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Genetic and Physiologic Analysis of a Formyl-Tetrahydrofolate Synthetase Mutant of *Streptococcus mutans*

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Previously we reported that transposon Tn*917* mutagenesis of *Streptococcus mutans* JH1005 yielded an isolate defective in its normal ability to produce a mutacin (P. J. Crowley, J. D. Hillman, and A. S. Bleiweis, abstr. D55, p. 258 *in Abstracts of the 95th General Meeting of the American Society for Microbiology 1995, 1995)*. In this report we describe the recovery of the mutated gene by shotgun cloning. Sequence analysis of insert DNA adjacent to Tn*917* revealed homology to the gene encoding formyl-tetrahydrofolate synthetase (Fhs) from both prokaryotic and eukaryotic sources. In many bacteria, Fhs catalyzes the formation of 10-formyl-tetrahydrofolate, which is used directly in purine biosynthesis and formylation of Met-tRNA and indirectly in the biosynthesis of methionine, serine, glycine, and thymine. Analysis of the *fhs* mutant grown anaerobically in a minimal medium demonstrated that the mutant had an absolute dependency only for adenine, although addition of methionine was necessary for normal growth. Coincidently it was discovered that the mutant was sensitive to acidic pH; it grew more slowly than the parent strain on complex medium at pH 5. Complementation of the mutant with an integration vector harboring a copy of *fhs* restored its ability to grow in minimal medium and at acidic pH as well as to produce mutacin. This represents the first characterization of Fhs in *Streptococcus*.

Streptococcus mutans, an organism associated with the causation of human dental caries, possesses features that enable it to colonize the host, accumulate on teeth, and ultimately produce a pathologic end result. Important structural features associated with virulence include surface adhesins and glucan or fructan capsules (for reviews, see references 26 and 41), which allow adherence to dental surfaces and coherence to other members of the plaque flora. Important physiologic features associated with virulence include the glycolytic conversion of dietary carbohydrates to high levels of lactic acid that contribute to low plaque pH levels and the ability to withstand these acidic conditions by low membrane permeability to protons and high activity of H^+/ATPase for increased proton extrusion (1a, 17). The ability to withstand low pH is a trait which also provides this organism a likely selective means by which to dominate its niche in the oral cavity. Its dominance in these microenvironments is thought to be aided by the ability of many strains of *S. mutans* to produce specific antimicrobial substances, referred to as mutacins (16), that are effective against other strains of *S. mutans* and related organisms. In some studies (16, 40) up to 80% of the strains tested had the ability to produce a mutacin.

In order to learn more about the genetic basis of virulence in this cariogenic streptococcus, we used the thermosensitive plasmid pTV1-OK, which harbors the nonconjugative transposon Tn*917*, to mutagenize the chromosome of this organism. In a previous publication (14), which describes the construction of plasmids used for transposition and for marker rescue of interrupted genes, we demonstrated random mutagenesis of *S. mutans*, even in a poorly transformable strain, and we were able to recover a number of genes involved in acid tolerance, glutamate biosynthesis (9), arginine biosynthesis, and putative chaperonin functions (15).

Some Tn*917* mutants displayed both acid sensitivity at pH 5 and an inability to produce mutacin (6, 14). The present study describes the characterization of one gene whose mutation is responsible for these pleiotropic effects. We discovered that a mutation in the gene (*fhs*) encoding formyl-tetrahydrofolate (formyl-THF) synthetase (Fhs) (EC 6.3.4.3), an enzyme central to de novo purine and amino acid biosynthesis (12, 38, 56), resulted in the loss of these key virulence-promoting traits. To our knowledge, this represents the first characterization of *fhs* in the streptococci.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1. *S. mutans* strains were subcultured routinely under aerobic conditions at 37°C in Todd-Hewitt broth (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Md.) supplemented with 0.3% yeast extract (THYE) and in brain heart infusion (BHI) broth (BBL). When required, antibiotics were used at the following concentrations: tetracycline, 15 μg/ml; erythromycin, 5 μg/ml; and kanamycin, 500 μg/ml. All streptococcal species, *Lactobacillus caseii* ATCC 7469, and *Staphylococcus aureus* Cowan used as indicator strains in the deferred-antagonism assay also were cultured aerobically in THYE at 37°C. When anaerobic cultivation was necessary, either an atmosphere composed of 85% nitrogen, 10% hydrogen, and 5% carbon dioxide or a GasPak Plus anaerobic system (BBL) was used. *Actinomyces naeslundii* PK19 was cultured in BHI broth under anaerobic conditions at 37°C as described above.

To evaluate sensitivity to acidic pH, *S. mutans* strains were cultured in THYE at pH 7.3 to mid-exponential phase (100 U with a green filter in a Klett colorimeter [Klett-Summerson, New York, N.Y.]) and then diluted 1:10 into prewarmed medium adjusted to pH 5.0 with HCl. Cultures were maintained at 37°C, and growth was measured with the Klett colorimeter until stationary phase was reached.

S. mutans JH1005, L22, and L22-6 were cultured anaerobically in the minimal medium of Carlsson (3) for determination of auoxotrophic properties. The medium (CMM) consisted of 0.1 M potassium phosphate (pH 7.0), 56 mM glucose, 0.36 mM cysteine-HCl, 10 mM ammonium sulfate, 0.81 mM magnesium sulfate, and a vitamin pool composed of 58 mM pyridoxine-HCl, 19 mM nicotinic acid, 0.02 mM biotin, 0.07 mM *p*-aminobenzoic acid, 0.15 mM thiamine-HCl, 0.53 mM riboflavin, and 2.52 mM calcium pantothenate. Adenine, guanine, and thymine (20 μg/ml), adenosine (40 μg/ml), and methionine (100 μg/ml) were added either singly or in combination to the minimal medium. A pool of 18 amino acids, not including cysteine, formulated by Terleckyj and Shockman (49) for growth of *Streptococcus* also was used to supplement the medium. Tubes containing complete CMM broth were covered with aluminum foil to prevent degradation of photosensitive vitamins and were preincubated for 16 to 24 h in an anaerobic chamber. Each 5 ml of equilibrated CMM was inoculated, in duplicate, with 0.25 ml of cells from 16-h THYE aerobic cultures that had been washed twice with CMM and resuspended to the original culture volumes with

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Mutans streptococci		
S. cricetus E49	Wild type	Lab collection
S. rattus BHT	Wild type	Lab collection
S. rattus BHT-2	Str^r (1 mg/ml) mutacin indicator strain	20
S. mutans NG8	Wild type	K. Knox
S. sobrinus SL1	Wild type	Lab collection
S. mutans V100	Wild type	Lab collection
S. mutans OMZ175	Wild type	Lab collection
S. sobrinus 27352	Wild type	ATCC[b]
S. downeii 33748	Wild type	ATCC
S. mutans JH1005	Mut^+ Tet^r (1 μg/ml)	21
S. mutans L22	*fhs*::Tn*917*/Erm^r Ade^- Mut^- pH 5^s	This study
S. mutans L22-6	*fhs*::Tn*917*::pAS25NCE (*fhs*$^+$ *dfp*::Tn*917*Δ*erm*::pVA981)/Fhs^+ Erm^r Tet^r Mut^+ pH 5	This study
Streptococcus salivarius		
27945		ATCC
25975		ATCC
PC1		Lab collection
Streptococcus mitis		
903		Lab collection
19950		ATCC
Streptococcus sanguis		
PC10		Lab collection
PC13		Lab collection
Challis e		Lab collection
Streptococcus pyogenes		
Manfredo		Lab collection
MC5		M. Cunningham
Lactobacillus caseii 7469		ATCC
Staphylococcus aureus Cowan		Lab collection
Actinomyces naeslundii PK19		W. Clark
Escherichia coli		
MC1061	F^- *araD139* Δ(*ara-leu*)*7696 galE15 galK16* Δ(*lac*)*X74 rpsL* (Str^r) *hsdR2* (r_K^- m_K^+) *mcrA mcrB1*	J. Hillman
RR1	F^- *mcrB mmr hsdS20* (r_B^- m_B^-) *leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20* (Sm^r) *supE441*	P. Youngman
Plasmids		
pTV1-OK	pWV01 *repA*(ts) Kan^r Tn*917* Erm^r	14
pUC19	Amp^r *lacZ*′	Lab collection
pPC193	*fhs*::Tn*917*::pUC19 Amp^r Erm^r	This study
pAS25NCE	*fhs*$^+$ *dfp*::Tn*917*Δ*erm*::pVA981	14

[a] Mut^+ and Mut^-, production and absence of a mutacin, respectively; pH 5^s, reduced ability to grow in media at pH 5; pH 5, normal ability to grow in media at pH 5; Ade^-, dependency on adenine for growth on CMM; Str^r, streptomycin resistance.
[b] ATCC, American Type Culture Collection.

CMM. The cultures were incubated anaerobically at 37°C for 48 h, and densities were measured with a Klett-Summerson colorimeter.

Escherichia coli MC1061 was cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl [pH 7.5] with 1 N NaOH) supplemented with ampicillin (150 μg/ml), tetracycline (15 μg/ml), or erythromycin (300 μg/ml). In general, cells were cultured aerobically for 16 h at 37°C with vigorous shaking.

Plasmid pTV1-OK is a *repA*(ts) derivative of the *Lactococcus lactis* cryptic plasmid pWVO1 (25) for temperature-dependent replication in both *S. mutans* and *E. coli*. It possesses a kanamycin resistance gene that is expressed in both *E. coli* and *S. mutans* and the transposon Tn*917*, which confers erythromycin resistance in streptococci and *E. coli* MC1061 (14).

Tn*917* mutagenesis and recovery of interrupted DNA. Mutagenesis of *S. mutans* JH1005 with pTV1-OK (Erm) was performed by the method of Gutierrez et al. (14). The percentage of total CFU that retained erythromycin resistance was determined by plating samples of the culture on BHI agar with and without erythromycin. The frequency of transposition coinciding with loss of pTV1-OK was determined by patching Erm^r colonies onto medium supplemented with kanamycin. Colonies which arose on BHI-erythromycin plates that were also Kan^s were screened for loss of mutacin activity by the deferred-antagonism assay described below.

Chromosomal DNA from transposon mutant L22 was restricted with *Eco*RI, which does not cut in the transposon, and ligated to *Eco*RI-linearized and dephosphorylated pUC19 DNA. Ligated DNA was used to transform *E. coli* MC1061, and recombinants were plated onto LB agar supplemented with ampicillin. After 16 to 24 h at 37°C, colonies were replica plated onto LB agar containing erythromycin and ampicillin and incubated for an additional 48 h at 37°C. Erm^r and Amp^r colonies were selected for plasmid isolation, and the presence of the transposon was confirmed by dot blot DNA hybridization with labeled pTV1-OK.

Transformations. Natural transformation of *S. mutans* JH1005 (wild type) and L22 (*fhs*::Tn*917*) was performed according to the method of Perry and Kuramitsu (36). Transformed cells were spread on THYE agar supplemented with 10% sucrose and the appropriate antibiotic(s). Plates were incubated anaerobically at 37°C for 48 h.

Genetic complementation of *fhs* mutant strain L22 was performed by electrotransformation (8) with 0.5 to 1.0 μg of integration vector pAS25NCE (14). Transformants were selected on tetracycline-agar.

E. coli MC1061 was transformed by using $CaCl_2$-competent cells as described by Maniatis et al. (30).

Southern hybridization analyses. Southern analysis of *Eco*RI-digested chromosomal DNA from *S. mutans* strains was performed according to the method of Southern (43). DNA probes were constructed by nick translation of pTV1-OK with biotin-14-dATP with the BioNick labeling kit (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer's instructions. Hybridized blots were developed with the Photogene Detection System (Bethesda Research Laboratories).

DNA preparations. Plasmid DNA preparations were performed by using a modified alkaline lysis-polyethelene glycol precipitation procedure (1).

Chromosomal DNA from streptococci was prepared as described previously (14).

DNA sequence analysis. Insert DNA from pPC193 was sequenced by the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research. Sequencing was accomplished by using the *Taq* DyeDeoxy Terminator and DyePrimer Cycle Sequencing protocols, developed by Applied Biosystems (Foster City, Calif.), with fluorescently labeled dideoxynucleotides and primers. The labeled extension products were analyzed on an Applied Biosystems model 373A DNA sequencer.

Oligonucleotide primers used for DNA sequencing were prepared by the DNA Synthesis Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research.

Homology searches of sequence databases were performed with the BLAST program from the National Centers for Biotechnology Information (Bethesda, Md.). Multiple amino acid sequence alignments were generated with the CLUSTAL W program (51) and displayed with SeqVu version 1.0.1 (Garvan Institute of Medical Research, Sydney, Australia).

Deferred-antagonism assay for mutacin production and sensitivity. Bioassay of mutacin activity was performed by using a modification of the assay described by Hillman et al. (21). The assay entailed stab inoculation of THYE agar plates with single colonies of the strains to be tested. The stabbed plates were incubated at 37°C for 16 h in a candle jar and then overlaid with 3 ml of molten THYE top agar (0.75% agar) containing 1 mg of streptomycin sulfate B per ml and a 10^{-6} dilution of the indicator stain *Streptococcus rattus* BHT-2 (Str^r). Overlaid plates were incubated for an additional 16 h at 37°C in a candle jar and scored for the presence of clear zones over individual stabs. For analysis of sensitivities of strains other than BHT-2 to the mutacin, no antibiotic was included in the agar overlays. Mutacin activity was apparent as clear zones surrounding each stab.

Preparation of cell lysates and assay for formyl-THF synthetase activity. Whole-cell lysates of *S. mutans* strains were prepared in duplicate according to the alumina grinding method of Vadeboncoeur et al. (52). Cell lysates were placed on ice and immediately assayed for Fhs activity. A portion of each extract was dialyzed against buffer containing 10 mM potassium phosphate (pH 7.5) and 1 mM EDTA for protein determination.

Fhs activity in cell lysates was determined according to the method of Rabinowitz and Pricer (37) as follows. In a 1-ml reaction volume, stock solutions were added to give final concentrations of 100 mM triethanolamine-HCl buffer (pH 8.0), 5 mM neutralized ATP (freshly prepared), 10 mM $MgCl_2$, 40 mM ammonium formate, and 2 mM DL-THF (Sigma Chemical Co., St. Louis, Mo.). The THF was freshly prepared in 1 M β-mercaptoethanol and neutralized with sodium hydroxide. Tubes in which extracts or THF were omitted served as controls. Tubes were preincubated at 37°C for 2 min, and then 10 μl of each cell lysate was added, undiluted, to tubes in triplicate. The tubes were incubated at 37°C for 20 min, and the reactions were stopped by the addition of 2 ml of 0.36 N HCl. Acidification with HCl stoichiometrically converts 10-formyl-THF produced in the reaction to 5,10-methenyl THF. The absorbance at 350 nm of samples was read at 15 min postacidification in a dual-beam spectrophotometer (model UV 160; Shimadzu, Columbia, Md.).

Specific activities were expressed as nanomoles of 10-formyl-THF produced per minute per milligram of crude extract protein, based upon a molar extinction coefficient of 24,900 $M^{-1} \cdot cm^{-1}$ for 5,10-methenyl-THF (42). Protein values were determined with the bicinchoninic acid protein assay kit (Sigma Chemical Co.) according to the manufacturer's instructions.

Nucleotide sequence accession number. The GenBank/EMBL accession number for the nucleotide and amino acid sequences of the *S. mutans* formyl-THF synthetase is U39612.

RESULTS

Transposon mutagenesis. Transformation of *S. mutans* JH1005 with 1 μg of the Tn*917*-bearing temperature-sensitive plasmid pTV1-OK resulted in recovery of 11 kanamycin-resistant clones. One was chosen for insertional mutagenesis by the resident transposon. After the temperature of the culture was shifted to 42°C to eliminate the plasmid, the frequency of transposition (Erm^r) was found to be 10^{-4}/cell. The frequency of replicon fusions (Kan^r Erm^r) was 10^{-6}/cell.

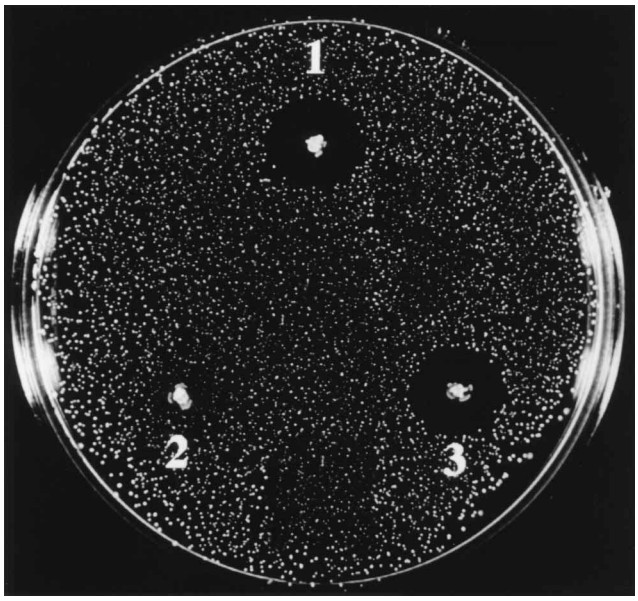

FIG. 1. Bioassay for mutacin production. *S. mutans* parent strain JH1005 (1), *fhs*::Tn*917* mutant strain L22 (2), and *fhs*$^+$-complemented strain L22-6 (3) were tested for production of mutacin by the deferred-antagonism assay described in Materials and Methods.

A pool of 1,750 Erm^r Kan^s transposon mutants was screened for loss of the ability to produce mutacin by inability to inhibit the growth of the indicator strain, *S. rattus* BHT-2. From this mutant pool, one mutacin-negative mutant, L22, was recovered. Figure 1 shows typical mutacin production by the parent strain JH1005, seen as a 15-mm-diameter zone of growth inhibition around the stab, and lack of this activity in mutant strain L22.

The presence of the transposon in the chromosome of L22 was confirmed by Southern analysis of *Eco*RI-restricted chromosomal DNA from this strain (not shown). One 9.1-kb fragment from restricted L22 chromosomal DNA hybridized with the biotinylated pTV1-OK DNA probe, while no hybridization was observed with the parental JH1005 DNA. This demonstrated that a single transposition event occurred to generate this mutant. Repeated subculturings of this strain in the absence of erythromycin did not result in loss of the transposon from its original site of insertion as evidenced by the phenotypic stability of the mutant in the absence or presence of erythromycin.

To demonstrate linkage of the Tn*917* mutation in strain L22 with the observed mutacin-negative phenotype, *Eco*RI-digested chromosomal DNA from L22 was used to transform the parent strain. This genetic backcross yielded a total of 17 recombinants, all of which were mutacin negative in the deferred-antagonism assay (not shown).

Mutant strain L22 was tested for growth inhibition of a variety of target strains (Table 2). Parent strain JH1005 served as the positive control. In all cases there was either a minimal amount of or no growth inhibition by L22, while JH1005 inhibited the growth of all indicator strains. This suggests that a single broad-spectrum mutacin activity is expressed by the par-

TABLE 2. Mutacin sensitivity assay

Indicator strain	Sensitivity[a] with test strain:	
	JH1005	L22
Mutans streptococci		
S. cricetus E49 (serotype *a*)	+/−	−
S. rattus BHT (serotype *b*)	+	−
S. mutans NG8 (serotype *c*)	+	−
S. sobrinus SL1 (serotype *d*)	+	−
S. mutans V100 (serotype *e*)	+	−
S. mutans OMZ175 (serotype *f*)	+	−
S. sobrinus ATCC 27352 (serotype *g*)	+	−
S. downeii ATCC 33748 (serotype *h*)	+	−
S. salivarius		
ATCC 27945	+	−
ATCC 25975	+	−
PC1	+	−
S. mitis		
903	+	−
ATCC 19950	+	−
S. sanguis		
PC10	+	+/−
PC13	+	+/−
Challis e	+	−
S. pyogenes		
Manfredo	+	−
MC5	+	−
Lactobacillus caseii ATCC7469	+	−
Staphylococcus aureus Cowan	+	−
Actinomyces naeslundii PK19	+	−

[a] Sensitivity to mutacin was determined as described in Materials and Methods. Indicator strains were evaluated as sensitive (+) (showing zones of 10 to 15 mm in diameter), insensitive (−), or slightly sensitive (+/−) (with zones of <5 mm in diameter) to the test strains JH1005 (wild type) and L22 (*fhs*::Tn*917*).

ent and that its production was eliminated in the mutant by means of a single Tn*917* insertion.

Recovery of the Tn*917*-interrupted gene. To identify the Tn*917*-inactivated gene associated with mutacin production in strain JH1005, chromosomal DNA from the mutant was restricted with *Eco*RI, which does not cut within Tn*917*, and ligated to similarly digested and dephosphorylated pUC19 (Amp^r). Although we have reported success with direct selection onto erythromycin-containing agar (14), our initial attempt at transformation of MC1061 with plasmids containing insert DNA from mutant strain L22, followed by direct selection on erythromycin, did not yield transformants. Therefore, a two-step approach was taken. Transformants initially were selected on ampicillin agar, followed by replica plating onto erythromycin agar. Ten Erm^r and Amp^r transformants were recovered in this manner. The presence of Tn*917*-containing DNA from one recombinant, PC193, was confirmed by hybridization of biotinylated pTV1-OK DNA with plasmid DNA from this strain (not shown).

Sequence analysis of *fhs*. Sequencing of Tn*917*-interrupted insert DNA harbored on plasmid pPC193 revealed integration of the transposon into an open reading frame (Fig. 2) with strong identity to *fhs*. This gene encodes formyl-THF synthetase (Fhs) from both prokaryotic and eukaryotic sources (Table 3). Fhs catalyzes the ATP-dependent formylation of THF to produce 10-formyl-THF used in the biosynthesis of purines and in one-carbon transfer reactions. The *Eco*RI insert fragment from pPC193 contained the entire *fhs* 1,670-bp open reading frame (Fig. 2). A putative promoter, ribosomal binding site, and rho-independent transcription terminator were identified and are indicated in Fig. 2. The 556-residue deduced amino acid sequence for Fhs also is shown in Fig. 2. Active Fhs in *Clostridium* spp. exists as a tetramer with a molecular mass of ~230,000 Da consisting of four 58,000-Da monomeric subunits (31, 53). The predicted molecular mass, based upon amino acid composition, for the *S. mutans* Fhs was 59,695 Da, nearly identical to that reported for the monomer from *Clostridium* as well as other bacterial species (22).

Additional flanking DNA was recovered with the *fhs* gene from *S. mutans* JH1005 and included 249 bp downstream of the TAA stop codon and approximately 2.8 kb of DNA upstream of the ATG start codon (not shown). Analysis of this upstream sequence revealed DNA homologous to *dfp*, a DNA flavoprotein gene first identified in *E. coli* (29). The DNA sequence encoding this protein, hypothesized to be involved in DNA biosynthesis and pantothenate metabolism (44, 45), was identical to the partial *dfp* sequence previously reported for *S. mutans* NG8 by Gutierrez et al. (14). This demonstrates that the *fhs* and *dfp* genes in these two *S. mutans* strains are linked.

Comparison of the deduced amino acid sequence of Fhs from *S. mutans* with those in protein sequence databases revealed strong homology to the enzyme from three clostridial species and to a partial sequence from *Zymomonas mobilis* (Table 3). The deduced *S. mutans* Fhs also had similar degrees of identity with the Fhs domain of the trifunctional C1-THF synthase from various eukaryotic sources (Table 3). Regions of highly conserved amino acids appear to be clustered primarily in the N-terminal halves of the molecules in prokaryotes (Fig. 3) and possibly correspond to functional domains.

Fhs activity assays. Enzyme assays for Fhs activity from crude cell lysates showed essentially no activity associated with mutant L22. The parent strain, JH1005, had a specific activity of 16.5 ± 3.6 nmol/min/mg of protein. This is comparable to the Fhs activity reported for a related species, *Enterococcus hirae* (formerly *Streptococcus fecaelis*) (22). Control tubes in which THF was left out had no activity.

Analysis of auxotrophy. Since a mutation in *fhs* would be expected to affect biosynthesis of purines and possibly thymine, methionine, serine, and histidine (Fig. 4), we predicted that the mutant might be auxotrophic for one or all of these compounds. Minimal medium plates containing only glucose, salts, cysteine, and a vitamin pool (not including folate) were streaked with both the parent and mutant strains and incubated anaerobically for 48 h. The mutant strain failed to grow, while the parent strain grew luxuriantly (not shown). When plates were supplemented with adenine, growth of the mutant was observed, although not at wild-type levels unless the medium was also supplemented with methionine. To obtain a more quantitative measure of the adenine requirement, strains were cultured in liquid minimal medium supplemented with adenine, methionine, guanine, and thymine, either alone or in combination. Growth after 48 h was measured with a Klett colorimeter (Fig. 5). These data demonstrated an absolute requirement by the mutant for supplemental adenine (Fig. 5). Adenine supplementation resulted in growth yields of the mutant to only about 50% of wild-type levels. Similar results were observed when adenosine was used in place of adenine (not shown). Addition of increasing concentrations of adenosine, up to 80 μg/ml, to the medium did not restore wild-type growth yields. Supplementation with methionine, guanine, and thy-

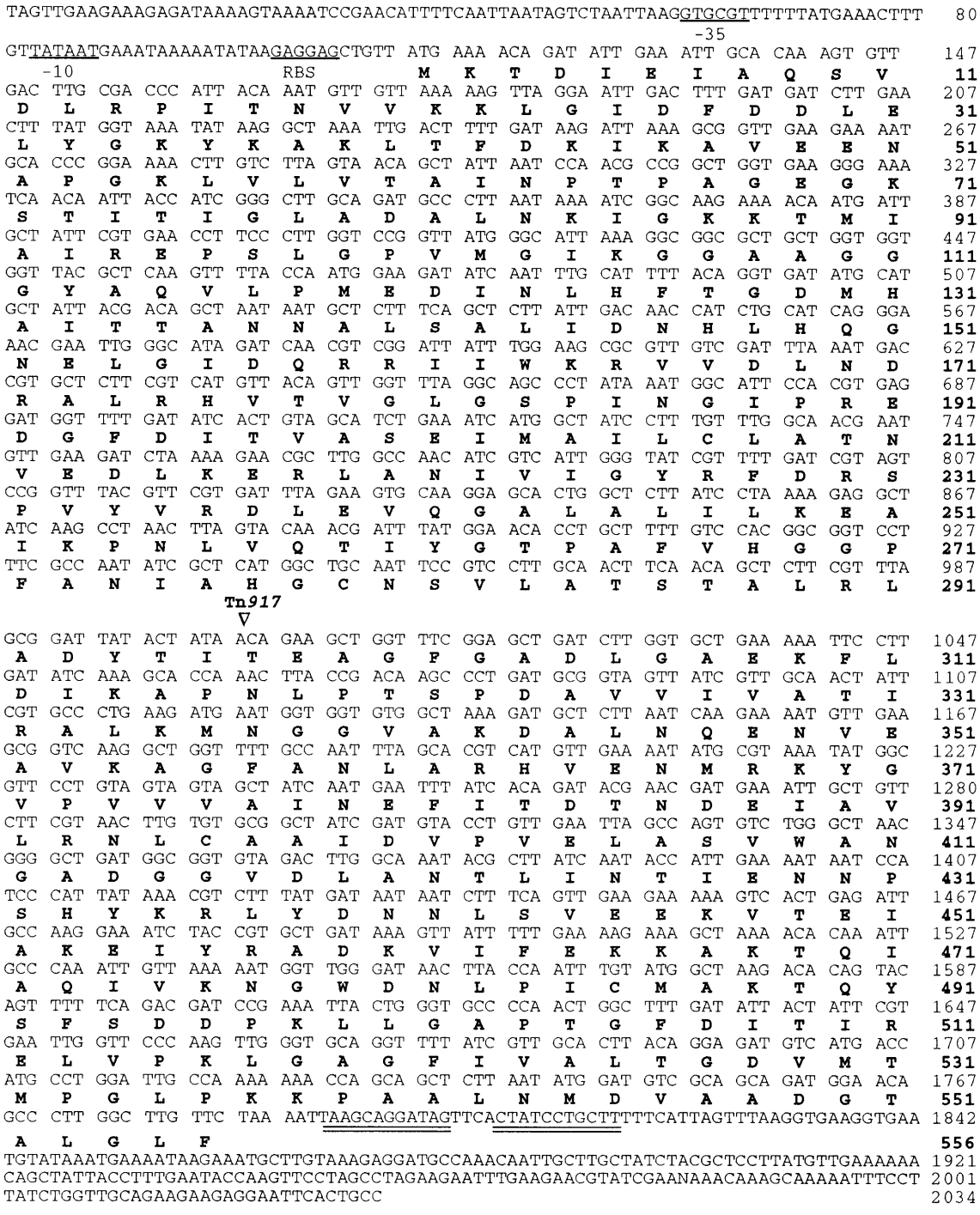

FIG. 2. DNA and amino acid sequences of *fhs* from *S. mutans*. RBS, putative Shine-Dalgarno sequence. Singly underlined areas represent putative −10 and −35 promoter sequences. The insertion site of Tn*917* is indicated by the arrowhead. Double underlines represent a putative transcriptional terminator.

mine alone failed to complement the auxotrophy. Interestingly, if both adenine and methionine were added together, a wild-type level of growth by the mutant was observed (Fig. 5). Adenine and guanine added together improved growth by 20% over that with adenine alone. No improvement in growth yield over that with adenine alone was seen when thymine and adenine were added together. The mutant did not grow in medium supplemented with a complete pool of amino acids. Taken together, these results demonstrate that mutant strain L22 is dependent upon adenine supplementation but also requires methionine for normal levels of growth.

The *fhs* mutation results in an acid-sensitive phenotype. In addition to the adenine-auxotrophic and mutacin-negative phenotypes, we found that upon passage of the parent JH1005 and mutant L22 strains growing in complex medium at pH 7.3 into the same medium at pH 5.0, the mutant was more sensitive to the acidity of the culture medium. Culture doubling times following passage to medium at pH 5 were 140 and 96

TABLE 3. Amino acid sequence similarities between Fhs from *S. mutans* and other sources

Organism	% Identity[a]	% Conservative substitutions[b]	% Total similarity	Accession no. (reference)
Prokaryotes				
C. thermoaceticum	45.3	12.7	58.0	P21164 (28)
C. cylindrosporum	52.3	8.7	61.0	Q07064 (39)
C. acidiurici	54.8	9.2	64.0	P13419 (55)
Z. mobilis[c]	47.5	10.5	58.0	X84019 (35)
Eukaryotes[d]				
Human (aa 317–460)	49.6	14.0	63.6	A31903 (24)
Rat (aa 317–460)	49.6	14.0	63.6	J05519 (50)
Spinach (aa 20–162)	50.3	11.2	61.5	A43350 (32)
Yeast (aa 344–489)	53.1	11.7	64.8	P07245 (46)

[a] Percentage of total amino acids that were identical.
[b] Percentage of total amino acids that were in similar charge groups.
[c] Only a partial sequence was available for *Z. mobilis.*
[d] Only the portion of the C1-THF synthase corresponding to the Fhs domain was used for comparison and is indicated in parentheses. aa, amino acids.

min for L22 and JH1005, respectively. Doubling times for the two strains at pH 7.3 were identical (46 min).

Analysis of *fhs* complementation. Systematic sequence analyses of several plasmids containing genes interrupted with Tn*917* and subsequently recovered with pTV21Δ2TetM, a hybrid vector constructed for marker rescue into *E. coli* of such mutated genes, identified one plasmid containing an intact native copy of *fhs* from *S. mutans* NG8 downstream from a Tn*917*-interrupted *dfp* gene (14). We used this plasmid, pAS25NCE, to complement strain L22 via electrotransformation. The *E. coli* recombinant strain harboring pAS25NCE expressed high levels of Fhs activity (not shown), whereas this species has been reported to lack the *fhs* gene entirely (54).

Successful complementation of the *fhs* mutation in L22 was verified by its reversion to a non-adenine-requiring phenotype while remaining Erm^r. The complemented mutant strain, L22-6, expressed wild-type levels (19.8 ± 1.9 nmol/min/mg of protein) of Fhs. In addition, strain L22-6 expressed a wild-type level of mutacin activity (Fig. 1) and demonstrated growth characteristics identical to those of the parent JH1005 strain in complex medium at pH 5.0. Taken together, these results suggest that the insertion of Tn*917* into *fhs* was directly responsible for all of the associated mutant phenotypes.

DISCUSSION

Transposon mutagenesis of *S. mutans* JH1005 was accomplished with the thermosensitive vector pTV1-OK, harboring the nonconjugative transposon Tn*917*. Previously we described

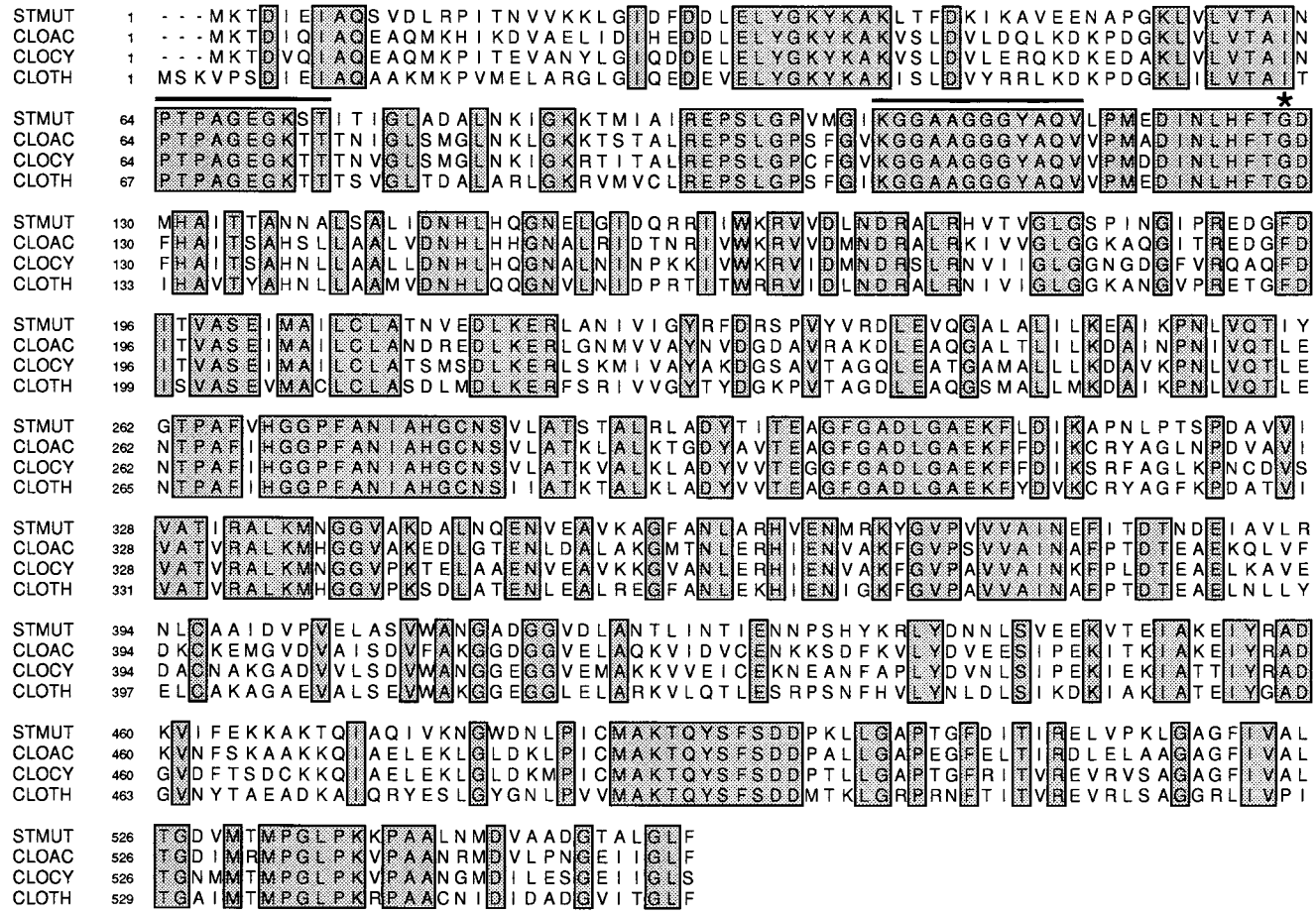

FIG. 3. Multiple amino acid sequence alignment of Fhs from *S. mutans* (STMUT), *Clostridium acidiurici* (CLOAC), *Clostridium cylindrosporum* (CLOCY), and *Clostridium thermoaceticum* (CLOTH). Regions of 100% identity are represented by boxed, shaded areas. The asterisk over residue Gly-128 locates the position of a point mutation generated in a PCR-amplified copy of *fhs* that resulted in conversion of this residue to a cysteine. Lines identify conserved regions thought to be involved in substrate binding.

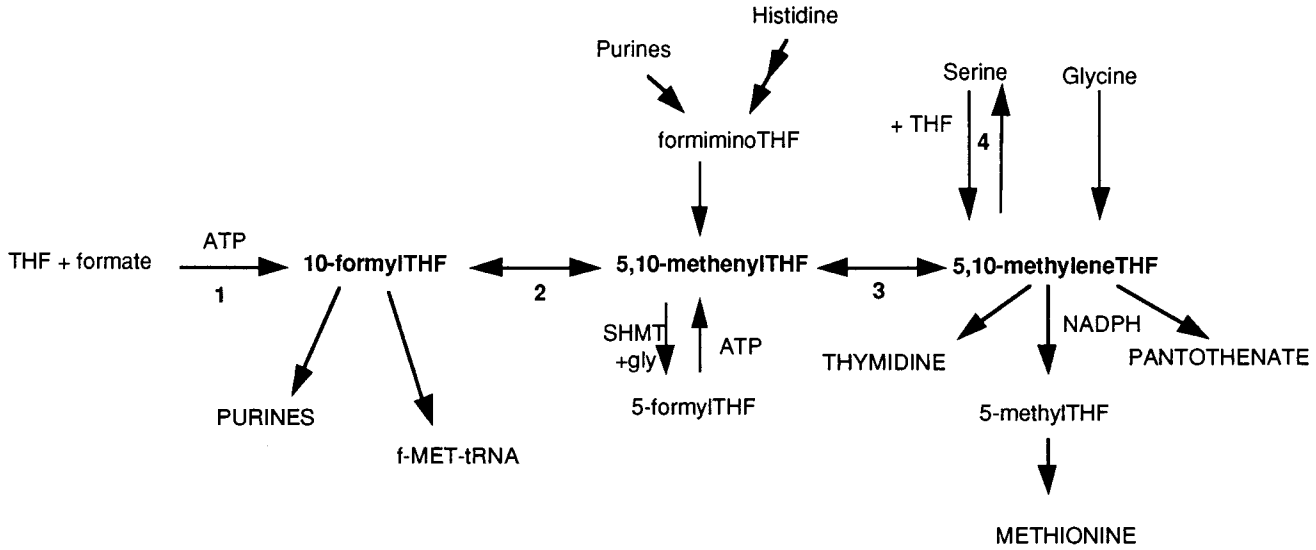

FIG. 4. General prokaryotic pathways of folate-mediated one-carbon transfer reactions. Enzymes: 1, formyl-THF synthetase (EC 6.3.4.3); 2, 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9); 3, 5,10-methylene THF dehydrogenase (EC 1.5.1.5); 4, SHMT (EC 2.1.2.1).

the construction and demonstrated the utility of this vector as a tool for random mutagenesis in this strain (13, 14). Other investigators have also reported success with this plasmid in mutagenizing both Lancefield group B (11) and group A (2) streptococci. Since the frequency of transposition in any given strain is not linked to its transformability, pTV1-OK is particularly useful for mutagenizing poorly transformable strains such as JH1005.

A consequence of Tn*917* mutagenesis of JH1005 and identification of a mutacin-negative mutant was recovery of the gene encoding formyl-THF synthetase, *fhs*. This enzyme catalyzes the formation of 10-formyl-THF from THF and formate in an ATP-dependent reaction. To date, folate metabolism in *S. mutans* is largely unknown, although this organism is known to synthesize folate de novo (3). Folate and its one-carbon derivatives are involved in both biosynthetic and catabolic pathways, and many of the reactions are common to both eukaryotes and prokaryotes (Fig. 4). Formyl-THF synthetase activity is widespread among prokaryotes and eukaryotes, with the notable exception of most genera of *Enterobacteriaceae*

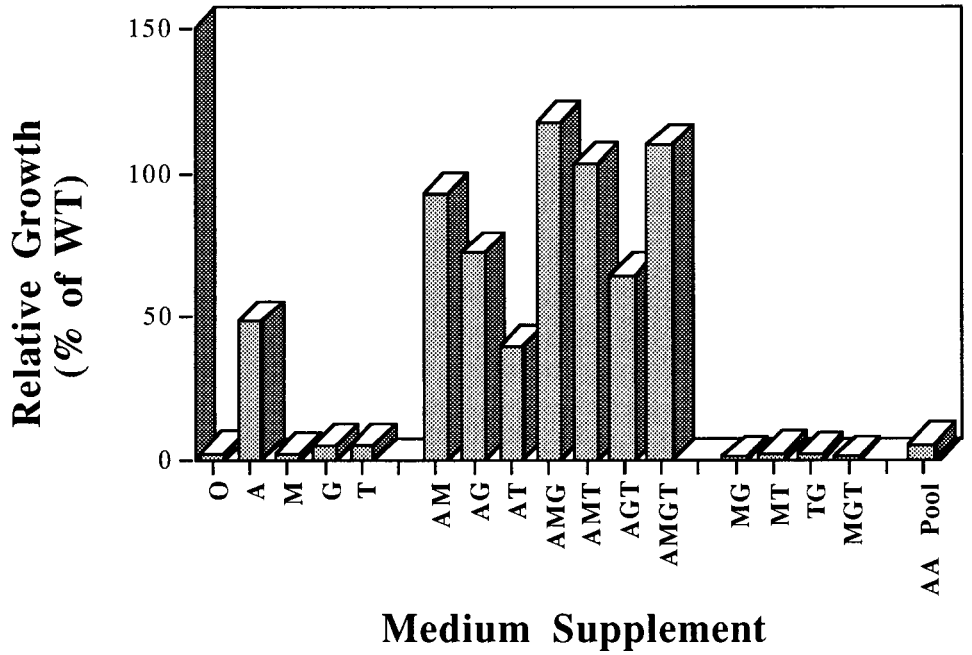

FIG. 5. Determination of auxotrophy in *fhs*-negative mutant L22. JH1005 (wild type [WT]) and L22 (*fhs*) were cultured anaerobically in minimal medium with adenine (A), methionine (M), guanine (G), and thymine (T), singly or in combination, and with a pool of 18 amino acids (AA Pool) at the concentrations specified in Materials and Methods. Growth of L22 is expressed as the mean percentage of the wild-type growth yield after 48 h in minimal medium with the indicated supplements.

(54). In this study, we sequenced cloned *fhs* from *S. mutans* and compared the sequence with those of genes from two genera of bacteria and a variety of eukaryotes. Strong similarities were noted. The deduced amino acid sequence of the *S. mutans* Fhs monomer corresponded to a protein nearly identical in size to the clostridial protein. Most of the sequence homology was clustered within the N-terminal halves of the streptococcal and clostridial molecules. In *Clostridium*, the ATP-binding domain is thought to be represented by the sequence ^{64}PTPAGEG KXT^{73}, where X is either S or T, and may represent the nucleotide-binding domain found in G-proteins and many other ATP-binding proteins (28). This sequence is present in the *S. mutans* Fhs molecule as well (Fig. 3). An additional glycine (G)-rich sequence which may represent a nucleotide-binding consensus sequence (^{105}KGGAAGGGY^{113}) is conserved in all *fhs* sequences, including that of *S. mutans.*

A consensus sequence for THF binding (4) has been proposed based upon sequence comparisons among a variety of THF-utilizing enzymes, although Fhs was not analyzed in that study. The putative consensus sequence includes XPS(X_2) P$(X)_{2-3}$G plus a D residue 25 or 26 residues carboxy terminal to it (4). This consensus sequence is located at residues 95 to 103 (EPSLGPX_2G) in *Clostridium* and *S. mutans*. The aspartate (D) residue is located at residue 129 in these species (Fig. 3). It has been suggested that this and other highly conserved regions may be involved with proper folding of monomeric subunits so that their association into a catalytically active tetramer may occur. Interestingly, in an early attempt to amplify *fhs* from strain JH1005 by PCR, a single point mutation that resulted in a lack of expressed Fhs activity was created. Integration of this mutant copy of *fhs* into strain L22 resulted in unsuccessful complementation; Fhs activity was expressed in neither this strain nor the *E. coli* recombinant strain harboring the defective PCR-amplified gene (7). The point mutation was identified to correspond to nucleotide 496 and resulted in a substitution of a cysteine for a glycine residue at position 128. This mutation is adjacent to the D residue of the consensus sequence. This finding suggests that residue Gly-128 also may be important for Fhs structure and/or function.

Predictably, the *fhs*-negative mutant had an absolute requirement for adenine, since 2 mol of 10-formyl-THF is required for production of 1 mol of IMP, the purine precursor. Results of medium supplementation experiments indicated that while addition of adenine or adenosine augmented growth in the mutant, wild-type growth levels were not achieved unless methionine was added as well. Methionine added alone to the medium did not compensate for the auxotrophy. Based upon the known pathways for interconversion of one-carbon THF intermediates in bacteria (Fig. 4), 10-formyl-THF can be converted to 5,10-methylene-THF, a precursor of methionine and an intermediate essential for methyl group transfer reactions. However, 5,10-methylene-THF is generated more predominantly from the conversion of serine to glycine catalyzed by serine hydroxymethyltransferase (SHMT) (Fig. 4) in many bacterial species. Medium supplementation of the *S. mutans fhs* mutant L22 with serine and glycine failed to alleviate requirements for adenine and methionine, indicating that adequate levels of 5,10-methylene-THF, and consequently all other THF intermediates, were not generated. This underscores the role of Fhs in providing necessary THF intermediates in *S. mutans* for the biosynthesis of a number of key metabolic intermediates, including adenine and methionine (and *S*-adenosylmethionine), as well as for methyl (formyl) group transfers.

An important question concerning formylation of Met-tRNA for initiation of protein synthesis remains. If 10-formyl THF is necessary for this process, as has been demonstrated in bacteria (10), how is the mutant L22 able to grow when adenine alone is used to supplement the minimal medium? Presumably addition of adenine allows for production of adenosine used for nucleic acid synthesis and for all ATP-requiring processes, but how is 10-formyl-THF generated, since addition of methionine alone or serine and glycine alone does not enable growth of this mutant? One answer may lie in reports that 5-formyl-THF, a bacterial growth factor found also in yeasts, rice bran, and liver extract (reviewed in reference 47), is converted to 10-formyl-THF in an ATP-dependent salvage reaction involving production of the intermediate 5,10-methenyl-THF. The enzyme that catalyzes this reaction, 5,10-methenyl-THF synthetase, is the only enzyme known to date which uses 5-formyl THF as a substrate (reviewed in reference 47). It was also reported that in the absence of NADP, 5-formyl-THF was formed by the irreversible hydrolysis of 5,10-methenyl-THF by SHMT, in the presence of glycine, to yield serine (reviewed in reference 47). Stover and Schirch (47) proposed that together SHMT and 5,10-methenyl-THF synthetase constitute an apparent futile cycle that maintains a level of 5-formyl-THF in the cell. If these reactions occur in *S. mutans*, though, one would not expect to see adenine auxotrophy, because enough 10-formyl-THF could be generated by compensatory reactions in the absence of Fhs. Alternatively, formylation of Met-tRNA for translation initiation may not be required in *S. mutans*. Clearly, further elucidation of the enzymes and pathways involved in interconversion and/or generation of THF intermediates, as well as their regulatory mechanisms, in *S. mutans* is necessary to provide answers to these questions.

An interesting finding in this study was that mutation of *fhs* resulted in the inability of cells to grow normally at an acidic pH (5.0). During the fermentation of glucose, large amounts of organic acids are produced by *S. mutans* and remain trapped in the extracellular polysaccharide matrix (23, 48). Tolerance to local high concentrations of acids is essential for *S. mutans* and effectively makes this a main virulence trait of this organism. *S. mutans* is able to carry out glycolysis at a pH of $\leq$4.0 (18, 19), well below the critical pH of 4.5 to 5.5 necessary for the beginning of enamel demineralization (27). The acid-sensitive phenotype in mutant L22 may be due to its inability to synthesize purines. Mutant L22 is totally dependent on exogenous adenine. Purine uptake from the medium may be rate limiting at a time when ATP demand is high. It is known that H^+/ATPase activity increases in response to acidic conditions for the purpose of proton extrusion (1a, 17). Presumably ATP demand would be highest under such conditions. However, we did not detect measurable differences in cellular ATP levels when we assayed both parent and mutant strains cultured in complex medium at pH 5 (5). In a related study of acid tolerance in *S. mutans* JH1005, using Tn*917* mutagenesis, we identified an acid-sensitive mutant (AS5) interrupted at the same genetic locus (*fhs*), based upon more limited sequence analyses of DNA flanking the transposon insertion site (14). These two independent studies done in this laboratory confirm key roles for this enzyme in the basic physiology and virulence of this oral streptococcus.

It is not obvious yet how Fhs in *S. mutans* is involved in mutacin synthesis or expression, although, based upon results from the genetic backcross experiment, a direct linkage between the mutation and the observed mutacin-negative phenotype was shown. It was reported recently that mutacin production in a lantibiotic-producing strain of *S. mutans* (33) involves the activity of a specific ATP-binding cassette transporter (34). If the mutacin expressed by JH1005 is a lantibiotic whose production involves a similar ATP-binding cassette

transporter, a mutation affecting the organism's ability to generate adequate ATP during growth in acidic medium could affect normal mutacin transport. Alternatively, biosynthesis and/or transport of the mutacin may involve one-carbon transfer reactions negatively affected by a mutation in *fhs*.

The study of virulence in *S. mutans* is complex, since many of the virulence factors attributed to this organism are the results of one or more of its normal metabolic processes. We hope that by recovering additional mutants defective in these and other virulence traits, such as acid tolerance or mutacin production, we will be able to better understand the relationships between pathogenicity and the physiology of this organism.

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REFERENCES

1. **Applied Biosystems, Inc.** 1991. User bulletin 18, October 1991. Applied Biosystems, Inc., Foster City, Calif.
1a. **Bender, G. R., S. V. W. Sutton, and R. E. Marquis.** 1986. Acid tolerance, proton permeabilities, and membrane ATPases of oral streptococci. Infect. Immun. **53:**331–338.
2. **Boyle, M. P.** Personal communication.
3. **Carlsson, J.** 1970. Nutritional requirements of *Streptococcus mutans*. Caries Res. **4:**305–320.
4. **Cook, R. J., R. S. Lloyd, and C. W. Wagner.** 1991. Isolation and characterization of cDNA clones for rat liver 10-formyltetrahydrofolate dehydrogenase. J. Biol. Chem. **266:**4965–4973.
5. **Crowley, P. J.** Unpublished result.
6. **Crowley, P. J., J. D. Hillman, and A. S. Bleiweis.** 1995. Generation of bacteriocin-negative mutants of *Streptococcus mutans* JH1005 using Tn*917*-transposon mutagenesis, abstr. D55, p. 258. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
7. **Crowley, P. J., J. A. Gutierrez, J. D. Hillman, and A. S. Bleiweis.** 1996. Phenotypic analysis of a formyl-tetrahydrofolate synthetase mutant of *Streptococcus mutans*. J. Dent. Res. **75:**96. (Abstr. 632.).
8. **Cvitkovitch, D. G., D. A. Boyd, and I. R. Hamilton.** 1995. Glucose transport by a mutant of *Streptococcus mutans* unable to transport sugars via the phosphoenolpyruvate-dependent phosphotransferase transport system. J. Bacteriol. **177:**2251–2258.
9. **Cvitkovitch, D. G., J. A. Gutierrez, and A. S. Bleiweis.** 1997. Role of the citrate pathway in glutamate biosynthesis by *Streptococcus mutans*. J. Bacteriol. **179:**650–655.
10. **Dickerson, H. W., E. Steers, Jr., B. G. Redfield, and H. Weissbach.** 1967. Methionyl soluble ribonucleic acid transformylase. I. Purification and partial characterization. J. Biol. Chem. **242:**1522–1525.
11. **Framson, P. E., V. Nizet, A. Nittayajarn, P. Youngman, and C. E. Rubens.** 1995. Mutagenesis of *Streptococcus agalactiae* (group B Streptococcus) with Tn*917*, abstr. H-152, p. 519. *In* Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
12. **Goldthwait, D. A., and G. R. Greenberg.** 1955. Some methods for the study of de novo synthesis of purine nucleotides. Methods Enzymol. **2:**504–519.
13. **Gutierrez, J. A., P. J. Crowley, J. D. Hillman, and A. S. Bleiweis.** 1995. New methods for mutagenesis of *Streptococcus mutans*. J. Dent. Res. **74:**178. (Abstr. 1331.)
14. **Gutierrez, J. A., P. J. Crowley, D. P. Brown, J. D. Hillman, P. Youngman, and A. S. Bleiweis.** 1996. Insertional mutagenesis and recovery of interrupted genes of *Streptococcus mutans* by using transposon Tn*917*: preliminary characterization of mutants displaying acid sensitivity and nutritional requirements. J. Bacteriol. **178:**4166–4175.
15. **Gutierrez, J. A.** Unpublished data.
16. **Hamada, S., and T. Ooshima.** 1975. Production and properties of bacteriocins (mutacins) from *Streptococcus mutans*. Arch. Oral Biol. **20:**641–648.
17. **Hamilton, I. R., and N. D. Buckley.** 1991. Adaptation by *Streptococcus mutans* to acid tolerance. Oral Microbiol. Immunol. **6:**65–71.
18. **Harper, D. S., and W. J. Loesche.** 1983. Effect of pH upon sucrose and glucose catabolism by various genogroups of *Streptococcus mutans*. J. Dent. Res. **62:**526–531.
19. **Harper, D. S., and W. J. Loesche.** 1984. Growth and acid tolerance of human dental plaque bacteria. Arch. Oral Biol. **29:**843–848.
20. **Hillman, J. D.** 1978. Lactate dehydrogenase mutants of *Streptococcus mutans*: isolation and preliminary characterization. Infect. Immun. **21:**206–212.
21. **Hillman, J. D., K. P. Johnson, and B. I. Yaphe.** 1984. Isolation of a *Streptococcus mutans* strain producing a novel bacteriocin. Infect. Immun. **44:** 141–144.
22. **Himes, R. H., and J. A. K. Harmony.** 1973. Formyltetrahydrofolate synthetase. Crit. Rev. Biochem. **1:**501–535.
23. **Hojo, S., M. Higuchi, and S. Araya.** 1976. Glucan inhibition of diffusion in plaque. J. Dent. Res. **55:**169.
24. **Hum, D. W., A. W. Bell, R. Rozen, and R. E. MacKenzie.** 1988. Primary structure of a human trifunctional enzyme. Isolation of a cDNA encoding methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase. J. Biol. Chem. **263:**15946–15950.
25. **Kok, J., J. M. B. M. van derVossen, and G. Venema.** 1984. Construction of plasmid cloning vectors of lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli*. Appl. Environ. Microbiol. **48:**726–731.
26. **Kuramitsu, H. K.** 1993. Virulence factors of mutans streptococci—role of molecular genetics. Crit. Rev. Oral Biol. Med. **4:**159–176.
27. **Larsen, M. J., and C. Bruun.** 1994. *In* A. Thystrup and O. Fejerskov (ed.), Textbook of clinical cariology, 2nd ed. p. 231–257. Munsgaard, Copenhagen, Denmark.
28. **Lovell, C. R., A. Przybla, and L. G. Ljungdahl.** 1990. Primary structure of the thermostable formyltetrahydrofolate synthetase from *Clostridium thermoaceticum*. Biochemistry **29:**5687–5694. (Erratum, **31:**1896, 1992.)
29. **Lundberg, L. G., H.-O. Thoresson, O. H. Karlstrom, and P. O. Nyman.** 1983. Nucleotide sequence of the structural gene for dUTPase of *Escherichia coli* K12. EMBO J. **2:**967–971.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **McKenzie, R. T., and J. C. Rabinowitz.** 1971. Cation-dependent reassociation of subunits of N^{10}-formyltetrahydrofolate synthetase from *Clostridium acidi-urici* and *Clostridium cylindrosporum*. J. Biol. Chem. **246:**3731–3736.
32. **Nour, J. M., and J. C. Rabinowitz.** 1992. Isolation and sequencing of the cDNA coding for spinach 10-formyltetrahydrofolate synthetase. Comparisons with the yeast, mammalian, and bacterial proteins. J. Biol. Chem. **267:**16292–16296.
33. **Novák, J., P. W. Caufield, and E. J. Miller.** 1994. Isolation and biochemical characterization of a novel lantibiotic mutacin from *Streptococcus mutans*. J. Bacteriol. **176:**4316–4320.
34. **Novák, J., H. Hirt, W. A. Woodruff, and P. W. Caufield.** 1996. Cloning, sequencing and expression of an ABC transporter involved in the production of the lantibiotic mutacin II in *Streptococcus mutans*, abstr. B-360, p. 217. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
35. **Peekhaus, N., B. Tolner, B. Poolman, and R. Kramer.** 1995. The glutamate uptake regulatory protein (Grp) of *Zymomonas mobilis* and its relation to the global regulator Lrp of *Escherichia coli*. J. Bacteriol. **177:**5140–5147.
36. **Perry, D., and H. K. Kuramitsu.** 1981. Genetic transformation of *Streptococcus mutans*. Infect. Immun. **32:**1295–1297.
37. **Rabinowitz, J. C., and W. E. Pricer, Jr.** 1963. Formyltetrahydrofolate synthetase. Methods Enzymol. **6:**375–379.
38. **Rabinowitz, J. C., and W. E. Pricer, Jr.** 1962. Formyltetrahydrofolate synthetase. I. Isolation and crystallization of the enzyme. J. Biol. Chem. **237:** 2898–2902.
39. **Rankin, C. A., G. C. Haslam, and R. H. Himes.** 1993. Sequence and expression of the gene for N10-formyltetrahydrofolate synthetase from *Clostridium cylindrosporum*. Protein Sci. **2:**197–205.
40. **Rogers, A. H.** 1976. Bacteriocinogeny and the properties of some bacteriocins of *Streptococcus mutans*. Arch. Oral Biol. **21:**99–104.
41. **Russell, R. R. B.** 1994. The application of molecular genetics to the microbiology of dental caries. Caries Res. **28:**69–82.
42. **Song, J. M., and J. C. Rabinowitz.** 1993. Function of yeast cytoplasmic C_1-tetrahydrofolate synthase. Proc. Natl. Acad. Sci. USA **90:**2636–2640.
43. **Southern, E.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
44. **Spitzer, E. D., H. E. Jimenez-Billinia, and B. Weiss.** 1988. β-Alanine auxotrophy associated with *dfp*, a locus affecting DNA synthesis in *Escherichia coli*. J. Bacteriol. **170:**872–876.
45. **Spitzer, E. D., and B. Weiss.** 1985. *dfp* gene of *Escherichia coli* K-12, a locus affecting DNA synthesis, codes for a flavoprotein. J. Bacteriol. **164:**994–1003.
46. **Staben, C., and J. C. Rabinowitz.** 1986. Nucleotide sequence of the *Saccharomyces cerevisiae* ADE3 gene encoding C1-tetrahydrofolate synthase. J. Biol. Chem. **261:**4629–4637.
47. **Stover, P., and V. Schirch.** 1993. The metabolic role of leucovorin. Trends Biochem. Sci. **18:**102–106.
48. **Tatevossian, A.** 1979. Diffusion of radiotracers in human dental plaque. Caries Res. **13:**154–162.
49. **Terleckyj, B., and G. D. Shockman.** 1975. Amino acid requirements of *Streptococcus mutans* and other oral streptococci. Infect. Immun. **11:**656–664.
50. **Thigpen, A. E., M. G. West, and D. R. Appling.** 1990. Rat C1-tetrahydrofo-

late synthase. cDNA isolation, tissue-specific levels of the mRNA, and expression of the protein in yeast. J. Biol. Chem. **265:**7907–7913.

51. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
52. **Vadeboncoeur, C., L. Thibault, S. Neron, H. Halvorson, and I. R. Hamilton.** 1987. Effect of growth conditions on levels of components of the phosphoenolpyruvate:sugar phosphotransferase system in *Streptococcus mutans* and *Streptococcus sobrinus* grown in continuous culture. J. Bacteriol. **169:** 5686–5691.
53. **Welch, W. H., D. H. Buttlaire, R. T. Hersh, and R. H. Himes.** 1971. The subunit structure of formyltetrahydrofolate synthetase. Biochem. Biophys. Res. Commun. **30:**255–261.
54. **Whitehead, T. R., M. Park, and J. C. Rabinowitz.** 1988. Distribution of 10-formyltetrahydrofolate synthetase in eubacteria. J. Bacteriol. **170:**995–997.
55. **Whitehead, T. R., and J. C. Rabinowitz.** 1988. Nucleotide sequence of the *Clostridium acidiurici* ("*Clostridium acidi-urici*") gene for 10-formyltetrahydrofolate synthetase shows extensive amino acid homology with the trifunctional enzyme C1-tetrahydrofolate synthase from *Saccharomyces cerevisiae*. J. Bacteriol. **170:**3255–3261.
56. **Whiteley, H. R., M. J. Osborn, and F. M. Hueenekens.** 1959. Purification and properties of the formate-activating enzyme from *Micrococcus aerogenes*. J. Biol. Chem. **234:**1538–1543.