-3′ (SphI site underlined) and 5′-CGAGCAGCTGTAGAGGGCCCCATCGAATCTGGAGTA-3′ (ApaI site underlined). The PCR products were cloned in the pGEMT Easy (Promega Life Science) vector, yielding pSL1 and pSL2, respectively, and sequenced. The syn Cm resistance cassette (7) was excised from plasmid pUC18SCATS and inserted downstream of the insert between the BamHI and SphI sites of pSL1, yielding plasmid pSL3. Then the insert of pSL2 was located downstream of the *cat* gene by insertion within the ApaI and SphI sites of pSL3. The resulting plasmid, pLB1, was transformed in *E. coli*, and transformants were selected for Cm resistance. Plasmid DNA was prepared using the Wizard DNA purification system (Promega Life Science), linearized, and transferred to *S. pneumoniae* strain 708 by transformation as described by Lacks and Greenberg (11). The transformed cells were mixed with BHI-blood agar with 3 $\mu g\ ml^{-1}$ of Cm, plated, and incubated at 37°C. As a result of the expected double crossover, the Cm resistance cassette replaced part of the chromosomal copy of the target gene, thereby creating a gene knockout (Fig. 1). Gene replacement in mutant clones was confirmed by PCR using primers that annealed outside the DNA region used for mutant construction. The *fabM* mutant of *S. mutans* was constructed by allelic replacement mutagenesis using the Km resistance cassette method described by Song et al. (17). Briefly a Km resistance cassette (904 bp) from *Staphylococcus aureus* ATCC 43300 was amplified with primers Kan-F (5′-AACAGTGAATTGGAGTTCGTCTTGTTATA-3) and Kan-R (5′-GCTTTTTAGACATCTAAATCTAGGTA-3). Two pairs of gene-specific primers, L-F/L-R and R-F/R-R, were used to amplify the left and right flanking regions of the target gene, generating PCR products that were 500 bp long. Primers L-R and R-F each consisted of 21 nucleotides (5-GACGAACTCCAATTCACTGTT-3 and 5-AGATTTAGATGTCTAAAAAGC-3, respectively) identical to a segment of the promoter region and the 3′ end of the Km^r gene plus 29 nucleotides of the *fabM* sequence. PCR DNA amplifications were performed as described by Song et al. (17), and the linear fused product was cloned in the pGEMT vector, yielding plasmid pLB2, and sequenced. Transformation and gene disruption of *S. mutans* were performed as described by Ahn et al. (3). To this end, overnight cultures were diluted 20-fold in prewarmed THYE supplemented with 5% heat-inactivated horse serum and incubated at 37°C until the optical density at 600 nm was approximately 0.2. Competence of the cultures was induced by addition of 1 μg of synthetic competence-stimulating peptide (3) and incubation for 20 min. Then

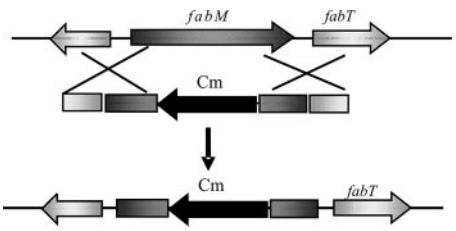

FIG. 1. Construction and structure of the FabM mutant. The *fabM* gene was disrupted with a Cm resistance cassette as outlined in Materials and Methods, and the structure of the chromosomal DNA of strain SH9 is diagrammed.

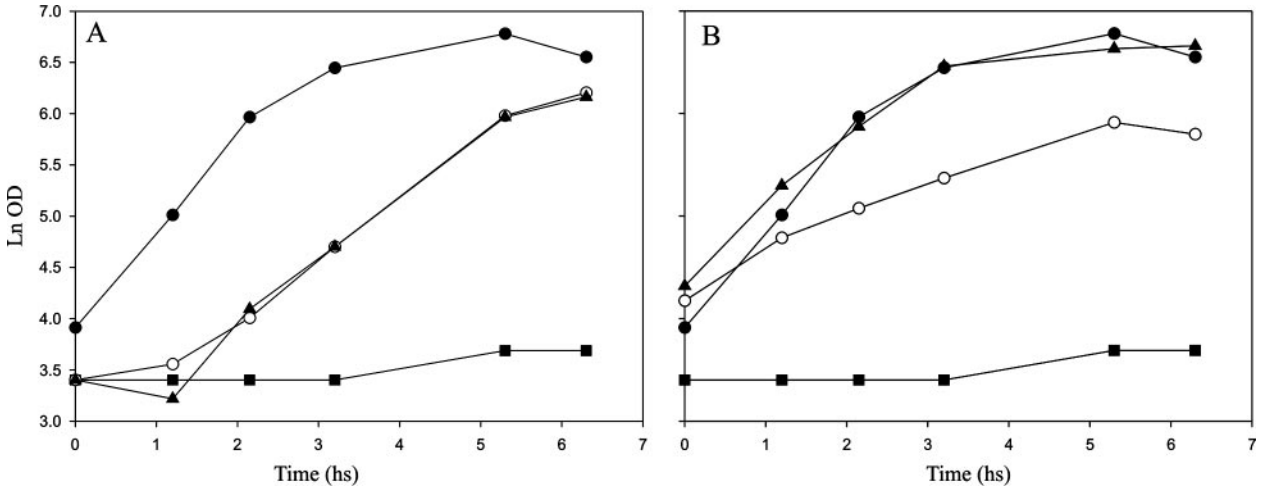

FIG. 2. Essentiality and growth phenotype of *S. pneumoniae fabM* mutant. (A) Cells of *S. pneumoniae* SH9 (*fabM*) were grown as described in the text. Cells were harvested, washed, and resuspended in fresh AGCHYE medium that was not supplemented (■) or was supplemented with 0.1 mM oleate (○) or 0.1 mM linoleic acid (▲). *S. pneumoniae* 708 (*fabM*$^+$) was grown in the same medium without supplements (●). (B) Growth curves for strains grown in AGCHYE medium without supplementation, including *S. pneumoniae* 708 (*fabM*$^+$) (●), SH9 (*fabM*/pDL278) (■), SH11 (*fabM*/p*fabM*$^+$) (▲), and SH13 (*fabM*/p*fabN*$^+$) (○). OD, optical density.

transformation was performed by incubating the cultures for 30 min with linearized plasmid pLB2 (Sp^r Km^r). To allow expression of the antibiotic resistance marker before plating, cultures were incubated for 2 h after addition of 0.5 ml of fresh THYE supplemented with 5% heat-inactivated horse serum. Transformants were selected by plating cultures on BHI-blood agar supplemented with 1 mg ml^{-1} Km and incubating the preparations at 37°C for 48 h. Gene replacement in mutant clones was confirmed by PCR. Genomic DNAs of mutant and wild-type strains were used as templates for PCR amplification with primers L-F and R-R to verify correct incorporation of the fused construct into the mutant genome.

Fatty acid analysis. For measurement of fatty acid biosynthesis cells were grown in AGCHYE medium to mid-exponential phase and labeled for one generation time with 2 μCi of [1-^{14}C]acetate. Lipids were extracted, converted to methyl esters, and separated into unsaturated and saturated fractions by chromatography on 10% silver nitrate-impregnated Silica Gel G plates (thickness, 0.5 mm; Analtech) developed with toluene at −20°C (1). Radioactivity on the plates was visualized using a Typhoon 9200 PhosphorImager screen and was quantified using ImageQuant software (version 5.2). Lipid species were identified by comigration with standards. To analyze the fatty acid profiles, streptococci were grown to mid-log phase in AGCHYE medium containing 0.8% sucrose at 37°C. Total cellular fatty acids were prepared by the method of Bligh and Dyer (6). The fatty acid methyl esters were prepared by transesterification of glycerolipids with 0.5 M sodium methoxide-methanol and then analyzed with a Perkin Elmer Turbo Mass gas chromatograph-mass spectrometer using a capillary column (30 m by 0.25 mm [inside diameter]) containing 100% dimethylpolysiloxane (PE-1; Perkin Elmer Co). Helium at a flow rate of 1 ml min^{-1} was used as the carrier gas, and the column temperature was programmed to increase at a rate of 4°C min^{-1} from 140 to 240°C. The spectra were recorded in the electron impact mode at 70 eV, using 1-s scans of *m/z* 40 to 400 (5). The SFAs and UFAs used as reference compounds were obtained from Sigma Chemical Co.

RESULTS AND DISCUSSION

***cis-trans* isomerase FabM is essential in *S. pneumoniae* and *S. mutans*.** The *fabM* gene of *S. pneumoniae* codes for the FabM enzyme proposed to be essential for UFA biosynthesis based on its vitro enzymatic activity (15). To directly test the role of this enzyme in the physiology of *S. pneumoniae*, we disrupted the *fabM* gene with a Cm resistance cassette. The resulting strain, designated SH9, did not exhibit a colony phenotype on BHI-blood agar plates, and it autolysed faster than the parental strain. More importantly, *fabM* mutants completely failed to grow in AGHYE complex medium unless it was supplemented with UFAs (Fig. 2). The growth defect of SH9 could be overcome by supplementing the medium with oleate or linoleate (Fig. 2A). On the other hand, SFAs were unable to support the growth of SH9 (data not shown), indicating that this strain is a UFA auxotroph.

To confirm the UFA auxotrophy of SH9, this strain was grown in AGCHYE medium supplemented with 0.1 mM oleic acid, and its fatty acid composition was determined by gas chromatography-mass spectrometry (Table 2). The membrane of wild-type strain 708 contained 89% UFAs, including three $C_{16:1}$ fatty acids with double bonds at positions Δ7, Δ9, and Δ11 and three $C_{18:1}$ fatty acids with double bonds at positions Δ9, Δ11, and Δ13 (Table 2), while the UFA content of the mutant

TABLE 2. Fatty acid compositions of total membrane lipid extracts from *S. pneumoniae* strains[a]

Fatty acid	% in *S. pneumoniae* strains		
	708	SH9[b]	SH11
n-$C_{12:0}$	0.16 ± 0.07	2.31 ± 0.68	0.62 ± 0.06
n-$C_{14:1}$	0.92 ± 0.02	0	0
n-$C_{14:0}$	1.29 ± 0.30	4.43 ± 1.12	5.51 ± 0.51
n-$C_{16:1\Delta7}$	4.29 ± 0.51	0	3.88 ± 0.10
n-$C_{16:1\Delta9}$	15.69 ± 1.39	tr	13.98 ± 0.45
n-$C_{16:1\Delta11}$	8.21 ± 0.26	0	3.68 ± 0.24
n-$C_{16:0}$	7.50 ± 1.65	9.25 ± 1.76	27.68 ± 2.45
n-$C_{18:1\Delta9}$	3.4 ± 0.73	83.29 ± 1.72	4.16 ± 0.28
n-$C_{18:1\Delta11}$	50.3 ± 0.71	0	33.92 ± 2.31
n-$C_{18:1\Delta13}$	6.53 ± 0.90	0	2.23 ± 0.16
n-$C_{18:0}$	1.67 ± 0.32	0.71 ± 0.21	4.1 ± 0.41
All UFAs	89.34 ± 4.52	83.69 ± 1.74	61.85 ± 3.54

[a] Cells were grown in AGCHYE medium to exponential phase at 37°C. The total lipids were extracted and transesterified to obtain fatty acid methyl esters, and products were identified by gas chromatography-mass spectrometry. The values are the means ± standard deviations of three independent experiments and are percentages of the total fatty acids.

[b] Cultures of SH9 were supplemented with 0.1 mM oleate.

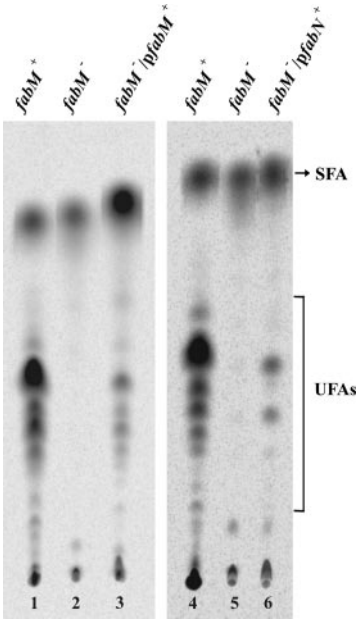

FIG. 3. [1-^{14}C]acetate labeling profiles of *S. pneumoniae* strains. Cultures were grown in AGCHYE medium with [1-^{14}C]acetate, lipids were extracted, and fatty acid methyl esters were prepared and separated by thin-layer chromatography on silver nitrate-impregnated silica plates. Lanes 1 and 4, strain 708 (*fabM*$^+$); lanes 2 and 5, strain SH9 (*fabM*); lane 3, strain SH11 (*fabM*/p*fabM*$^+$); lane 6, strain SH13 (*fabM*/p*fabN*$^+$).

strain membrane was 84% and the membrane was composed almost exclusively of oleic acid. In addition, strain SH9 produced a larger amount of shorter-chain $C_{12:0}$ and $C_{14:0}$ saturated fatty acids than the parental strain (6% versus 1%). We verified that the UFA composition changes arose from alterations in de novo fatty acid synthesis by labeling strains 708 and SH9 with [1-^{14}C]acetate. The lipids were extracted, and the distribution of label in the fatty acyl methyl esters was determined by argentation chromatography. As shown in Fig. 3, the parent strain (lanes 1 and 4) incorporated radioactivity into both SFAs and UFAs, whereas SH9 (lanes 2 and 5) incorporated radioactivity only into SFAs. These results provided a direct demonstration that SH9 failed to synthesize UFAs.

We confirmed that the deficiency in UFA synthesis of strain SH9 was due to the absence of a functional pneumococcal FabM protein by introducing into strain SH9 a plasmid that expressed the wild-type *fabM* gene. Expression of *fabM* in strain SH9 (strain SH11) completely eliminated its growth deficiency in AGCHYE medium (Fig. 2B) and reestablished the synthesis of UFAs (Fig. 3, lane 3). As shown in Table 2 and Fig. 3, strain SH11 synthesized a smaller amount of UFAs than wild-type strain 708. This was likely due to the fact that the His-tagged version of FabM expressed by strain SH11 (Table 1) was less active than native FabM. In any case, we concluded from these data that *fabM* is an essential gene in *S. pneumoniae* and that the absence of FabM was the sole cause for the biochemical and growth phenotypes exhibited by strain SH9.

It has recently been reported that a *fabM* mutant of *S. mutans* is viable, although it exhibited an increased doubling time compared to the wild-type parent strain (10). This result

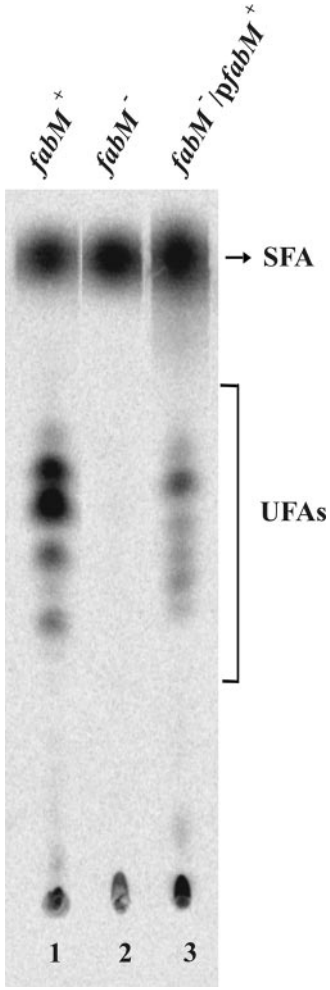

FIG. 4. [1-^{14}C]acetate labeling profiles of *S. mutans* strains. Labeling of fatty acids and chromatography on silver nitrate-impregnated silica plates were performed as described in the legend to Fig. 3. Lane 1, strain U159 (*fabM*$^+$); lane 2, strain SA20 (*fabM*); lane 3, strain SA21 (*fabM*/p*fabM*$^+$).

could indicate that *S. mutans* has the ability to synthesize other fatty acids that partially overcome the deficiency of UFAs in the absence of the *fabM* gene product. To test this possibility, we constructed the *S. mutans* SA20 strain, which is a *fabM* insertional null mutant derivative of the UA159 streptococcal strain (see details in Materials and Methods). Mutant strain SA20 was still able to generate small colonies on BHI-blood agar, but we found that it required supplementation with UFAs for growth in AGCHYE complex medium (data not shown). SFAs did not cure the growth defect of SA20 in AGCHYE medium, indicating that this strain is an UFA auxotroph. The inability of the *fabM* strain to synthesize UFAs was verified by in vivo labeling of the fatty acids with [1-^{14}C]acetate, followed by argentation chromatography. While SA20 synthesized only SFAs (Fig. 4, lane 2), the control strain synthesized both SFAs and UFAs (Fig. 4, lane 1). We also examined the membrane lipid composition of the *S. mutans* strains by gas chromatography-mass spectrometry analysis (Table 3). We found that like *S. pneumoniae*, *S. mutans* UA159 synthe-

TABLE 3. Fatty acid compositions of total membrane lipid extracts from *S. mutans* strains[a]

Fatty acid	% in *S. mutans* strains		
	SMU159	SA20[b]	SA21
n-$C_{12:0}$	0.64 ± 0.14	5.94 ± 0.17	3.23 ± 0.07
n-$C_{14:1}$	0	0	0.6 ± 0.07
n-$C_{14:0}$	4.2 ± 1.10	4.28 ± 0.80	10.53 ± 0.84
n-$C_{16:1\Delta7}$	2.07 ± 0.27	0	2.21 ± 0.27
n-$C_{16:1\Delta9}$	0.91 ± 0.27	tr	1.75 ± 0.03
n-$C_{16:1\Delta11}$	1.09 ± 0.10	0	1.12 ± 0.23
n-$C_{16:0}$	37.4 ± 2.2	43.08 ± 2.20	46.68 ± 3.2
n-$C_{18:1\Delta9}$	3.69 ± 0.41	18.86 ± 0.35	2.08 ± 0.35
n-$C_{18:1\Delta11}$	20.75 ± 0.61	0	7.28 ± 0.18
n-$C_{18:1\Delta13}$	0.81	0	0
n-$C_{18:0}$	13.34 ± 1.7	10.49 ± 1.12	19.36 ± 1.12
n-$C_{20:1}$	13.2 ± 0.7	13.96 ± 1.73	2.81 ± 0.23
n-$C_{20:0}$	1.85 ± 0.3	2.85 ± 0.30	2.12 ± 0.27
All UFAs	42.52 ± 2.46	32.82 ± 2.08	17.25 ± 1.29

[a] Cells were grown in AGCHYE medium to exponential phase at 37°C. The total lipids were extracted and transesterified to obtain fatty acid methyl esters, and products were identified by gas chromatography-mass spectrometry. The values are the means ± standard deviations of three independent experiments and are percentages of the total fatty acids.

[b] Cultures of SA20 were supplemented with 0.100 mM oleate.

sized straight-chain saturated and monounsaturated fatty acids, but the carbon atom chain lengths of the UFAs were predominately 18 and 20 (Table 3). As shown in Table 3, when SA20 was grown in AGCHYE medium supplemented with oleate, the UFA content of the mutant strain was 19% oleate and 14% $C_{20:1}$, the elongation product of oleic acid. Although these data are fully consistent with the conclusion that *fabM* is an essential gene in *S. mutans*, they are not in agreement with recent results of Fozo and Quivey (10), who reported that a *fabM S. mutans* strain, UR117, is able to grow in complex medium without a UFA supplement. Since these experiments were performed in TY rich medium, we reexamined whether the UFA auxotrophy of SA20 could be relieved in this medium. However, this was not the case, since in TY medium SA20 was still unable to grow without addition of exogenous UFAs (data not shown). We cannot readily explain why our results differ from those of Fozo and Quivey (10). A possible explanation is that the growth medium used in the experiment of Fozo and Quivey contained contaminant fatty acids that partially met the UFA requirement of UR117. In agreement with this possibility, it was reported that UR117 contains almost 40% nonidentified fatty acids in its membranes (10). These unknown fatty acids could in some way partially support the growth of UR117.

The isolation of UFA growth-dependent *fabM* null mutants of *S. pneumoniae* demonstrated that FabM is essential for growth and cell viability of this bacterium and that the role of UFAs cannot be filled by other fatty acids synthesized by this organism. In addition, under the conditions used in this work we found that *fabM* is also essential in *S. mutans*, indicating that FabM is the only enzyme involved in the control of membrane fluidity in streptococci.

Functional replacement of *S. pneumoniae* FabM by *E. faecalis* FabN. The gram-positive bacterium *E. faecalis* has a fatty acid composition very similar to that of *E. coli*, but it lacks FabA, FabB, and FabM homologues (18). However, it contains a protein, FabN, which is a bifunctional dehydratase/isomerase capable of introducing a double bond into a growing acyl chain (18). It has been demonstrated that FabN can produce UFAs in *E. coli fabA* cells, but the low level of UFAs produced was unable to support growth (18). Here we investigated whether expression of the FabN enzyme corrected the FabM deficiency of *S. pneumoniae* SH9. To do this, we transformed *S. pneumoniae* SH9 with plasmid pDL*fabN* containing the *fabN* coding sequences from *E. faecalis*. The resulting transformant, strain SH13 (Table 1), was selected on a BHI-blood agar plate containing the appropriate antibiotics (see Materials and Methods) and tested for growth in AGCHYE medium in the absence of a UFA supplement. As shown in Fig. 2B, *fabN* was able to complement the growth defect of the SH9 *fabM* mutant. Analysis of the de novo synthesis of [1-^{14}C]acetate-labeled fatty acids by argentation thin-layer chromatography and quantification of the spots revealed that strain SH13 produced about 22% UFAs. The UFAs synthesized by SH13 were identified as $C_{16:1\Delta9}$ and $C_{18:1\Delta11}$ (Fig. 3, lane 6), which are identical to the UFAs produced by *E. faecalis* (18). These data indicate that FabN functionally replaces the *S. pneumoniae* FabM protein. Our experiments also demonstrate that in *S. pneumoniae* FabM-deficient cells expressing FabN, the synthesis of UFAs was not strongly decreased by competition for *trans*-2-decenoyl-ACP between FabN and the host enoyl-ACP reductase FabK. This was not the case in *E. coli*, where FabN was unable to complement the UFA auxotrophy of a *fabA* mutant strain because the FabI enoyl reductase efficiently competed with FabN for *trans*-2-decenoyl-ACP (14). It should be noted that the *fabN*-complemented *fabM* SH13 strain synthesized about 20% of the UFAs synthesized by the wild-type strain (Fig. 3). This appears to indicate that *S. pneumonie* requires smaller amounts of UFAs for growth than *E. coli*. Therefore, this observation could also explain the finding that *fabN* functionally complements the growth of *S. pneumonie* but not the growth of *E. coli*. In any case, it is clear from our work that UFA auxotrophs of *S. pneumoniae* could be helpful in the identification of novel genes involved in the anaerobic synthesis of UFAs in organisms that lack recognizable homologues of FabA or FabM genes in their genomes.

ACKNOWLEDGMENTS

We gratefully acknowledge Ying-Jie Lu for sending plasmid pDL278*fabN* and Charles Rock for helpful discussions and suggestions. We also thank Monica Hourcade for technical support and Antonio Uttaro for critically reading the manuscript.

This work was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina) and Agencia de Promoción Científica y Tecnológica (FONCYT, Argentina). S.A. and D.D.M. are Career Investigators at CONICET. D.D.M. is an International Research Scholar of the Howard Hughes Medical Institute. The work at the Centro de Investigaciones Biológicas was supported by the Spanish Fondo de Investigación Sanitaria (grant PI040808 to P.L.).

REFERENCES

1. **Aguilar, P. S., J. E. Cronan, Jr., and D. de Mendoza.** 1998. A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. J. Bacteriol. **180:**2194–2200.
2. **Aguilar, P. S., and D. de Mendoza.** 2006. Control of fatty acid desaturation: a mechanism conserved from bacteria to humans. Mol. Microbiol. **62:**1507–1514.
3. **Ahn, S. J., Z. T. Wen, and R. A. Burne.** 2006. Multilevel control of competence development and stress tolerance in *Streptococcus mutans* UA159. Infect. Immun. **74:**1631–1642.

4. **Ajdic, D., W. M. McShan, R. E. McLaughlin, G. Savic, J. Chang, M. B. Carson, C. Primeaux, R. Tian, S. Kenton, H. Jia, S. Lin, Y. Qian, S. Li, H. Zhu, F. Najar, H. Lai, J. White, B. A. Roe, and J. J. Ferretti.** 2002. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc. Natl. Acad. Sci. USA **99:**14434–14439.
5. **Altabe, S. G., P. Aguilar, G. M. Caballero, and D. de Mendoza.** 2003. The *Bacillus subtilis* acyl lipid desaturase is a delta5 desaturase. J. Bacteriol. **185:**3228–3231.
6. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **31:**911–917.
7. **Claverys, J.-P., A. Dintilhac, V. Ekaterina, E. V. Pestova, B. Martin, and D. A. Morrison.** 1995. Construction and evaluation of new drug-resistance cassettes for gene disruption mutagenesis in *Streptococcus pneumoniae*, using an *ami* test platform. Gene **164:**123–128.
8. **Cronan, J. E., C. H. Birge, and P. R. Vagelos.** 1969. Evidence for two genes specifically involved in unsaturated fatty acid biosynthesis in *Escherichia coli*. J. Bacteriol. **100:**601–604.
9. **Cronan, J. E.** 2006. A bacterium that has three pathways to regulate membrane lipid fluidity. Mol. Microbiol. **60:**256–259.
10. **Fozo, E. M., and R. G. Quivey.** 2004. The *fabM* gene product of *Streptococcus mutants* is responsible for the synthesis of monosaturated fatty acids and is necessary for survival at low pH. J. Bacteriol. **186:**4152–4158.
11. **Lacks, S. A., and B. Greenberg.** 2001. Constitutive competence for genetic transformation in S*treptococcus pneumoniae* caused by mutation of a transmembrane histidine kinase. Mol. Microbiol. **4:**1035–1045.
12. **LeBlanc, D. J., L. N. Lee, and A. Abu-Al-Jaibat.** 1992. Molecular, genetic, and functional analysis of the basic replicon of pVA380-1, a plasmid of oral streptococcal origin. Plasmid. **28:**130–145.
13. **Lu, Y. J., Y. M. Zhang, and C. O. Rock.** 2004. Product diversity and regulation of type II fatty acid synthases. Biochem. Cell Biol. **82:**145–155.
14. **Lu, Y. J., S. W. White, and C. O. Rock.** 2005. Domain swapping between *Enterococcus faecalis* FabN and FabZ proteins localizes the structural determinants for isomerase activity. J. Biol. Chem. **280:**30342–30348.
15. **Lu, Y. J., S. W. White, and C. O. Rock.** 2006. Transcriptional regulation of fatty acid biosynthesis in *Streptococcus pneumoniae*. Mol. Microbiol. **59:**551–566.
16. **Marrakchi, H., K. H. Choi, and C. O. Rock.** 2002. A new mechanism for anaerobic unsaturated fatty acid formation in *Streptococcus pneumoniae*. J. Biol. Chem. **277:**44809–44816.
17. **Song, J. H., K. S. Ko, J. 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.
18. **Wang, H., and J. Cronan.** 2004. Functional replacement of FabA and FabB proteins of *Escherichia coli* fatty acid synthesis by *Enterococcus faecalis* FabZ and FabF homologues. J. Biol. Chem. **279:**34489–34495.