***Streptococcus mutans* ffh, a gene encoding a homologue of the 54 kDa subunit of the signal recognition particle, is involved in resistance to acid stress**

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The ability of *Streptococcus mutans*, a bacterial pathogen associated with dental caries, to tolerate rapid drops in plaque pH (acidurance), is considered an important virulence factor. To study this trait, Tn917 mutants of *S. mutans* strain JH1005 which display acid sensitivity have been isolated and partially characterized. In this paper, the characterization of one of these mutants, AS17, is reported. Preliminary sequence analysis revealed that the transposon insertion in AS17 occurred in the intergenic region of a two-gene locus which has been named *sat* for secretion and acid tolerance. This locus displays a high degree of homology to the *ylxM*–*ffh* operon of *Bacillus subtilis*. The *sat*⁺ locus was cloned by complementation of a conditional *Escherichia coli* *ffh* mutant with an *S. mutans* genomic library. Sequencing of the complementing clone identified the intact *ylxM* and *ffh* genes as well as a partial ORF with homology to the *proUopuAC* gene of *B. subtilis* which encodes the binding protein of the ProU/OpuA osmoregulated glycine betaine transport system. RNA dot blot experiments indicated steady-state levels of *ffh* mRNA in the mutant that were approximately eightfold lower compared to parental levels. This suggests a partial polar effect of the *sat*–1::Tn917 mutation on *ffh* expression. Upon acid shock (pH 5), wild-type *ffh* mRNA levels were found to increase approximately four- to eightfold compared to unstressed (pH 7.5) levels. Mutant levels remained unaltered under the same conditions. Experiments designed to investigate the origins of the acid-sensitivity of the mutant revealed a lack of an acid-adaptive/tolerance response. Assays of proton-extruding ATPase (H⁺/ATPase) specific activity measured with purified membranes derived from acid-shocked AS17 showed twofold lower levels compared to the parent strain. Also, AS17 was found to be unable to ferment sorbitol although it was able to grow in glucose and a variety of other sugar substrates. These findings suggest that Ffh may be involved in the maintenance of a functional membrane protein composition during adaptation of *S. mutans* to changing environmental conditions.

Keywords: *Streptococcus mutans*, *ffh* homologue, acid tolerance, Tn917 mutagenesis

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Abbreviations: ATR, acid tolerance response; GSP, general secretory pathway; SRP, signal recognition particle.

The GenBank accession number for the DNA sequence of the 3 kb DNA fragment containing the *ylxM*–*ffh* genes is U88582.

INTRODUCTION

Streptococcus mutans plays a major role in the aetiology of human dental caries. This bacterium is able to demineralize dental enamel by generation of a localized acidic environment in plaque by rapid glycolysis of dietary sugars to lactic acid. In addition to its acidogenicity, *S. mutans* is able to tolerate exposure to continual and rapid cycles of acid shock. *In vivo* pH measurements have demonstrated that the pH of plaque can drop from pH 7.0 to values ranging from 3.0 to 4.0 in less than 20 min following the intake of carbohydrates (Imfeld & Lutz, 1980; Yamada *et al.*, 1980; Mormann & Muhlemann, 1981; Schachtele & Harlander, 1984; Jensen & Wefel, 1989). Early work by Stephan (1944) demonstrated that rampant caries is associated with plaque acidification as well as tolerance of the microflora to low pH. Acidurance, or resistance to acid, is therefore regarded as a major virulence factor in *S. mutans*. The mechanisms that are utilized by *S. mutans* to tolerate these cyclic and often prolonged pH reductions are poorly understood; however, recent work suggests that *S. mutans* has both constitutive and acid-inducible mechanisms that act to enhance its ability to tolerate rapid pH drops (Svensater *et al.*, 1997).

Genetic studies of the acid-protective mechanisms in *S. mutans* have resulted in the identification of genes that, when inactivated, result in acid-sensitive phenotypes. Using Tn916 mutagenesis, Yamashita *et al.* (1993) identified an acid-sensitive mutant of *S. mutans* GSS deficient in diacylglycerol kinase, an enzyme involved in phospholipid metabolism and, therefore, in maintenance of membrane architecture and composition. This mutant was also sensitive to high osmolarity and high temperature. We have been involved in similar research but have used transposon Tn917 to isolate acid-sensitive mutants of *S. mutans* (Gutierrez *et al.*, 1996). This small and relatively stable mutagenic element was delivered in pTV1-OK, a broad-host-range, conditional temperature-sensitive (T_s) replication vector for transformation-independent mutagenesis. Our work resulted in the isolation of a variety of acid-sensitive mutants, suggesting that acidurance in *S. mutans* is a multifactorial phenomenon (Gutierrez *et al.*, 1996; Crowley *et al.*, 1997). One such mutant (AS17) was recovered with the transposon insertion in the intergenic region between the *S. mutans* *ylxM-ffh* homologues. We have named this locus *sat* (for secretion and acid tolerance; Gutierrez *et al.*, 1996). In *Bacillus subtilis* (Honda *et al.*, 1993) this operon encodes YlxM, a putative 13.2 kDa protein of unknown function which displays no significant homology to other known proteins in databases. YlxM contains helix–turn–helix motifs characteristic of DNA-binding proteins (Samuelsson *et al.*, 1997). The other protein in the operon is Ffh, the fifty-four kDa subunit homologue of the eukaryotic signal recognition particle (SRP), a chaperonin which is also found in *Escherichia coli* and is involved in protein translocation and membrane biogenesis (reviewed by Lührink & Dobberstein, 1994).

In an attempt to understand the molecular basis of the acid-sensitive phenotype of AS17, we cloned and sequenced the wild-type *sat* locus. Further, we present data here that suggest a role for Ffh in acidurance in *S. mutans*.

METHODS

Bacteria and cultivation conditions. *E. coli* MC1061 [Δ (*araA-leu*)7697 *araD139* Δ (*codB-lac*)3 *galE15* *galK16* *mcrA0* *relA1* *rpsL150* *spoT1* *mcrB9999* *hsdR2*], DH5 α [*F'* F80*dlac*(*lacZ*) M15/*endA1* *hsdR17* *supE44* *thi1* *recA1* *gyrA96* *relA1* Δ (*lacZYA-argF*)U169] and WAM113 [*F'* *araD139* Δ (*argF-lac*)U169 *rpsL150* *relA* *deoC1* *ptsF25* *rbsR* *flb5301* *ffh-1::kan* *l araB-ffh*⁺] (Phillips & Silhavy, 1992), were grown either in liquid or on solid (1.5% agar) Luria–Bertani (LB) medium. For WAM113, LB was supplemented with 0.2% L-arabinose as indicated. *S. mutans* strains LT11 (wild-type, obtained from R. Russell, University of Newcastle upon Tyne, UK), JH1005 [parent strain; Tet^r (1 mg ml⁻¹) (Hillman *et al.*, 1987)], AS17 (*sat-1::Tn917*; Gutierrez *et al.*, 1996) and AX1 (*icd-1::Tn917*; Cvitkovitch *et al.*, 1997) were grown in the following liquid and solid (2.0% agar) media: Blood agar; Todd–Hewitt containing 0.3% yeast extract (THYE) at pH 4.7–7.5, with the pH adjusted by addition of NaOH or HCl; tryptone yeast extract medium, containing 1% tryptone, 0.5% yeast extract, 0.1–1.0% (w/v) glucose (TYEG), galactose, lactose, sorbitol, mannitol or sucrose. Medium consisting of 2% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% Na₂HPO₄, 0.002% bromocresol purple and 1.0% of the above-mentioned sugars was used to study fermentation of carbon sources (TYEBP). In experiments to establish the killing pH and adaptation to acid tolerance, the TYEG (0.1% glucose) medium was supplemented with 40 mM K₂HPO₄/citrate buffer to the appropriate pH (TYPC) such that the pH did not vary by more than 0.2 pH units. In liquid media, cultures of *S. mutans* were incubated either aerobically or anaerobically in a chamber using a gas mixture of 10% CO₂, 15% H₂ and 75% N₂ without agitation. Cultivation on solid media was performed in either jars containing a GasPak for anaerobic growth (BBL) or the anaerobic chamber. Antibiotics included ampicillin (50 µg ml⁻¹ for *E. coli*), kanamycin (75 µg ml⁻¹ for *E. coli* and 500 µg ml⁻¹ for *S. mutans*), erythromycin (Em, 300 µg ml⁻¹ for *E. coli* and 10 µg ml⁻¹ for *S. mutans*), chloramphenicol (Cm, 12.5 µg ml⁻¹ for *E. coli*) and spectinomycin (Sp, 100 µg ml⁻¹ for *E. coli* and 1000 µg ml⁻¹ for *S. mutans*).

Cloning of the intact *sat* locus (*ylxM-ffh*) from *S. mutans*.

The *ylxM-ffh*⁺ genes of JH1005 were cloned by complementation of an *ffh* conditional-lethal mutant of *E. coli*, strain WAM113. This mutant harbours a chromosomal *ffh* knock-out mutation (*ffh-1::kan*) and an intact *ffh* gene under the control of an L-arabinose-inducible promoter (*l araB-ffh*⁺). To clone the *S. mutans* *ylxM-ffh*⁺ genes, WAM113 was transformed with a *Sau*III JH1005 genomic library in pVA891 (Macrina *et al.*, 1983) selecting for Em^r and Cm^r derivatives able to grow independently of 0.2% L-arabinose.

Nucleotide sequencing and analyses. Nucleotide sequencing of the *sat* locus in plasmids pUC-AS17 (*sat-1::Tn917*) and pJG301 (*ylxM-ffh*⁺) was carried out at the DNA Sequencing Core Laboratory of the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR). Sequence analyses were carried out with MacVector v3.5 (Kodak) software and programs BLASTN, BLASTX, CLUSTAL W and SeqVu available via the Internet.

Backcross and complementation experiments. In order to establish that the insertion of Tn917 was responsible for the observed acid-sensitive phenotype, a genetic backcross was performed. For this purpose, chromosomal DNA isolated from AS17 was used to transform *S. mutans* strain NG8 as described previously (Gutierrez *et al.*, 1996). Em^r transformants were isolated and assessed for their sensitivities to acid when compared to the parent strains NG8 and JH1005 and mutant AS17. One such transformant was designated AS17N. In an attempt to complement the *ffh* mutation in *S. mutans* strains AS17 and AS17N, a 2.5 kb DNA fragment containing *ylxM-ffh* was amplified by PCR using an upstream primer (bp 384–400 upstream of the translational start codon) and downstream primer (reverse and complement of bp 59–76 downstream of the *ffh* translational stop codon). Plasmid pJG301 (Gutierrez *et al.*, 1996) served as the template. The amplified product was gel-purified and ligated to *Sma*I-digested *S. mutans*-*E. coli* shuttle vector pDL278 (Dunny *et al.*, 1991) to generate plasmid pPC300, which was used to transform *E. coli* strain WAM113 (*ffh*) and *S. mutans* strains AS17 and AS17N.

RNA isolation and RNA dot blotting. To isolate *S. mutans* RNA, 18 h THYE cultures were subcultured 1:10 (v/v) into 50 ml pre-warmed THYE media supplemented with 20 mM D,L-threonine. Cells were grown to OD₆₀₀ ranging from 0.3 to 0.5. When studying the levels of *ffh* mRNA expressed under different pH values, cells were grown in TYPC containing 20 mM D,L-threonine at pH 7.5 and pH 5.0 as described below for preparation of acid-shock *S. mutans* fractions. In preparing total RNA from each strain we found that AS17 grew equally well in medium with or without D,L-threonine. We did not detect any difference in the degree of cell lysis during the RNA preparations between the parent and mutant, and equivalent yields of RNA from the parent and mutant were obtained from a specific volume of cells. The method of Lunsford (1995) was followed for isolation of total streptococcal RNA, except that we omitted glycine, and used 500 U mutanolysin (Sigma) ml⁻¹ instead of lysozyme. Lysates were subjected to selective RNA isolation using the TRIZOL reagent (Gibco-BRL) according to the manufacturer's instructions. RNA preparations were treated with 20 units RQ1 DNase (Promega) for 30 min at 37 °C, extracted with TRIZOL and chloroform, and precipitated with ethanol. *ffh* mRNA levels were detected by RNA dot blotting using the Genius non-radioactive nucleic acid labelling and detection system (Boehringer Mannheim). A DNA probe was generated by amplifying an internal segment of *ffh* by PCR using primer 5' CTGCGTGATAT-CAAGC 3' as the forward primer and either 5' AGGCT-AACTGATAAAGC 3' or 5' CCTTCATCAACTTCGAG 3' as the reverse primer, with pJG301 as the template. The probe was labelled directly during PCR using digoxigenin-dUTP. Comparable amounts of total RNA were applied to the membrane as determined by both spectrophotometric quantification and visualization of ethidium-bromide-stained agarose gels. Also, a parallel blot was run as a control to detect *ldh*-specific mRNA using *ldh* primers 5' TAAGGAGATG-TTTAG 3', 5' GGAATTACTACAGTG 3' with p10-5 (Hillman *et al.*, 1990) as the template.

Acid sensitivity and adaptation to acid in AS17. Preliminary studies involved comparing the abilities of the acid-sensitive mutant AS17 and the parent strain JH1005 to grow on solid (2% agar) THYE media at pH 7.5–4.0. The effects of pH on the growth of AS17 were evaluated in liquid THYE media at pH 7.5 and pH 5.0. JH1005 and AS17 were grown to mid-exponential phase (Klett value = 100) in THYE broth at pH 7.5. Cultures were subcultured 1:10 (v/v) into fresh medium

at pH 7.5 and pH 5.0 and their growth was recorded using a Klett colorimeter (Klett–Summerson).

The mutant AS17 was tested for its ability to activate an exponential phase acid tolerance response (ATR) by determining whether a pre-conditioning period at a sublethal acid pH resulted in survivors following exposure to a pH found to kill non-conditioned control cells maintained at pH 7.5. The killing pH was established by inoculating tubes containing fresh medium buffered at pH values between 7.5 and 3.0 with exponential-phase cells (~10⁶ cells) grown anaerobically in TYPC at pH 7.5. The culture tubes were incubated for 3 h in an anaerobic chamber and aliquots were removed, diluted and quantified by spiral plating (Spiral Systems) on blood agar as described previously (Svensater *et al.*, 1997). Surviving c.f.u. were counted after 48 h and compared to counts obtained with the original suspension. For the adaptation-to-acid experiment, cells were grown to exponential phase in TYPC at pH 7.5 and harvested by centrifugation. Aliquots were transferred to the same medium buffered at pH values from 6.0 to 3.0 and incubated for 2 h at 37 °C. Following the 2 h adaptation period, all of the cultures were rapidly acidified to the appropriate 'killing pH' with HCl and incubation was continued for 3 h. The number of survivors was determined by plating dilutions of the acidified cultures on blood agar. Cell recovery was calculated by comparison with control cells incubated at pH 7.5 during the 2 h adaptation period. Adaptation was considered valid only when the pH 7.5 control sample was devoid of survivors.

Preparation of acid-shocked *S. mutans* membrane fractions and assay for H⁺/ATPase activity. Fifty millilitre 16 h cultures of *S. mutans* strains JH1005 and AS17 were grown in TYPC-pH 7.5 medium and used to inoculate 1 l pre-warmed TYPC-pH 7.5 medium. The cultures were grown to mid-exponential phase at 37 °C for 5–6 h (OD₆₀₀ 0.3–0.4). Bacterial cells were harvested by centrifugation for 10 min at 10000 g at room temperature and suspended in 500 ml TYPC medium at pH 5.0 (acid shock) or pH 7.5. The cultures were incubated at 37 °C for 2 h, placed on ice for 20 min, then the cells were harvested by centrifugation for 10 min at 10000 g, washed twice in ice-cold 50 mM Tris/maleate buffer (pH 6.0) containing 20 mM MgCl₂ and 0.1 mM PMSF (TM buffer) and frozen at –70 °C. Membrane fractions were prepared using a slight modification of the alumina grinding method of Vadeboncoeur *et al.* (1991). Frozen cells were ground for 20 min in a –20 °C mortar with alumina [2 g alumina (g wet cells)⁻¹] and 1–2 ml TM buffer. The mixture was centrifuged for 3 min at 4000 g to remove the alumina and the supernatant was centrifuged at 16000 g for 20 min to remove intact cells and cell debris. The supernatant was centrifuged at 100000 g for 18 h and the pelleted membranes were rinsed briefly in TM buffer and resuspended in 0.5 ml TM buffer. Protein concentrations were determined using the bicinchoninic acid protein assay (Sigma) with BSA as the standard.

H⁺/ATPase activity was assayed in membrane fractions by measuring the release of inorganic phosphate (P_i) from ATP by the method of Bender *et al.* (1986). Reaction mixtures contained (final concentrations): 50 mM Tris/maleate (pH 6.0), 20 mM MgSO₄, 5 mM ATP and 50 µg membrane protein in a final volume of 0.5 ml. After incubation at 37 °C for 5 min, the reaction was stopped by the addition of 25 µl 0.2 M HCl and placing the samples on ice for 10 min. Samples were centrifuged at 15000 g in a bench-top microfuge for 10 min. The amount of P_i released was measured in 400 µl of the reaction mixture by a modification (Weisman & Pillegi, 1974) of the Fiske–Subbarow method using an inorganic phosphorous determination kit (Sigma). Negative controls and

background samples included reaction mixtures devoid of membrane protein or ATP. The reported activities were mean values obtained from duplicate assays of three independent membrane preparations.

RESULTS

Cloning and sequence analysis of the *sat* locus (*ylxM*–*ffh*)

In a previous study (Gutierrez *et al.*, 1996), we reported the cloning of a 9.0 kb *Eco*RI chromosomal fragment in plasmid pUC18 from the acid-sensitive transposon mutant AS17. This plasmid, pUC-AS17 (Fig. 1), contained the *sat-1::Tn917* element and adjacent streptococcal DNA. Sequence analyses revealed that the insertion occurred in the intergenic region between the *ylxM* and *ffh* genes, which in *B. subtilis* form an operon (Honda *et al.*, 1993). Comparisons with sequences deposited in GenBank indicated that clone pUC-AS17 contained about one-third of the 5' end of the *ffh* ORF at the *erm*-proximal end of Tn917, and, at the *erm*-distal end of the transposon, the complete *ylxM* gene. Upstream from *ylxM*, sequences were found that were homologous to the *proU/opuA* operon of *B. subtilis* (Kempf & Bremer, 1995), specifically to the 3' half of the *proV/opuAA* gene and to unlinked segments of the complete downstream *proW/opuAB* and *proX/opuAC* genes. The *proU/opuA* operon encodes an osmo-regulated ABC-dependent transport system involved in the uptake of compatible solutes including glycine betaine.

To clone the intact *sat* locus, we transformed an *E. coli* *ffh* conditional mutant, WAM113, with a JH1005 genomic library in the low-copy-number vector pVA891 and selected for Em^r Cm^r clones able to grow under non-permissive conditions (i.e. in the absence of the inducer L-arabinose). Out of a pool of approximately 2000 Em^r and Cm^r transformants isolated on media supplemented with L-arabinose, we identified two clones able to grow on LB without arabinose. An approximately 16 kb plasmid, pJG301 (Fig. 1), was isolated from one of the arabinose-independent WAM113 derivatives for further characterization. Reintroduction of pJG301 into WAM113 was found to complement the *ffh* lesion in 100% of the screened Cm^r and Em^r clones.

Restriction enzyme analysis of pJG301 and pUC-AS17 indicated that the two clones shared common fragments. Successful sequencing of pJG301 using *ffh*-specific primers confirmed that we had indeed cloned the intact *sat* (*ylxM*–*ffh*) locus. Both strands of a 3000 bp fragment including the *ylxM*–*ffh* genes were sequenced (accession no. U88582). We also found a partial ORF 5' proximal to the *ylxM* gene with a predicted amino acid sequence that displayed similarity (61%) to a segment (residues 60–181) of *B. subtilis* OpuAC (Kempf & Bremer, 1995), the membrane-bound, substrate-binding protein of an ABC-type permease. There appeared to be a transcriptional terminator at the end of the putative *S. mutans* *opuAC* and before the 5' end of *ylxM*. Similar inverted repeats were also found downstream from *ffh*

which could serve as rho-independent terminators. Also, several putative –35 and –10 sequences were found upstream from the *ylxM* gene but not in the *ylxM*–*ffh* intergenic region. This suggests that *ylxM* and *ffh* constitute an operon similar to that of *B. subtilis* (Honda *et al.*, 1993). The insertion site of Tn917 was at bp 934 of the sequence and appears to be in the intergenic region between the *ylxM* and *ffh* genes of the *sat* locus.

In *B. subtilis* and *Mycoplasma pneumoniae*, *ylxM* encodes a 13.2 kDa protein of unknown function which appears to exist in operons encoding gene products involved in protein secretion (Honda *et al.*, 1993; Himmelreich *et al.*, 1996). The predicted amino acid sequences of YlxM from *B. subtilis* and *M. pneumoniae* share extensive similarity (76 and 54%, respectively) with that from *S. mutans*.

The deduced amino acid sequence for *S. mutans* *ffh* predicts a polypeptide of approximately 57 kDa, larger than the *B. subtilis* (49 kDa) and *E. coli* (50 kDa) homologues and similar to that of *Saccharomyces cerevisiae* (60 kDa). Extensive similarity was revealed between the predicted amino acid sequences of Ffh from *S. mutans* and *B. subtilis* (76%), *E. coli* (68%) and *Sacc. cerevisiae* (59%). A high degree of homology also exists around the predicted functional domains of the Ffh of *S. mutans*: the G domain encompassing the N-terminal region (amino acid residues 1 through 296) which contains three putative GTP-binding sites (amino acid residues 108–115, 191–194 and 249–252) known to be essential in GTPase activity and Ffh function *in vivo* in *E. coli* (Samuelsson *et al.*, 1995) via interaction with the Ffh receptor FtsY (Miller *et al.*, 1994); and the alpha-helical M domain located at the C-terminal region (amino acid residues 297–516) which, in *E. coli*, binds the small RNA subunit and the signal sequence of the transported proteins (Jensen *et al.*, 1994).

Plasmid pPC300, the pDL278 (Sp^r) derivative containing the PCR-derived *sat* locus, was also able to complement the *E. coli* *ffh* mutant WAM113. Exhaustive attempts were made using both electroporation and natural transformation protocols to transform the *S. mutans* *ffh* mutant AS17 with pPC300, but without success. Backcrosses into the transformable strain NG8 using DNA from mutant strain AS17 yielded Em^r transformants with a frequency of co-transfer of the acid-sensitive phenotype of 100% (Gutierrez *et al.*, 1996) confirming the role of the *sat* locus in acid tolerance. One such convertant, AS17N, also was used in transformation experiments with pPC300, as well as the parent plasmid pDL278, again without success.

The *sat-1::Tn917* insertion in AS17 results in reduced *ffh* mRNA levels

It was not apparent why the *sat-1::Tn917* insertion was not lethal in AS17 since *ffh* is an essential gene in *B. subtilis*, *E. coli* and *Schizosaccharomyces pombe* (Phillips & Silhavy, 1992; Honda *et al.*, 1993; Walter & Johnson, 1994). To study the effect of the Tn917 insertion on *ffh* gene expression, we compared the levels

Fig. 1. Genetic structure of the *sat* (*ylxM-ffh*) locus of *S. mutans*. pUC-AS17 contains the Tn917 (5.2 kb) insertion of AS17 (*sat::Tn917*) in a 9.2 kb *EcoRI* fragment. pJG301 (*Em^r*) contains the intact *sat⁺* locus as a 14.5 kb *SauIII*A chromosomal fragment in pVA891(*Em^r*) and pPC300 (*Sp^r*) contains the intact *sat⁺* locus as a 2.5 kb PCR-amplified fragment in pDL278 (*Sp^r*).

Fig. 2. (a) Levels of *ffh* expression in JH1005 (parent) and AS17 (*sat-1::Tn917*) grown at pH 7.5 as determined by RNA dot blotting. (b) Levels of *ffh* expression in JH1005 acid-shocked at pH 5 or maintained at pH 7.5. (c) Levels of *ldh* expression in JH1005 and AS17 at pH 7.5 and JH1005 at pH 5.0. Two-fold serial dilutions of total RNA, starting with 10 µg for (a) and (b) and 2.5 µg for (c), were applied to the membranes and probed as described in Methods.

of *ffh* mRNA in JH1005 and AS17 by dot blot analysis. Approximately eightfold lower levels of *ffh* were detected in mutant AS17 than in the parent grown at pH 7.5 (Fig. 2a). Similar results were obtained with RNA samples isolated from three separate cultures. In this experiment, JH1005 and AS17 were grown in the absence of *Em*. To eliminate the possibility that the residual *ffh* message detected in the mutant samples had originated from a population of cells that had lost the transposon in the absence of antibiotic selective pressure, the level of *ffh* message was compared between cultures of AS17 grown in both the presence and absence of erythromycin. No difference in levels of *ffh* message was observed (not shown). Further, in order to assess *ffh* message levels in AS17 compared with the parent strain JH1005, which does not contain the transposon and could not be grown in the presence of erythromycin, RNA also was analysed from strain AX1 (Cvitkovitch *et al.*, 1997). This is an *icd::Tn917* mutant that survives

the selective agent, and whose *ffh* levels would be unaffected by Tn917. Levels of *ffh* message in AX1 were similar to JH1005 while AS17 again showed an approximate four- to eightfold decrease (not shown). We have shown here that *ldh* is expressed at similar levels in pH 7.5 and 5.0-grown JH1005 cells (Fig. 2c) and so have probed blots with labelled *ldh* DNA as an internal control to ensure that equivalent amounts of RNA isolated from both AS17 and JH1005 pH 7.5-grown cells had been applied to the membranes. Nearly identical reactivity with the *ldh* probe was observed for both strains. Taken together, these data indicate that the Tn917 insertion in the *sat* locus of AS17 decreases but does not eliminate *ffh* mRNA expression.

ffh is induced under acidic shock

The levels of *ffh* mRNA in JH1005 and AS17 expressed from cells grown and maintained at pH 7.5 and cells

Fig. 3. Acid sensitivity of AS17 (*sat-1::Tn917*; right half of plates) compared to JH1005 (parent; left half of plates) in THYE agar at (a) pH 7.5, (b) pH 5.0, (c) pH 4.8 and (d) pH 4.7.

shocked at pH 5 were also analysed by RNA dot blotting as described above. Acid-shocked JH1005 cells exhibited an approximate four- to eightfold increase in *ffh* expression compared to the non-shocked (pH 7.5) cells (Fig. 2b). There was no observable difference in RNA levels between shocked and non-shocked AS17 cells (not shown).

Effect of acid pH on the growth and survival of AS17

To characterize the acid-sensitive phenotype of AS17 in more detail, we analysed the ability of AS17 to grow on solid THYE buffered at pH values ranging from pH 7.0 to pH 4.7 at 37 °C for 48 h. We observed that compared to the parent JH1005, AS17 showed increased sensitivity to acid (Fig. 3b–d). Whereas JH1005 could grow on solid medium at pH 4.7 (Fig. 3d), the mutant grew only poorly at pH 4.8 (Fig. 3c) and not at all at pH 4.7 (Fig. 3d). The revertant AS17N generated by genetic back-cross into *S. mutans* strain NG8 also showed a similar impairment of growth at low pH relative to the parental strain (not shown). We also studied the acid-sensitive phenotype of AS17 in liquid medium by transferring cells previously grown at pH 7.5 to medium at pH 5.0. We observed a substantial reduction of the growth rate of the mutant as compared to the parent strain (Fig. 4; generation times of 6.3 h and 4.3 h, respectively). In addition, there was a lower relative yield of mutant cells grown at pH 5 (final Klett value of 95) as compared to the parent (final Klett value of 140). Because this difference was not observed when the strains were cultured at pH 7.5 (Fig. 4; generation times of 1.6 h and 1.3 h, respectively) the change in growth rate between the mutant and parent appears to be specific for acidic growth conditions.

Fig. 4. Acid sensitivity of AS17 (*sat-1::Tn917*) compared to JH1005 (parent) in THYE broth after acid shock at pH 5.0. □, JH1005-pH 7.5; ◇, JH1005-pH 5; ○, AS17-pH 7.5; △, AS17-pH 5.

Strains of *S. mutans* are known to show enhanced acid tolerance after growth in acidic environments at pH values approaching 5.0 (Belli & Marquis, 1991; Bender *et al.*, 1986). As a consequence, we were interested in determining whether the parent strain JH1005 also exhibited an ATR and, if so, whether the acid-sensitive mutant AS17 had lost its ability to adapt to a low external pH. The adaptation test is based on the principle that prior exposure of cells to low pH conditions them to increased survival at a killing pH compared to non-conditioned cells maintained at pH 7.5

Fig. 5. Survival and adaptation of AS17 (*sat-1::Tn917*) compared to JH1005 (parent) and LT11 (wild-type control) in buffered media at acidic pH values. (a) Determination of the killing pH. JH1005 (□) and AS17 (■) were grown in TYEPC at pH 7.5. (b) Adaptation to acid tolerance of LT11 (□), JH1005 (■) and AS17 (*). Recovery was calculated from parallel incubations of cells at pH 7.5 during the 2 h adaptation period.

(Svensater *et al.*, 1997). As seen in Fig. 5(a), the parent strain JH1005 was present in greater numbers at pH values 6.0–5.0 compared to cells maintained at pH 7.5. The percentage of parent strain survivors declined when cells were incubated at pH values below pH 5.0 with 100% killing occurring at pH 3.0. Unlike the parent, the mutant showed lower cell numbers when incubated at pH values lower than pH 7.5 with 100% killing occurring at pH 3.5.

Using this information, the strains were tested for adaptation to increased acid tolerance by inoculating pH 7.5-grown exponential-phase cells into fresh TYPC medium buffered at pH values above the killing pH and incubating the cells for 2 h at 37 °C to permit adaptation. This was followed by a 3 h exposure at the killing pH before the cells were plated on blood agar to detect survivors. As a control, *S. mutans* LT11, an organism also killed at pH 3.0 and known to exhibit an ATR (Svensater *et al.*, 1997), was included in the experiment.

Table 1. H⁺/ATPase activities in membrane fractions of normally grown and acid-shocked *S. mutans* parent strain JH1005 and acid-sensitive mutant AS17

<i>S. mutans</i> strain	pH shock condition	Mean enzyme activity*
JH1005	7.5	135 ± 4
JH1005	5.0	224 ± 24†
AS17	7.5	135 ± 18
AS17	5.0	106 ± 16

* One unit of activity was defined as 1 nmol P_i liberated min⁻¹ (mg protein)⁻¹ ± SE.

† Significantly greater activity than in JH1005 at pH 7.5 (*P* < 0.05).

As seen in Fig. 5(b), a 2 h exposure of cells of *S. mutans* JH1005 and LT11 to pH 5.5 and 5.0 induced a system that enhanced cell survival during a subsequent 3 h exposure to the killing pH of 3.0. Survivors were not detected in the control culture incubated at pH 7.5, or those incubated at pH 6.0 or at values below 5.0. Contrary to these results, survivors were not observed in a similar experiment carried out with the mutant AS17 at its own killing pH of 3.5. This indicates that an external pH of 5.5–5.0 provided a stimulus for the induction of an ATR in the parent strain JH1005, but not in the mutant. This suggests involvement of the *sat* locus in the adaptation process.

Mutant AS17 exhibits a defect in the H⁺/ATPase activity at acidic pH values

Because acidurance is dependent on the membrane-embedded H⁺/ATPase (Kobayashi *et al.*, 1986; Sutton & Marquis, 1986) and since Ffh is involved in the localization of membrane proteins (Degier *et al.*, 1997; Ulbrandt *et al.*, 1997), we assayed for H⁺/ATPase activity in mutant membranes, particularly under acidic growth conditions. The results of the H⁺/ATPase assays performed on membrane fractions of *S. mutans* parent strain JH1005 and acid-sensitive mutant AS17 are shown in Table 1. While we found no apparent differences between the specific activities measured in membranes isolated from cells grown and maintained at pH 7.5, the specific activities detected in membrane fractions prepared from pH 5.0 acid-shocked wild-type cells were significantly greater (*P* < 0.05) than mutant cells. As has been previously reported for other wild-type *S. mutans* strains (Hamilton & Buckley, 1991), we found the activities in JH1005 to increase nearly twofold in response to the pH 5.0 acid shock. On the other hand, AS17 activities remained essentially unchanged and were twofold lower than parental, pH 5.0 membranes. These results suggest that Ffh may be involved in the assembly of a functional H⁺/ATPase particularly under acid shock, conditions that would require increased amounts of H⁺/ATPase to maintain pH regulation.

Effects of the *sat::Tn917* mutation on sugar fermentation of AS17

To assess the effects of reduced Ffh expression on the ability to ferment sugars possibly due to impaired function of sugar transport permeases, we tested for differences in the fermentation profiles of mutant and parent strains grown anaerobically on TYEBP agar with 1.0% glucose, galactose, lactose, mannitol and sorbitol. Mutant AS17 was unable to ferment sorbitol, while fermentations of all other sugars tested remained unaffected when compared to the parent strain (not shown). Growth in liquid media confirmed that AS17 cannot utilize sorbitol as a carbon source.

DISCUSSION

Prokaryotes and eukaryotes employ two main routes for protein translocation into or across biological membranes: the general secretory pathway (GSP), which is dependent on the Sec proteins (SecB/A/Y/E/D/G), and the SRP or Ffh-dependent pathway (Bernstein *et al.*, 1989; Akimaru *et al.*, 1991; Hann & Walter, 1991; Lührink *et al.*, 1992; Phillips & Silhavy, 1992; Economou & Wickner, 1994; Lührink & Dobberstein, 1994; Walter & Johnson, 1994; Lutke, 1995; Nishiyama *et al.*, 1996). The SRP-like complexes of *E. coli* and *B. subtilis* are ribonucleoproteins consisting of the nascent polypeptide, Ffh (a GTP-binding protein), and a small cytoplasmic RNA molecule (scRNA in *B. subtilis* and 4-5S RNA in *E. coli*) (Phillips & Silhavy, 1992; Honda *et al.*, 1993; Lührink & Dobberstein, 1994; Nakamura *et al.*, 1994; Hauser *et al.*, 1995; Lutke, 1995). Ffh is a bifunctional protein with two domains: the G domain (located at the N-terminus) which binds GTP and an alpha-helical M domain (located at the C-terminus) which binds the RNA subunit and signal sequence of the protein being translocated (Lührink & Dobberstein, 1994; Lührink *et al.*, 1994; Miller *et al.*, 1994; Bunai *et al.*, 1996). It has been proposed that the Ffh-RNA complex functions as a chaperonin specific for certain nascent pre-proteins (Ribes *et al.*, 1990; Phillips & Silhavy, 1992; Lührink & Dobberstein, 1994; Lutke, 1995; Ng *et al.*, 1996). Recent studies suggest that there is little overlap in protein substrates translocated via the GSP and SRP pathways, although in some cases non-cytosolic proteins can be targeted by either route (Macfarlane & Muller, 1995; Chen *et al.*, 1996; Ng *et al.*, 1996; Degier *et al.*, 1997). Experimental evidence suggests that translocation of proteins whose expression is constitutive occurs via the GSP, while proteins expressed via regulated genes could utilize the more discriminatory and efficient Ffh-dependent route to allow for rapid responses to environmental changes (Lutke, 1995; Chen *et al.*, 1996).

In other microbial systems, *ffh* is a gene essential for viability (Phillips & Silhavy, 1992; Honda *et al.*, 1993; Walter & Johnson, 1994). *In vivo* studies in *E. coli* and *B. subtilis* show that depletion of either Ffh or the RNA component causes defects in protein secretion, changes in cell morphology and cell death (Jensen *et al.*, 1994;

Lührink *et al.*, 1992; Ng *et al.*, 1996; Shibata *et al.*, 1995). It is perhaps fortuitous then that insertion of the transposon occurred in the region between *ylxM* and *ffh* in AS17, the result of which was a viable organism, albeit one with decreased tolerance to acid. Preliminary primer extension data suggest that only one promoter, upstream of *ylxM*, drives the expression of both *ylxM* and *ffh* in the JH1005 parental cells (Gutierrez *et al.*, 1997), suggesting that these genes form an operon in *S. mutans*, as has been reported for *B. subtilis* (Honda *et al.*, 1993). Since the transposon insertion in mutant AS17 is in the intergenic region between *ylxM* and *ffh* and causes a reduction, rather than total elimination of *ffh* gene expression, this suggests the presence of promoter-like sequences upstream from the *ffh* translational start site that are responsible for the observed low-level *ffh* transcription. Confirmation that *ffh* of *S. mutans* is an essential gene will require the construction of an *ffh* conditional mutant similar to the *E. coli* strain WAM113.

The association of *ffh* with acid sensitivity was demonstrated by backcross experiments in which the mutated *sat* locus was introduced into wild-type *S. mutans*. Complementation of mutant AS17 with the wild-type locus was attempted but not achieved. The inability to transform the acid-sensitive mutant AS17 with plasmid pPC300 containing wild-type *ffh* was an interesting observation. This plasmid was constructed in an attempt to reintroduce *ffh* into the mutant to restore the parental phenotype. This experiment would have solidified the linkage of *ffh* to the observed phenotypic properties of AS17. Plasmid pPC300 was able to complement the *ffh* mutation in the *E. coli* *ffh* mutant WAM113; however, no transformants of *S. mutans* strains AS17 and AS17N could be isolated despite exhaustive attempts. It is attractive to speculate that these strains are no longer genetically competent due to a lowered level of a membrane-associated component involved in transformation that is dependent on full *ffh* function. Indeed, we were not able to effect marker rescue of the *sat::Tn917* locus from AS17 by using our vector pTV21Δ2TetM and resorted to conventional gene cloning (Gutierrez *et al.*, 1996). AS17 was the only one of our several characterized transposon mutants that was not amenable to this transformation-mediated approach.

Another interesting finding from this study was that AS17 was unable to ferment sorbitol, a defining physiological characteristic of *S. mutans*. It is likely that integration of the EII_{sorb}, an integral membrane protein of the phosphotransferase system required for sorbitol transport, or other membrane proteins needed for transport or metabolism of this sugar alcohol is affected in the mutant. Why galactose (non-phosphotransferase system sugar), lactose and mannose (phosphotransferase system sugars) fermentations are not affected is unknown. The assembly mechanism obviously is not universal for sugar permeases, but is likely specific to sorbitol transport/metabolism. A protein specific to sorbitol metabolism and one which may be affected by

impaired Ffh expression is sorbitol-6-phosphate dehydrogenase (Carlsson, 1986).

We investigated the possibility that expression of the *sat* operon is subjected to regulation by environmental signals. Our data showed elevated *ffh*-specific mRNA levels in the parental cells grown in batch culture at pH 5.0 as compared with pH 7.5-grown cells. Analysis of mutant cells revealed detectable but lower levels of *ffh* mRNA at each pH when compared to the parent (see Fig. 2). Also, data presented in this paper demonstrate that the mutant is unable to respond to an environmental signal (growth at pH 5.0–5.5) for adaptation to acid shock, a condition that may lead to cell death. A normal response to such a signal could involve the accelerated translation and translocation of H⁺/ATPase subunits into the cytoplasmic membrane of an adapting cell. It is tempting to speculate that the Ffh-deficient mutant AS17 may be unable to respond to this signal as a result of its inability to translocate a sufficient amount of the membrane-bound H⁺/ATPase necessary for extrusion of protons. In this regard, Ffh could be an integral player in the ATR of *S. mutans* since the ATR or any stress response involves the transcription, translation and functional activity of a specific subset of stress proteins. That is to say, if components are made but do not function due to inefficient localization in the cell membrane, the response is therefore not elicited (Rall *et al.*, 1996). The existence of other *sat*-specific environmental signals, such as nutrient availability or osmolarity, is not yet known. Future research in our laboratories will include the construction of a *sat* (*ylxM*)–*lacZ* heterodiploid fusion strain in *S. mutans* to study *ffh* regulation under a variety of growth conditions. Increased expression of Ffh would be expected to enable rapid alterations in membrane composition required to adjust to environmental stress which may not be possible via the more time-consuming GSP.

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