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

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Previously we reported that transposon Tn917 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 Tn917 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 Tn917, 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 Tn917 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.

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 $\mu g/ml$; erythromycin, 5 $\mu g/ml$; and kanamycin, 500 $\mu g/ml$. 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 obic 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

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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 ¹ (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
S. downeii 33748	Wild type	ATCC
S. mutans JH1005	Mut Tet $(1 \mu g/ml)$	21
S. mutans L22	fhs:: In917/Erm' Ade Mut pH 5 ^s	This study
S. mutans L22-6	<i>fhs</i> ::Tn917::pAS25NCE (<i>fhs</i> ⁺ <i>dfp</i> ::Tn917 Δ <i>erm</i> ::pVA981)/Fhs ⁺ Erm ¹ Tet ⁴ 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
		4TCC
Lactobaculus casell /469		AICC
Staphylococcus aureus		Lab collection
Cowan		
Actinomyces naeslundii PK19		W. Clark
Escherichia coli		
MC1061	F^- araD139 Δ (ara-leu)7696 galE15 galK16 Δ (lac)X74 rpsL (Str ^r) hsdR2 ($\mathrm{r_k}^ \mathrm{m_k}^+$)	J. Hillman
	mcrA mcrB1	
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 <i>repA</i> (ts) Kan ^r Tn917 Erm ^r	14
pUC19	$\operatorname{Amp}^{\mathrm{r}} lacZ'$	Lab collection
pPC193	fhs::Tn917::pUC19 Amp ^r Erm ^r	This study
pAS25NCE	$fhs^+ dfp::Tn917\Delta erm::pVA981$	14

TABLE 1.	Bacterial	strains	and	plasmids	used	in	this study
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^{*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 μ g/ml), tetracycline (15 μ g/ml), or erythromycin (300 μ g/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 pWV01 (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 Tn917, which confers erythromycin resistance in streptococci and *E. coli* MC1061 (14).

Tn917 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 EcoRI, which does not cut in the transposon, and ligated to EcoRI-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:*:Tn917) 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 μ g 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 EcoRI-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^T). 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 MgCl₂, 40 mM ammonium formate, and 2 mM pL-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 · 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 μ g of the Tn917-bearing temperature-sensitive plasmid pTV1-OK resulted in recovery of 11 kanamycin-resistant



FIG. 1. Bioassay for mutacin production. S. mutans parent strain JH1005 (1), fhs::Tn917 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.

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.

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 Tn917 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::*Tn917).

ent and that its production was eliminated in the mutant by means of a single Tn917 insertion.

Recovery of the Tn917-interrupted gene. To identify the Tn917-inactivated gene associated with mutacin production in strain JH1005, chromosomal DNA from the mutant was restricted with EcoRI, which does not cut within Tn917, 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 Tn917-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 Tn917-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-

	'I''I'GA.	AGAA	AGAG.	АТАА.	AAGT.	ΑΑΑΑ	TCCG.	AACA	TTTT	СААТ	TAAT	AGTC	TAAT	TAAG	GTGC	<u>GT</u> TT'	TTTA	TGAA	ACTTT	80
GT <u>T</u>	ATAA	<u>T</u> GAA.	АТАА	AAAT	ATAA	GAGG.	<u>ag</u> ct(GTT 1	ATG .	AAA	ACA (ЗАТ .	ATT	GAA	-35 ATT (GCA	CAA	AGT	GTT	147
<i>.</i>	-10					RBS	~~~~	~~~	М	ĸ	T	D	I	E	I	A	Q	S	v	11
GAC	TTG T.	CGA	CCC	A'I''I' T	ACA TT	AA'T'	GTT V	GTT	AAA	AAG	TTA	GGA	ATT	GAC	TTT	GAT	GAT	CTT	GAA	207
CTT	TAT	GGT	AAA	TAT	AAG	GCT	AAA	TTG	ACT	TTT	GAT	AAG	ATT	AAA	GCG	с GTT	GAA	GAA	ם ידע מ	267
L	Y	G	ĸ	Y	ĸ	A	ĸ	L	т	F	D	ĸ	I	ĸ	A	v	E	B	N	51
GCA	CCC	GGA	AAA	CTT	GTC	TTA	GTA	ACA	GCT	ATT	AAT	CCA	ACG	CCG	GCT	GGT	GAA	GGG	AAA	327
		G ATTT	X	ם שידר	occ.		CCA	T CAT	A		N אסרד	P אאא	T ATC	P	A AAC	G AAA		G ATTC	K N TETT	71
S	T	Ï	T	I	G	L	A	D	A	L	N	K	I	G	K	K	T	M	Ĩ	91
GCT	ATT	CGT	GAA	CCT	TCC	CTT	GGT	CCG	GTT	ATG	GGC	ATT	AAA	GGC	GGC	GCT	GCT	GGT	GGT	447
A	I	R	E	P	S	L	G	P	v	М	G	I	ĸ	G	G	A	A	G	G	111
GGT	TAC	GCT	CAA	GTT	'I''I'A	CCA	ATG	GAA	GAT	ATC	AA'I'	TTG	CAT	TTT	ACA	GGT	GAT	ATG	CAT	507
GCT	ATT	ACG	ACA	GCT	AAT	AAT	GCT	CTT	TCA	GCT	CTT	ע הידי א	GAC	AAC	CAT	CTG	CAT	CAG	GGA	567
A	I	т	т	A	N	N	A	L	s	A	L	I	D	N	Н	L	н	Q	G	151
AAC	GAA	TTG	GGC	ATA	GAT	CAA	CGT	CGG	ATT	ATT	TGG	AAG	CGC	GTT	GTC	GAT	TTA	AAT	GAC	627
N	E		G			Q	R	R	I	I	W	K	R	V	V	D	L	N	D	171
R	GCT A	C111	R	CAT u	v	ACA T	v	GGT	TTA T.	GGC	AGC C	D	ATA	AAT	GGC	A'1''1' T	CCA	CGT	GAG	687
GAT	GGT	TTT	GAT	ATC	ACT	GTA	GCA	TCT	GAA	ATC	ATG	GCT	ATC	CTT	TGT	TTG	GCA	ACG	а Таа	747
D	G	F	D	I	т	v	A	S	E	I	M	A	I	L	С	L	A	T	N	211
GTT	GAA	GAT	CTA	AAA	GAA	CGC	TTG	GCC	AAC	ATC	GTC	ATT	GGG	TAT	CGT	TTT	GAT	CGT	AGT	807
V	E	D		K	E	R	L	A	N	I	V	I	G	Y	R	F	D	R	S	231
P	U GIT	TAC V	U GIT	R	GAT	TTA T.	GAA	GTG V	CAA	GGA	GCA	CTG T.	GCT	CTT	ATC	CTA T.	AAA	GAG	GCT	867
ATC	AAG	CCT	AAC	TTA	GTA	CAA	ACG	ATT	TAT	GGA	ACA	ССТ	GCT	TTT	GTC	CAC	GGC	GGT	CCT	927
I	ĸ	P	N	L	v	Q	т	I	Y	G	Т	P	A	F	v	H	G	G	P	271
TTC	GCC	AAT	ATC	GCT	CAT	GGC	TGC	AAT	TCC	GTC	CTT	GCA	ACT	TCA	ACA	GCT	CTT	CGT	TTA	987
F	A	N	I	A	H 1-01	, G	С	N	S	v	L	A	т	S	т	A	L	R	L	291
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GCG	GAT	TAT	ACT	ATA	ACA	GAA	GCT	GGT	TTC	GGA	GCT	GAT	CTT	GGT	GCT	GAA	AAA	TTC	CTT	1047
A	GAT D	TAT Y	ACT T	ATA I	ACA T	GAA E	GCT A	GGT G	TTC F	GGA G	GCT A	GAT D	CTT L	GGT G	GCT A	GAA B	AAA K	TTC F	CTT L	1047 311
A GAT	GAT D ATC	TAT Y AAA	ACT T GCA	ATA I CCA	ACA T AAC	GAA E TTA	GCT A CCG	GGT G ACA	TTC F AGC	GGA G CCT	GCT A GAT	GAT D GCG	CTT L GTA	GGT G GTT	GCT A ATC	GAA E GTT	AAA K GCA	TTC F ACT	CTT L ATT	1047 311 1107
A GAT D CGT	GAT D ATC I GCC	ТАТ Ү ААА К СТG	ACT T GCA A AAG	ATA I CCA P ATG	ACA T AAC N AAT	GAA E TTA L GGT	GCT A CCG P GGT	GGT G ACA T GTG	TTC F AGC S GCT	GGA G CCT P AAA	GCT A GAT D GAT	GAT D GCG A GCT	CTT L GTA V CTT	GGT GTT V AAT	GCT A ATC I CAA	GAA E GTT V GAA	AAA K GCA A AAT	TTC F ACT T	CTT L ATT I GAA	1047 311 1107 331 1167
A GAT D CGT R	GAT D ATC I GCC A	TAT Y AAA K CTG L	ACT T GCA A AAG K	ATA I CCA P ATG M	ACA T AAC N AAT N	GAA E TTA L GGT G	GCT A CCG P GGT G	GGT G ACA T GTG V	TTC F AGC S GCT A	GGA G CCT P AAA K	GCT A GAT D GAT D	GAT D GCG A GCT A	CTT L GTA V CTT L	GGT GTT V AAT N	GCT A ATC I CAA Q	GAA E GTT V GAA E	AAA K GCA A AAT N	TTC F ACT T GTT V	CTT L ATT L GAA B	1047 311 1107 331 1167 351
A GAT D CGT R GCG	GAT D ATC I GCC A GTC	TAT Y AAA K CTG L AAG	ACT T GCA A AAG K GCT	ATA I CCA P ATG M GGT	ACA T AAC N AAT N TTT	GAA E TTA L GGT GCC	GCT A CCG P GGT G AAT	GGT G ACA T GTG V TTA	TTC F AGC S GCT A GCA	GGA G CCT P AAA K CGT	GCT A GAT D GAT D CAT	GAT D GCG A GCT A GTT	CTT L GTA V CTT L GAA	GGT GTT V AAT N AAT	GCT A ATC I CAA Q ATG	GAA E GTT V GAA E CGT	AAA K GCA A AAT N AAA	TTC F ACT T GTT V TAT	CTT L ATT I GAA E GGC	1047 311 1107 331 1167 351 1227
A GAT D CGT R GCG A	GAT D ATC I GCC A GTC V	TAT Y AAA K CTG L AAG K	ACT T GCA A AAG K GCT A	ATA I CCA P ATG M GGT G	ACA T AAC N AAT N TTT F	GAA E TTA L GGT GCC A	GCT A CCG P GGT G AAT N	GGT G ACA T GTG V TTA L	TTC F AGC S GCT A GCA A	GGA G CCT P AAA K CGT R	GCT A GAT D GAT D CAT H	GAT D GCG A GCT A GTT V	CTT L GTA V CTT L GAA E	GGT GTT V AAT N AAT N	GCT A ATC I CAA Q ATG M	GAA E GTT V GAA E CGT R	AAA K GCA A AAT N AAA K	TTC F ACT T GTT V TAT Y	CTT L ATT I GAA B GGC G	1047 311 1107 331 1167 351 1227 371
A GAT D CGT R GCG A GTT V	GAT D ATC I GCC A GTC V CCT P	TAT Y AAA CTG L AAG K GTA Y	ACT T GCA A AAG K GCT A GTA Y	ATA I CCA P ATG M GGT G GTA Y	ACA T AAC N AAT N TTT F GCT	GAA E TTA GGT GCC A ATC T	GCT A CCG P GGT G AAT N AAT	GGT G ACA T GTG V TTA L GAA	TTC F AGC S GCT A GCA A TTT F	GGA G CCT P AAA K CGT R ATC	GCT A GAT D GAT D CAT H ACA	GAT D GCG A GCT A GTT V GAT	CTT L GTA V CTT L GAA E ACG	GGT GTT V AAT N AAT N AAT	GCT A ATC I CAA Q ATG M GAT	GAA E GTT V GAA E CGT R GAA	AAA K GCA A AAT N AAA K ATT	TTC F ACT T GTT V TAT Y GCT	CTT L ATT I GAA E GGC G GTT	1047 311 1107 331 1167 351 1227 371 1280 291
A GAT D CGT R GCG A GTT V CTT	GAT D ATC I GCC A GTC V CCT P CGT	TAT Y AAA CTG L AAG K GTA Y AAC	ACT T GCA AAG CT A GCT A GTA V TTG	ATA I CCA P ATG GGT G GTA V TGT	ACA T AAC N AAT N TTT F GCT A GCG	GAA E TTA GGT GCC A ATC I GCT	GCT A CCG P GGT G AAT N AAT N ATC	GGT G ACA T GTG V TTA L GAA E GAT	TTC P AGC S GCT A GCA A TTT P GTA	GGA G CCT P AAA K CGT R ATC I CCT	GCT A GAT D GAT D CAT H ACA T GTT	GAT D GCG A GCT A GTT V GAT D GAA	CTT L GTA V CTT L GAA E ACG T	GGT GTT V AAT N AAT N AAC N GCC	GCT A ATC I CAA Q ATG M GAT D AGT	GAA E GTT V GAA E CGT R GAA E GTC	AAA K GCA A AAT N AAA K ATT I TGG	TTC F ACT T GTT V TAT Y GCT A GCT	CTT L ATT GAA B GGC G GTT V AAC	1047 311 1107 331 1167 351 1227 371 1280 391 1347
A GAT D CGT R GCG A GTT V CTT L	GAT D ATC I GCC A GTC V CCT P CGT R	TAT Y AAA CTG L AAG K GTA Y AAC N	ACT T GCA AAG K GCT A GTA V TTG L	ATA I CCA P ATG GGT G GTA V TGT C	ACA T AAC N AAT N TTT F GCT A GCG A	GAA E TTA GGT GCC A ATC I GCT A	GCT A CCG P GGT G AAT N AAT N ATC I	GGT G ACA T GTG V TTA L GAA E GAT D	TTC P AGC S GCT A GCA A TTT F GTA V	GGA G CCT P AAA K CGT R ATC I CCT P	GCT A GAT D GAT D CAT H ACA T GTT V	GAT D GCG A GCT A GTT V GAT D GAA B	CTT L GTA V CTT L GAA B ACG T TTA L	GGT GTT V AAT N AAT N AAC N GCC A	GCT A ATC I CAA Q ATG M GAT D AGT S	GAA E GTT V GAA E GAA E GTC V	AAA K GCA A AAT N AAA K ATT I TGG W	TTC F ACT T GTT V TAT Y GCT A GCT A	CTT L ATT GAA B GGC G GTT V AAC N	1047 311 1107 331 1167 351 1227 371 1280 391 1347 411
A GAT D CGT R GCG A GTT V CTT L GGG	GAT D ATC I GCC A GTC V CCT P CGT R GCT	TAT Y AAA K CTG L AAG K GTA Y AAC N GAT	ACT T GCA A AGG CT A GTA V TTG L GGC	ATA I CCA P ATG GGT GGT GTA V TGT C GGT	ACA T AAC N AAT N TTT F GCT A GCG A GCG	GAA E TTA L GGT GCC A ATC I GCT A GAC	GCT A CCG P GGT G AAT N AAT N AAT I TTG	GGT G ACA T GTG V TTA GAA GAA GAT D GCA	TTC F AGC S GCT A GCA TTT F GTA V AAT	GGA G CCT P AAA K CGT R ATC CCT P ACG	GCT A GAT D CAT H ACA T GTT V CTT	GAT D GCG A GCT A GTT V GAT D GAA B ATC	CTT L GTA V CTT L GAA E ACG T TTA L AAT	GGT GTT V AAT N AAT N AAC N GCC A ACC	GCT A ATC I CAA Q ATG M GAT D AGT S ATT	GAA E GTT V GAA E CGT R GAA E GTC V GAA	AAA K GCA A AAT N AAA K ATT I TGG W AAT	TTC F ACT T GTT V TAT Y GCT A GCT A AAT	CTT L ATT GAA B GGC GGT V AAC N CCA	1047 311 1107 331 1227 371 1280 391 1347 411 1407
A GAT D CGT R GCG A GTT V CTT L GGG G G G	GAT D ATC I GCC A GTC V CCT P CGT R GCT A	TAT Y AAA K CTG L AAG K GTA V AAC N GAT D	ACT T GCA AAG CT A GCT A TTG L GCC G G C	ATA I CCA P ATG GGT GGT GTA V TGT C GGT GGT	ACA T AAC N AAT TTT F GCT A GCG A GCG A GTA V	GAA E TTA GGT GGT GCC A ATC I GCT A GAC D	GCT A CCG P GGT G AAT N AAT N AAT I TTG L	GGT G ACA T GTG V TTA L GAA E GAT D GCA A	TTC F AGC S GCT A GCA TTT F GTA V AAT N	GGA G CCT P AAA K CGT R ATC I CCT P ACG T	GCT A GAT D CAT H ACA T GTT V CTT L	GAT D GCG A GCT A GTT V GAT D GAA B ATC I	CTT L GTA V CTT L GAA E ACG T TTA L AAT N	GGT GTT V AAT N AAT N AAC N GCC A ACC T	GCT A ATC I CAA Q ATG M GAT D AGT S ATT I	GAA E GTT V GAA E CGT R GAA E GTC V GAA E	AAA K GCA AAT N AAA K ATT I TGG W AAT N	TTC F ACT T GTT V TAT Y GCT A GCT A AAT N	CTT L ATT J GAA B GGC GTT V AAC N CCA P	1047 311 1107 331 1227 371 1280 391 1347 411 1407 431
GCG A GAT D CGT R GCG A GTT V CTT L GGG G G G C C T C C S	GAT D ATC I GCC A GTC V CCT P CGT R GCT A CAT H	TAT Y AAA K CTG L AAG K GTA V AAC N GAT D TAT Y	ACT T GCA AAG K GCT A TTG L GGC G AAA K	ATA I CCA P ATG GT GT GTA V TGT C GGT GGT C GT B	ACA T AAC N AAT TTT F GCT A GCG A GCG A GTA V CTT L	GAA E TTA GGT GCC A ATC I GCT A GAC D TAT Y	GCT A CCG P GGT G AAT N AAT I TTG L GAT D	GGT G GTG V TTA L GAA E GAT D GCA A A A T N	TTC F AGC S GCT A GCA TTT F GTA V AAT N AAT	GGA GCT P AAA K CGT R ATC I CCT P ACG T CTT L	GCT A GAT D CAT H ACA T GTT V CTT L CAT S	GAT D GCG A GTT V GAT D GAT B ATC I GTT V	CTT L GTA V CTT L GAA B ACG T TTA L AAT N GAA B	GGT GTT V AAT N AAT N AAC N GCC A ACC T GAA	GCT A ATC I CAA Q ATG M GAT D AGT S ATT I AAA	GAA GTT V GAA E GTC V GAA E GTC V GAA C V GAA	AAA K GCA AAT N AAA K AAT I GG W AAT N ACT	TTC F ACT T GTT V TAT Y GCT A GCT A AAT N GAG	CTT L ATT J GAA B GGC G GTT V AAC N CCA P ATT T	1047 311 1107 331 1167 351 1227 371 1287 391 1347 411 1407 431 1467 451
GCG A GAT D CGT R GCG A GTT V CTT GGG G G CC S GCC	GAT D ATC I GCC A GTC V CCT P CGT R GCT A CAT H AAG	TAT Y AAA CTG L AAG K GTA V AAC N GAT D TAT Y GAA	ACT T GCA A AAG K GCT A U TTG L GCC G AAA K ATC	ATA I CCA P ATG GGT GGT GGT C GGT C GGT C C C T AC	ACA T AAC N AAT TTT F GCT A GCG A GCG A GCG CTT L CGT	GAA E TTA GGT GCC A ATC I GCT GCT A GAC D TAT Y GCT	GCT A CCG P GGT GAT AAT AAT AAT I AAT I TTG GAT GAT	GGT G GTG V TTA L GAA E GAA D GCA A A A T N AAA	TTC F AGC GCT A GCA TTT F GTA V AAT N AAT S GTT	GGA G CCT P AAA K CGT R ATC CCT P ACG CTT L ATT	GCT A GAT D CAT H ACA T GTT V CTT L TCA S TTT	GAT D GCG A GCT V GAT D GAA E ATC I GAA GAA	CTT GTA V CTT L GAA B ACG T TTA L AAT N GAA B AAG	GGT GTT V AAT N AAT N AAC N GCC A ACC T GAA B AAA	GCT A ATC I CAA Q ATG M GAT D AGT S ATT I AAA K GCT	GAA GTT V GAA E GAA GTC V GAA E GTC V AAA	AAA K GCA AAT N AAA K AAT I GG W AAT N ACT T ACA	TTC F ACT T GTT V TAT Y GCT A GCT A AAT N GAB	CTT L ATT GAA B GGC G GTT V AAC N CCA P ATT	1047 311 1107 331 1227 371 1227 371 1247 411 1407 431 1467 451 1527
GCG A GAT D CGT R GCG A GTT CTT L GGG G C TCC S GCC A	GAT D ATC I GCC A GTC V CCT P CGT R GCT A CAT H AAG K	TAT Y AAA CTG L AAG K GTA V AAC N GAT D TAT Y GAA B	ACT T GCA A AAG GCT A GCT C GC G AAA K ATC I	ATA I CCA P ATG GGT GGT GGT C CGT R TAC Y	ACA T AAC N AAT TTT F GCT A GCG A GTA V CTT L CGT R	GAA E TTA GGT GCC A ATC I GCC D TAT Y GCT A	GCT A CCG P GGT GAT N AAT N AAT N AAT I TTG GAT D GAT D	GGT G GTG V TTA GAA GAT D GCA A A A T N AAA K	TTC F AGC GCT A GCA TTT F GTA V AAT N AAT N GTT V	GGA G CCT P AAA K CGT R ATC I CCT P ACG T CTT L ATT I	GCT A GAT D CAT H ACA T GTT V CTT L CAT S TTT F	GAT D GCG A GCT V GAT D GAA E ATC I GAA E GAA E SAA E	CTT L GTA V CTT L GAA E ACG T TTA L AAT S AAT S AAG K	GGT GTT V AAT N AAT N AAC N GCC A ACC T GAA E AAA K	GCT A ATC I CAA Q ATG M GAT D AGT S AGT I AAA K GCT A	GAA GTT V GAA E GTC V GAA E GTC V AAA K	AAA GCA AAT N AAA K ATT I TGG W AAT N ACT T ACA T	TTC F ACT T TAT Y GCT A GCT A AAT N GAG E CAA	CTT L ATT GAA B GGC G GTT V AAC N CCA P ATT I ATT I	1047 311 1107 331 1227 371 1280 391 1347 411 1407 431 1467 451 1527 471
GCG A GCG CGT GCG A GCG A GTT CTT L GCG G G CC A GCC A GCC	GAT D ATC I GCC A GTC V CCT P CGT R GCT A CAT H AAG K CAA	TAT Y AAA K CTG L AAG K GTA V AAC N GAT D TAT Y GAA B ATT	ACT T GCA AAG K GCT GTA V TTG GGC G AAA K ATC I GTT	ATA I CCA P ATG G G TA G G TA C G G T C C G G T C C R TAC Y AAA	ACA T AAC N AAT TT F GCT A GCG A GCG A CTT L CGT R AAT	GAA E TTA GGT GCC A ATC I GCT A GAC D TAT Y GCT A GGT	GCT A CCG P GGT GAT N AAT N AAT N ATC I TTG GAT D GAT D TGG	GGT G ACA T GTG V TTA L GAA E GAT D GCA A A A A A X K GAT	TTC F AGC S GCT A TTT F GTA V AAT N ATT N GTT V AAC	GGA G CCT P AAA K CGT R ATC I CCT P ACG CTT L ATT I TTA	GCT A GAT D CAT H ACA T GTT CAT CTT L TCA S TTT F CCA	GAT D GCG A GCT A GAT D GAA B ATC I GAA C T V GAA B ATT	CTT L GTA V CTT L GAA E ACG TTA L AAT N GAA E AAG K TGT	GGT G TT V AAT N AAT N AAT SCC A ACC T GAC B AAA K ATG	GCT A ATC I CAA Q ATG ATG AGT D AGT S ATT I AAA K GCT A GCT	GAA E GTT V GAA E GTC V GAA E GTC V GAA E GTC V AAA K AAG	AAA GCA AAT N AAA K ATT I TGG W ATT I TGG AAT ACT T ACA	TTC F ACT T TAT Y GCT A GCT A AAT N GAG E CAA Q CAG	CTT L ATT GAA B GGC GTT V AAC N CCA P ATT I ATT I TAC	1047 311 1107 331 1227 371 1280 391 1347 411 1407 431 1467 451 1587
GCG A GAT CGT R GCG A GTT CTT L GCG G G C C TCC S GCC A GCC A C C TCC	GAT D ATC G G C C T C G T C G T C G T C C T R G C T A C A T C A C C C C C C C C C C C C	TAT Y AAA K CTG L AAG K GTA V AAC N GAT D TAT Y GAA B ATT I I	ACT T GCA AAG K GCT GTA V TTG GGC GGC AAA K ATC I GTT V	ATA I CCA P ATG G G G G G G G G G G T C G G T C C G G T C C A S G G T A C A S G T A C A S G G T A C A S G G A C A S G G A S G G A S G G A S G G G A G G G G	ACA T AAC N AAT TT F GCT A GCG A GCG A GTA V CTT L CGT R AAT N	GAA E TTA GGT GCC A ATC I GCT A GAC D TAT Y GCT A GGT G	GCT A CCG P GGT GAT N AAT N AAT I TTG GAT D GAT D TGG	GGT G ACA T GTG V TTA L GAA E GAT D GCA A A A A A A A C A T D GAT D C C C C C C C C C C C C C C C C C C	TTC F AGC S GCT A GCA A TTT F GTA V AAT N GTT V AAC N C TT V AAC	GGA G CCT P AAA K CGT R ACG CCT P ACG T CTT L ATT I TTA L	GCT A GAT D CAT H ACA T GTT CAT TCA S TTT F CCA P	GAT D GCG A GCT A GTT D GAT GAA B ATC I GAA B ATT I I	CTT L GTA V CTT L GAA B ACG TTA L AAT N GAA B AAG K TGT C	GGT G TT V AAT N AAT N AAT SCC A ACC T GAC T AAA K ATG M	GCT A ATC I CAA Q ATG M GAT D AGT S ATT I AAT S GCT A GCT A	GAA E GTT V GAA E GTC V GAA E GTC V GAA E GTC V AAA K AAG	AAA GCA AAT N AAA K AAT T TGG W AAT N ACT T ACA T	TTC F ACT T TAT GCT GCT A AAT N GAG E CAA Q CAG Q	CTT L ATT GAA B GGC GT V AAC N CCA P ATT I ATT I TAC Y	1047 311 1107 331 1227 371 1280 391 1347 411 1407 431 1467 451 1587 491
GAT GAT CGT R GCG A GCG CTT L GGG GCC A GCC A GCC A GCC A GCC	GAT D ATC G G C C C C C C C C C C C C C C C C C	TAT Y AAA CTG L AAG K GTA V AAC N GAT D TAT GAA B ATT I CA S S A TAT	ACT T GCA A GCT GCT C GCC G GCC G GCC G GCC G GCC G GCC G GCC G GCC G GCC G GCC G GCC C GCC C A C C C C	ATA I CCA P ATG GGT GGT GGT C GGT C GGT R CGT R TAC Y AAA K GAT	ACA T AAC N AAT F GCT A GCT A GCA GTA V CTT L CGT R AAT N CCG	GAA E TTA GGT GCC A ATC I GCT A GAC D TAT Y GGT GGT GGT A A A K	GCT A CCG P GT G AAT N AAT N AAT TTG GAT D GAT D TGG W A TTG	GGT GACA T GTG GTT GAA E GAT D GCA A AAT N AAA K CTG CTG C T	TTC F AGC S GCA A TTT F GTA V AAT N GTT V AAC N GC C C C C C C C C C C C C C	GGA G CCT P AAA K CGT R ACC CTT CTT L ATT L GCC CTT L	GCT A GAT D CAT H ACA T T CAT TCA S TTT F CCA P CCA	GAT D GCG A GTT V GAT D GAT D GAT E ATC I GTT V GAA E ATT I ATT	CTT L GTA V CTL GAA E ACG TTA L AAT SAA E AAG K TGT C GCC	GGT G TT V AAT N AAT N AAT N GCC A ACC T GAA B AAC TT F	GCT A ATC I CAA Q ATG ATG A GAT D AGT A AAA K GCT A GCT A GCT A C T	GAA E GTT V GAA E GTC QAA E GTC V GAA E GTC V AAA K AAG K AT	AAA K GCA AAT N AAT T TGG W AAT T TGG W AAT T ACA T ACA T ACA	TTC F ACT T TAT GCT A GCT A CAG CAG CAG Q ATT	CTT L ATT J GAA B GGC G GT V AAC N CCA P ATT I ATT I TAC Y CGT	1047 311 1107 331 1227 371 1280 391 1347 411 1407 431 1467 451 1527 491 1647 51
A GAT D CGT R GCG A GTT C CTT L GGG G G C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C C T C	GAT D ATC G G C C C T C C T C C T C C T C C T T T G C C T T T T	TAT Y AAAA CTG CTG AAG TAT Y AAC N GAT Y GAA B ATT TCA S GTT GTT	ACT T GCA A AGG GCT A GCT C GCC G C C C C C C C C C C C C C C	ATA I CCA P ATG GGT GGT GGT C CGT R CGT R CGT R CGT R CGT R CGT AAA AAA	ACA T AAC N AAT TTTT F GCT A GCG A GCA CTTT L CCTT R AAT N CCG P TTG P TTG	GAA E TTA GGT G GCC A ATC I GCT A GCT Q GCT Q C C A AAA K GGT GGT	GCT A CCG P GGT N AAT N AAT TTG GAT D GAT D GAT D TTG GAT C GAT C GAT C GAT C GAT C GAT C GAT C GAT C GAT C G G C G G C G G C G C G C G C G G C G C G C G C G C G C G C G C G C G C G C G C G C G C G C C G C G C C G C C G C C G C C C C G C	GGT GACA T GTG GTG GAT C GAT D GCA A AAT N AAA K GAT C TG GGT	TTC P AGC S GCT A GCA A TTT F GTA V AAT N GTA V AAC N GTT TTT	GGA G CCT P AAA CGT R ATC CT P ACG T CTT L ATT ATC CTT L ATT A CTT A CTT A CTT	GCT A GAT D GAT D CAT H ACA T CTT CTT L CTT CTT F CCA P CCA GTT GTT GTT	GAT D GCG A GTT V GAT D GAT C GAT GAT C GAT S ATC I GAT C A ATT C GAT GAT C GAT C GAT C C C C C C C C C C C C C C C C C C C	CTTT L GTA V CTTT GAA E ACG T AAG TTL AAT B GAA E AAG GGC GCTT	GGT GTT V AAT N AAT N AAC N GCC T GAA B AAC TT T R ATG M TTT P ACA	GCT A ATC CAA Q GAT D AGT S ATT I AAA K GCT A GCT A GGA	GAA E GTT V GAA E CGT R GAC V GAB GTC V AAA K AAG K AAT I GAT	AAA K GCA A AAT N AAA K AATT I GC W AAT N ACT T ACA T ACA T ACA T GTC	TTCC F ACT T GTT V GCT A ACT A GCT A ACT A CAG Q CAG Q ATT I ATG	CTT L ATT J GAA B GGC G GT V AAC N CCA P ATT I ATT I TAC Y CGT R ACC	1047 311 1107 331 1227 371 1280 391 1347 411 1407 431 1467 451 1527 491 1647 517 1707
A GAT D CGT R GCG A GTT V CTT L L GGG G G C C S GCC A A GCC A A GCC A B GCC A C GT C CT R C CT C T C CT R C C C C	GAT D ATC I GCC C C C C C C C C C C C C C C C C	TAT Y AAA CTG CTG GTA K AAC O AAC V AAC N GAT TAT Y GAA B ATT I TCA S GTT V	ACT T GCA AAG GCT TTG C GCC C AAA K ATC GCT C C C C C C C C C C C C C C C C C	ATA I CCA P ATG GGT GGT C C GGT C C C GT R C C C C T ACA B AAA B AAG AAA K	ACA T AAC N AAT TTT F GCT A GCG A GCG A CTT L CGT R AAT N CCG P TC G C T T G L	GAA E TTA GGT GCC A C GCC I GCC I GCT A C C TAT Y GCT A C GCT A C GCT C G G C C C C C C C C C C C C C C	GCT A CCG P GGT G AAT N AAT TTG C GAT D GAT D GAT D C GAT C G A C G A A C G A A A C G G T C G G T C G G T C G G T C G G T C C G G T C C G G T C C G G T C C G C C C C	GGT GGG GTG GTG TTA L GAT D GCA A AAT N AAA K GAT S GAT G GTG G G G G G G G G G G G G G G G	TTC F AGCC GCT GCA A TT F GTA V AAT N GTT N GTT S GT TTT F	GGA G CCT P AAA CGT R ATC CT P ACG T CTT L ATT I GCC A T TTA L GCC T	GCT A GAT D CAT H ACA T CAT CAT C TT CA S TTT F CCA S TTT F CCA P CCA S TTT F CCA S TTY F CCA S	GAT D GCG GCT GTT V GAA B GTT V GAA B GTT V GAA ATT I ACT T GCA A	CTTT L GTA V CTT GAA E ACG T TTA AAT B AAG K T C GGC G CTT L	GGT GT V AAT N AAT N GCC A A CC T GAA B AAC T T T F ACA T	GCT A ATC I CAA Q ATG M ATG D AGT I AAA K GCT A GCT Q GCT Q GCT Q GCT GCT C A C A C A C A C A C A C A C A C A C	GAA E GTT V GAA E GTC V GAB E GTC V AAA E GTC V AAA K AAG K ATT I GAT D	AAA K GCA A AAT N AAA K AATT T G W AAT N ACT T ACA T ACA T GTC V	TTC F ACT T T GTT S GCT A ACT A ACT A CAG CAG CAG Q ATT I ATG M	CTT L ATT J GAA B GGC G GT V AAC N CCA P ATT I TAC Y CGT R ACC T	1047 311 1107 331 1227 371 1280 391 1347 411 1467 451 1587 491 1647 511 1707 531
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A GAT D CGT R GCG G G G CTT L GGG G CTT L GGG G C C T C C T C C T C C T C C T C C T C C T C C T C C T C C T C C C T C	GAT D ATC I GCC V CCT P CCT P CCT R CCT R CCT H AAG CAA TTT F TTG L CCT P	TAT Y AAA CTG CTG GTA Y AAG TAT Y GAA S GTT Y GGA GTT Y CGGA	ACT T GCA AAG GCT AC TTG GCC C GCC G AAA K ATC GTT GTT GCC D CCC P TTG L	ATA I CCA P ATG GGT GGT C GGT TGT C GGT R TAC GGT R AAA K GAT D AAG K CCA	ACA T AAC N AAT TTT F GCT A GCG A GCG A GCG CTT L CGT R AAT CCG T T G C T T G C G T A X X X X X X X X X X X X X X X X X X	GAA E TTA GGT GCC A GCC I GCC I GCT A GCT C GCT C GCT A GCT C GCT A C GCT C A AAA K C GCT A AAA	GCT A CCG P GT G AAT N AAT N AAT TTG GAT D GAT D GAT CGA TTG GCA A CCA P	GGT G ACA T GTG V TTA E GAT D GCA A AAT N AAA K GAT D CTG G GCT G GCA A	TTCC F AGCC GCT GCA A TTT F GTA V AAT N GTT N GTT G GT TTT F GCT A	GGA G CCT P AAA K CGT R ACCG CCT P ACCG T CCTT L ATT L GCC A CCT T TTA L GCC I U CTT L	GCT A GAT D CAT H ACA T CAT T CAT T CAT T CAT C T T C CAT C CAT C CAT C CAT T C CAT T C CAT T C CAT C C CAT C CAT C CAT C CAT C CAT C C C C	GAT D GCG A GTT V GAT D GAA B GTT V GAA B GTT V GAA I GTT V GAA A TT GCA A ATT GCA	CTTT L GTA V CTT GAA E ACG T TTA AAT TTA AAG GGA GGC GC CTT L GAT D	GGT G GTT V AAT N AAT N GCC A A ACC T GAA B AAA K GAA B AAA T TTT F ACA T T V V	GCT A ATC I CAA Q ATG M ATG D AGT S ATT I AAA K GCT A GCT Q GCA GCA A	GAA E GTT V GAA E GTC V GAA E GTC V AAA K AAG K AATT I GAA	AAA K GCA A AAT N AAA K AAA T TGG W AAT T ACA T ACA T ACA T C GTC V GAT D	TTC F ACT T GTT Y GCT A A GCT GAG GAG Q ATT I ATG Q GGA GGA GGA GGA	CTT L ATT GAA GGC GT V AAC N CCA P ATT I ATT I ATT X CGT R ACC T ACA T	1047 311 1107 331 1227 371 1227 371 1280 391 1347 411 1407 451 1527 471 1527 471 1647 511 1767 551
A GAT D CGT R GCG G G G CTT L GGG G CTT L GGG G C C T C C T C C T C C T C C T C C T C C T C C T C C T C C C T C	GAT D ATC I GCC V CCT P CCT R CCT R CCT A CAT H AAG CAA TTT F TTG L CCT P TTG CCT	TAT Y AAA CTG CTG GTA V AAC TAT Y GAA B ATT I TCA S GTT V GGA GCC	ACT T GCA AAG GCT A GCT C GCC G AAA K ATC GTT G C C C C C C C C C C C C C C C C	ATA I CCA P ATG GGT GGT C GGT C GGT C CGT R TAC C GGT R TAC C GGT R TAC C CGT R TAC C C T C C C T C C C T C C C C T C	ACA T AAC N AAT TTT F GCT A GCG A GCG A GCG CTT L CGT T C G T A AT N CCG P T T G C T T T T T T T T T T T	GAA E TTA GGT GCC A GCC I GCC I GCT A C GCT C C GCT C C GCT A A A C C C C C C C C C C C C C C C C	GCT A CCG P GT G AAT N AAT N AAT TTG G GAT D GAT D GAT CGA W TTA CCA P TTAG CCA	GGT G GTG V TTA G GAT D GCA A AAT N AAA K GAT D CTG G GCA C GGA C A GCA C A GCA	TTC F AGCC GCT GCA A TTT F GTA V AAT N GTT N GGT GCT A ACC A ACC A ACC A ACC A A CCA A CCA CCA A CCA CCA CCA CCA CCA CCA CCA CCA CCA	GGA G CCT P AAA K CGT R CCT P ACG CTT L ACT L GCC A CTT L CTT L CTT L CTT L CTT L CTT CTT L	GCT A GAT D CAT H ACA T CAT H ACA T CAT T C TTC C C T T C C T T C C A C T T C C T T C C T T C C T T C C T T C C T T C C T C T C C C T C	GAT D GCG A GTT V GAT D GAA B GTT V GAA B GTT V GAA I GTT V GAA A TC GCT C CCCCC	CTTT L GTA V CTT GAA E ACG T TTA AAT TTA AAG C TTA C GGC CTT L GAT D CTTTT	GGT G GTT V AAT N AAT N AAT N GCC A A ACC T GAA B AAA K GAA T TTT F ACA T TTT T F C C V TTCA	GCT A ATC I CAA Q ATG M GATG D AGT S ATT I AAA K GCT A GCT A GCT A GCT A GCT A C CAA C C A T ATG C A ATG A T C A A T G A T G A T G A A T G A A T G A A T G A A T G A A G A T G A A T G A A T G A A T G A A T G A A T G A A T G A A T G A T G A A T G T A A T G A T G A T G A T G T A A A T A A A T A T	GAA E GTT V GAA E GTC V GAA E GTC V AAA K AAG K AATT I GAT I C GAA	AAA K GCA A AAT N AAA K AAA T TGG W AAAT T ACA T ACA T ACA T C C C V GAT D AGGTC	TTC F ACT T GTT Y GCT A A GCT A A GGA GGA Q ATT I ATG GGA GGA GGAAG	CTT L ATT J GAA GGC G GT V AAC N CCA P ATT I TAC Y CGT R ACC T ACA T GTGAA	1047 311 1107 331 1227 371 1227 371 1280 391 1347 411 1407 451 1527 471 1527 491 1647 511 1767 551 1842
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GCG A GAT D CGT R GCG G GT CTT L GGG G CTT L GGG G CTT L GGG G C CTT L GGG G C CTT L GGG G C CTT L CTT L CGTT CTT L CTT CTT C T C CTT C CTT C CTT C CTT C CTT C	GAT D ATC I GCC A GTC V CCT P CCT A CAT A AG CAT TTG CAT TTG CTT CCT CCT CCT CCT CCT TTG CTT CTT	TAT Y AAA K CTG GTA GTA K AAC N AAC N AAC N TAT Y GAA AATT I TCA S GTT Y GGA GC GC GC GC ATGAA	ACT T GCA A AAG GCT A GCT C GCC G AAA K ATC C C C C C C C C C C C C C C C C C C	ATA I CCAA P ATG GGT G GGT C CGT T C C GGT C CGT R T ACC A AA AA AA AA CCA P TTC F AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	ACA T AAC N AAT TTT F GCT A GCG A GCG A GCG CCTT L CGT T CGT T CGT T T G C T T G C T T T G C C T T T T	GAA E TTA G G G G C C A C G C C C C C C C C C C	GCT A CCG P GT G AAT N AAT N AAT TTG G GAT D GAT D GAT CGA TGG W TTA CCA P TTAAG	GGT G GTG V TTA E GAT D GCA A AAT N AAA K GAT D CTG G CTG GCA CAGGA CAGGA	TTC F AGCC GCT GCA A TTT F GTA V AAT N GTT N GGT GCT A ACC A ACC A ACC A ACC A A A C A A C A A C A A C A A C A A C A A A C A A A C A A A C A A A A A A A A A A A A A	GGA G CCT P AAA K CGT R ACCG T CCT P ACCG T CTT L ATT L GCC CTT L CTT L CTT L CTT CTT CTT CTT CTT	GCT A GAT D CAT H ACA T CAT H ACA T CAT T CAT C CAT F CCA P CCA P CCA T T CA T T CAT T CAT T CAT T CAT CA	GAT D GCG A GTT V GAT D GAA B GTT V GAA B GTT V GAA C T GTT T C A C C C C G C T G C T C C C C C C C C C C	CTTT L GTA V CTT CTTT L GAA E ACG T TTA AAG T C CTTT C GGC CTT L GAA CTT TTA C CTT C CTT C CTT CTT	GGT GT V AAT N AAT N AAT N GCC A ACC A C A A C C C A A T T T C C A A C C C A A C C C C	GCT A ATC I CAA Q ATG M GAT J AGT A GCT A GCT A GCT A GCT A GCT A GCT A CCAA A C CAA A C CAA A C CAA A C A C	GAA E GTT V GAA E GTC V GAA E GTC V AAA K AATT I GCA TTTAA	AAA K GCA A AAT N AAA K AAA T TGG W AAT T ACA T ACA T ACA T C GTC V GAT D AGGTV V	TTCC F ACT T GTT V V TAT Y GCT A A GGA GGA CAG Q ATT I A ATG GGA GGA GGA GGA GGA GGA	CTT L ATT J GAA GGC G GT V AAC N CCA N CCA N ATT I TAC Y CGT R ACC T ACA T GTGAA	1047 311 1107 331 1227 371 1227 371 1280 391 1347 411 1407 431 1467 451 1527 471 1527 471 1647 511 1767 551 1842 556 1921 2001

FIG. 2. DNA and amino acid sequences of flvs from S. mutans. RBS, putative Shine-Dalgarno sequence. Singly underlined areas represent putative -10 and -35 promoter sequences. The insertion site of Tn917 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 wildtype 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

Tn917 and subsequently recovered with pTV21 Δ 2TetM, 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 Tn917-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 \pm 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 Tn917 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 Tn917. Previously we described

STMUT	1	MKTDIEIAQS VDL RPITN VVKKLGID FDDLELYGKYKAKLTFDKIKA VEENAPGKLVLVTAIN
CLOAC	1	MKTDIQIAQEAQMKHIKD VAELIDIHEDDLELYGKYKAKVSLDVLDQLKDKPDGKLVLVTAIN
CLOCY	1	MKTDVQIAQEAQMKPITEVANYLGIQDDELELYGKYKAKVSLDVLERQKDKEDAKLVLVTAIN
CLOTH	1	MSKVPSDIELAQAAKMKPVMELARGLGIQEDEVELYGKYKAKISLDVYRRLKDKPDGKLILVTAI
STMUT	64	PTPAGEGKSTITIGLADALNKIGKKTMIAIREPSLGPVMGIKGGAAGGGYAQVLPMEDINLHFTGD
CLOAC	64	PTPAGEGKTTTNIGLSMGLNKLGKKTSTALREPSLGPSFGVKGGAAGGGYAQVVPMADINLHFTGD
CLOCY	64	PTPAGEGKTTTNVGLSMGLNKIGKRTITALREPSLGPCFGVKGGAAGGGYAQVVPMDDINLHFTGD
CLOTH	67	PTPAGEGKTTTSVGLTDALARLGKRVMVCLREPSLGPSFGIKGGAAGGGYAQVVPMEDINLHFTGD
STMUT	130	MHAIITTAN NALSALIDNH LHQGNELGIDQR RIIWKRVVDLNDRALRHVTVGLGSPINGIPREDGFD
CLOAC	130	FHAITSAHSLUAALVDNH LHHGNALRIDTNRIVWKRVVDMNDRALRKIVVGLGGKA OGITREDGFD
CLOCY	130	FHAITSAHNLLAALUDNH LHQGNALNINPKKIVWKRVIDMNDRSLRNVIIGLGGNGDGFVRQA QFD
CLOTH	133	IHAVTYAHNLLAAMVDNH LQQGNVLNIDPRTITWRRVIDLNDRALRNIVIGLGGKA NGVPRETGFD
STMUT	196	ITVASEIMAILCLATN VEDLKERLAN I VIGYRFDRSPVY VRDLEVOGALALI LKEAI KPNLVQTI Y
CLOAC	196	ITVASEIMAILCLAND REDLKERLGNMV VAYN VDGDAVRAKDLEACGAL TLI LKDAI NPNIVQTLE
CLOCY	196	ITVASEIMAILCLATSMSDLKERLSKMI VAYAKDGSAVTAGOLEATGAMALLLKDAV KPNLVQTLE
CLOTH	199	ISVASEVMACLCLASDLMDLKERFSRI VVGYTYDGKPVTAGDLEACGSMALLMKDAI KPNLVQTLE
STMUT	262	GT PAF VHGG PFANIAHGCNSVLATSTALRLADYT IT EAG FGADLGAEKFLDIKAPNLPTSPDAVVI
CLOAC	262	NTPAFIHGG PFANIAHGCNSVLATKLALKTGDYAVTEAG FGADLGAEKFFDIKC RYAGLNPDVAVI
CLOCY	262	NTPAFIHGG PFANIAHGCNSVLATKVALKLADYVVTEGG FGADLGAEKFFDIKS RFAGLKPNCDVS
CLOTH	265	NTPAFIHGG PFANIAHGCNSIIATKTALKLADYVVTEAG FGADLGAEKFYDVKC RYAG FKPDATVI
STMUT	328	VATIIRALKMNGG VAKDALNQEN VEAVKAGFAN LARHVENMRKYG VPVVVAINEFITDTNDEIAVLR
CLOAC	328	VATVRALKMHGG VAKEDLGTENLDALAKGMTNLERHIENVAKFG VPSVVAINAFPTDTEAEKQLVF
CLOCY	328	VATVRALKMNGG VPKTELAAEN VEAVKKG VANLERHIENVAKFG VPAVVAINKFPLDTEAELKAVE
CLOTH	331	VATVRALKMHGG VPKSDLATENLEALREGFANLEKHIENIGKFG VPAVVAINAFPTDTEAELNLLY
STMUT	394	N LCA A I D V PVE L AS VWANGA DGG V D LAN T L I N T I EN N PSH Y K RLYD N N LS VE EK V T EI AK EI Y RAD
CLOAC	394	D KC KEMG V D VA I S D VFAKGG DGG V E LAQ K V I D V CEN KKSD F K VLYD V E ES I PEKI T K I AK EI Y RAD
CLOCY	394	D ACN A KG A D V V LS D VWANGG EGG V EMAKKV V E I CEKN E AN F A PLYD V N LS I PEKI E K I ATT I Y RAD
CLOTH	397	E LCA KAG A E VA LS E VWAKGG EGG L E LAR K V L Q T LES R PSN F H VLYN LD LS I KD KI A KI AT EI Y GAD
STMUT	460	KVI FEKKAKTQI AQIVKNGWDNLPICMAKTQYSFSDDPKLLGAPTGFDITIRELVPKLGAGFIVAL
CLOAC	460	KVNFSKAAKKQI AELEKLGLDKLPICMAKTQYSFSDDPALLGAPEGFELTIRDLELAAGAGFIVAL
CLOCY	460	GVDFTSDCKKQI AELEKLGLDKMPICMAKTQYSFSDDPTLLGAPTGFRITVREVRVSAGAGFIVAL
CLOTH	463	GVNYTAEADKAIQRYESLGYGNLPVVMAKTQYSFSDDMTKLGRPRNFTITVREVRLSAGGRLIVPI
STMUT	526	TGD VMTMPGLPKKPAALN MDVAADGTALGLF
CLOAC	526	TGD IMRMPGLPKVPAAN RMDVLPNGEIIGLF
CLOCY	526	TGN MMTMPGLPKVPAAN GMDILESGEIIGLS
CLOTH	529	TGAIMTMPGLPKRPAACN IDIDADGVITGLF

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 Tn917 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 ⁶⁴PTPAGEG KXT⁷³, 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 nucleotidebinding consensus sequence (105KGGAAGGGY113) 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 (EPSLGPX₂G) 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 MettRNA 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 ≤ 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 Tn917 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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