Streptococcal viability and diminished stress tolerance in mutants lacking the signal recognition particle pathway or YidC2

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The signal recognition particle (SRP)-translocation pathway is conserved in all three domains of life and delivers membrane and secretory proteins to the cytoplasmic membrane or endoplasmic reticulum. We determined the requirement in the cariogenic oral pathogen *Streptococcus mutans* of the three universally conserved elements of the SRP pathway: Ffh/SRP54, scRNA, and FtsY/SR α . Previously, we reported that insertional interruption of *S. mutans* *ffh* was not lethal, but resulted in acid sensitivity. To test whether *S. mutans* could survive extensive disruption of the SRP pathway, single and double deletions of genes encoding Ffh, scRNA, and FtsY were generated. Without environmental stressors, all mutant strains were viable, but unlike the wild-type, none could initiate growth at pH 5.0 or in 3.5% NaCl. Survival of challenge with 0.3 mM H₂O₂ was also diminished without *ffh*. Members of the YidC/Oxa1/Alb3 family are also ubiquitous, involved in the translocation and assembly of membrane proteins, and have been identified in prokaryotes/mitochondria/chloroplasts. Two genes encoding YidC homologs, YidC1 and YidC2, are present in streptococcal genomes with both expressed in *S. mutans*. Deletion of YidC1 demonstrated no obvious phenotype. Elimination of YidC2 resulted in a stress-sensitive phenotype similar to SRP pathway mutants. Mutants lacking both YidC2 and SRP components were severely impaired and barely able to grow, even in the absence of environmental stress. Here, we report the dispensability of the cotranslational SRP protein translocation system in a bacterium. In *S. mutans*, this pathway contributes to protection against rapid environmental challenge and may overlap functionally with YidC2.

protein translocation | streptococcus | Ffh | FtsY | membrane biogenesis

The signal recognition particle (SRP) is a highly conserved ribonucleotide–protein complex involved in cotranslational membrane targeting of signal-peptide-bearing proteins (1). The SRP pathway is conserved in all domains of life and has been considered crucial for the vitality of all organisms (2–6). *Saccharomyces cerevisiae* represents the only known example of an organism able to endure mutations within the SRP pathway (7). Mutants are exceedingly sick and adapt physiologically by repression of protein synthesis, induction of heat shock genes, and dramatic slow-down of growth (8). Comparative phylogenetic analyses demonstrate that genomes of Eukaryotes, Archaea, and Bacteria encode at a minimum the SRP components SRP54 protein, known in Eubacteria as Ffh (54-kDa homolog), and a 4.5 or 7S small cytoplasmic RNA (scRNA) (5). The third universally conserved element of the pathway is the SRP receptor subunit (SR α), or in *Escherichia coli*, FtsY (9).

The bacterial SRP translocation pathway and protein secretion has been studied in *E. coli* and *Bacillus subtilis*; however, little is known in other bacteria, including the streptococci. As the major etiological agent of human dental caries (reviewed in ref. 10), *Streptococcus mutans* is well studied at the genetic and physiologic level. Major factors contributing to pathogenicity include acidogenicity and aciduricity (reviewed in ref. 11). The

unique biological nature of *S. mutans* allows it to ferment a wide range of dietary carbohydrates and, within minutes after sugar consumption, lower the surrounding plaque pH to below pH 4.0. This rapid acidification is followed by expression of numerous proteins, including chaperones and membrane proteins, in a process necessary for cellular viability referred to as the acid-tolerance response (12, 13). Transposon mutagenesis in *S. mutans* resulted in several acid-sensitive mutants (14) and one mutation mapped to *ffh* within the *sat* (secretion and acid tolerance) operon (15). Surprisingly, interruption of *ffh* was not lethal (16), in contrast to what was reported for *E. coli* and *B. subtilis* (3, 17), but did render *S. mutans* sensitive to acidic pH.

In Eubacteria, the SRP is composed of Ffh and scRNA. This ribonucleotide–protein complex delivers incompletely translated substrates to the membrane-associated receptor protein, FtsY, for completion of translation and translocation into or through the membrane (4). The targeting path is determined as the nascent peptide chain emerges from the ribosome (18). The SRP specifically recognizes and binds to highly hydrophobic signal peptide sequences on the ribosome (19), and then the SRP/ribosome complex targets to the receptor FtsY (9, 20) via a high-affinity GTP-dependent interaction. The complex is then transferred to the translocon resulting in release of the SRP from the ribosome–nascent chain complex. Translation resumes and the polypeptide is directed through the translocon. GTP hydrolysis drives dissociation of the SRP from its receptor allowing it to initiate a new round of targeting (21). The majority of integral membrane proteins are channeled via a translocon comprised of two heterotrimeric complexes, SecYEG and SecDFYajC (reviewed in ref. 22).

Members of the YidC/Oxa1/Alb3 family have been identified in prokaryotes, mitochondria, and chloroplasts (23). These proteins are believed to serve as membrane-localized chaperones involved in translocation and assembly of a large number of membrane proteins (23, 24) and function in concert with the Sec translocon (23, 25, 26), as well as independently (27, 28). Recently, Oxa2 was identified in mitochondria of *Neurospora crassa* and functionally complemented the related yeast protein Cox18 (29). Domain swapping and complementation experiments using engineered variants of *E. coli* YidC suggest that the ribosome binding function associated with a C-terminal α -helical coiled-coil tail present in mitochondrial Oxa1 can be transferred, but appending this tail to YidC destroys its ability to complement Oxa2/Cox18 (30). Studies suggest that Oxa2/Cox18/YidC act primarily on completely synthesized substrates, whereas Oxa1 primarily mediates cotranslational translocation

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Abbreviations: SRP, signal recognition particle; scRNA, small cytoplasmic RNA; SR, SRP receptor; Td, doubling time.

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In light of the phylogenetically ubiquitous conservation of Ffh, scRNA, and FtsY and the survival of *S. mutans* in the absence of a complete Ffh protein, this study was undertaken to evaluate the essentiality of the *S. mutans* SRP pathway. The contributions of the *S. mutans* YidC homologs to viability and resistance to environmental stress were also evaluated singly and in combination with SRP genes. We provide evidence that, in *S. mutans*, the SRP pathway is uniquely nonessential under nonstress conditions, and clearly enables the organism to contend with rapid environmental challenges. Elimination of *yidC2*, but not *yidC1*, also resulted in a stress-sensitive phenotype and simultaneous disruption of the SRP pathway and YidC2 rendered *S. mutans* severely impaired and barely able to grow even under nonstress conditions. Our results suggest the involvement of SRP-cotranslational translocation in *S. mutans*' ability to maintain a membrane composition resistant to varying environmental stressors and that YidC2 may serve at least partially redundant functions with the SRP pathway.

Materials and Methods

Bacterial Strains and Growth Conditions. Bacterial strains are listed in Table 2, which is published as supporting information on the PNAS web site. Mutants were derived from *S. mutans* NG8 (33). Mutations of *ffh* (SMU.1060), *ftsY* (SMU.744), or scRNA (base pairs 293148–293220 of the genomic sequence; GenBank accession no. AE014133) in *S. mutans* UA159 had identical phenotypes to those in NG8 (not shown). *S. mutans* was grown in Todd–Hewitt broth (BBL, Becton Dickinson Microbiology System, Cockeysville, MD) supplemented with 0.3% (wt/vol) yeast extract (THYE) at 37°C with 5% CO₂. Kanamycin (500 µg/ml plates or 100 µg/ml broth) and erythromycin (10 µg/ml) were used as antibiotics. *E. coli* was grown in LB broth supplemented with 300 µg/ml erythromycin when appropriate.

DNA Methods. Standard methods were used to isolate and manipulate plasmid DNA. *S. mutans* genomic DNA was isolated as described (14). Cloning, restriction endonuclease digestions, and DNA ligations were carried out according to standard protocols. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. PCRs used iQ Supermix (Bio-Rad).

Construction of Mutant Strains. Nonpolar deletion mutants were constructed by PCR ligation mutagenesis (34). Genes were replaced with an antibiotic marker cassette [kan (16) or erm (35)] without introducing transcriptional stops or disrupting the translational reading frame. Sequences flanking the replaced genes were amplified by using *S. mutans* genomic DNA as template and appropriate primers (Table 3, which is published as supporting information on the PNAS web site). PCR-amplified fragments were digested via restriction sites engineered into the primers and ligated to the marker cassette. Ligated DNA fragments were gel-purified (Qiagen, Valencia, CA), served as templates for a second round of PCR amplification and the products used to transform *S. mutans* (36). Allelic replacement mutants were selected by antibiotic resistance and confirmed by PCR (Fig. 3, which is published as supporting information on the PNAS web site). Replaced genes and loci are shown schematically in Fig. 4, which is published as supporting information on the PNAS web site. For construction of double mutant strains $\Delta ftsY\Delta ffh$ and $\Delta scRNA\Delta ffh$, the kan^r-resistant single mutant strains AH307 ($\Delta ftsY$) or AH312 ($\Delta scRNA$) were

transformed with a linear DNA amplicon containing the *ffh* deletion construct harboring the erythromycin resistance cassette. Double mutants were selected on erythromycin-containing agar plates and verified by PCR (Fig. 3).

Construction of *yidC1* and *yidC2* mutant strains was carried out as described above for the SRP mutants with *Erm* used as the selection marker. For *yidC*/SRP-pathway mutant strains, $\Delta(ylxM\text{-}ffh\text{-}satC)::kan$ and $\Delta ftsY::kan$ fragments were PCR-amplified by using genomic DNA from AH316 or AH307 as templates. Resulting linear DNA fragments were used to transform AH374 and AH398. Double mutant strains were selected on THYE agar plates containing kanamycin and erythromycin and confirmed by PCR (Fig. 3).

Complementation of the *ftsY* Mutation. To complement the *ftsY* mutation, *ftsY* was PCR-amplified by using primers AH14F and AH1R (Table 3). Because *ftsY* is the fourth *orf* in a putative operon, genomic DNA from mutant strain AH308 $\Delta(orf1-orf2-orf3)$, which maintains the upstream promoter sequence, followed by the kan-resistance gene and *ftsY* structural gene, was used as the template. The PCR amplicon was ligated to the EcoRV site of the *Streptococcus-E. coli* shuttle vector pDCerm (37), and the resulting plasmid, (*erm*^r and kan^r), was introduced into the *ftsY* null mutant, AH307, and NG8 by natural transformation. Transformants were selected on erythromycin.

RNA Isolation and RT-PCR to Identify scRNA. Total RNA from 10-ml exponential phase cultures of *S. mutans*, lysed with the Mini Bead-Beater (Biospec Products), was isolated by using the RNeasy Mini kit (Qiagen). RNA was treated with RQ1 RNase-free DNase (Promega) and repurified through RNeasy columns.

RT-PCR was used to confirm the presence of scRNA in total RNA samples, using primer PC62R (Table 3) and SuperScript II RNase H⁻ Reverse Transcriptase (Invitrogen) for the reverse transcription reaction and primers PC62F and PC62R and iQ Supermix (Bio-Rad) for the PCR. Genomic DNA from strain NG8 and total RNA from strains NG8 and AH312 served as control templates for PCR.

Bacterial Growth Under Acid, Osmotic, and Oxidative Stress. *S. mutans* was grown for 48 h on THYE-agar (pH 7.0) with antibiotic at 37°C in air with 5% CO₂, then passaged into 10 ml of THYE (pH 7.0) and incubated overnight. Cultures were diluted 1:20 in THYE pH 7.0 without antibiotic and grown to O.D.₆₀₀ of 0.3–0.4. A 100-well Bioscreen C plate (Labsystems, Helsinki, Finland) was filled with 350 µl of prewarmed media (THYE pH 7.0 or 5.0; pH 7.0 with 3.5% NaCl; or pH 7.0 with 0.3 mM H₂O₂). Twenty-microliter culture volumes were added to the wells and grown for 16 h with absorbance recorded every 20 min. Wells containing media only served as background controls. Doubling time (Td) was calculated by measuring the slope of the logarithmic growth phase (38) and the formula: $Td = (t_2 - t_1) \ln(2) / (\ln(OD_2) - \ln(OD_1))$. Results represent an average of five replicates with standard deviation of the mean. Each experiment was performed two to four times. Statistical significance was determined by using Student's *t* test and a *P* value of <0.05.

Membrane Preparation and H⁺/ATPase Activity. Preparation of bacterial membranes and measurement of H⁺/ATPase enzymatic activity were performed as described (39). ATPase-specific activities of membrane fractions prepared from cultures grown at pH 7.0 or 5.0 (acid shock) of the wild-type and mutant strains were calculated and expressed as nmol of Pi released per min per mg of total protein. The assay was performed twice with duplicate samples in each assay. Statistical significance was determined by using Student's *t* test, *P* < 0.05.

Table 1. Summary of doubling times for *S. mutans* wild-type and SRP-pathway and *yidC*-mutant strains

Strain	Description	Growth conditions			
		Nonstress	pH 5.0	3.5% NaCl	0.3 mM H ₂ O ₂
NG8	wild-type	44.07 ± 0.74	79.11 ± 0.68*	93.28 ± 2.18*	50.17 ± 1.04
MK4	<i>ffh</i> ::kan	57.61 ± 1.85 [†]	0 ± 0* [†]	0 ± 0* [†]	62.91 ± 1.81 [†]
AH329	Δ <i>ffh</i>	66.10 ± 1.31 [†]	0 ± 0* [†]	0 ± 0* [†]	84.04 ± 1.39 ^{§¶}
AH316	Δ (<i>ylxM</i> - <i>ffh</i> - <i>satC</i>)	82.34 ± 6.08 [†]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	87.97 ± 1.94 [§]
AH307	Δ <i>ftsY</i>	63.06 ± 0.80 [†]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	70.86 ± 2.67 [‡]
AH308	Δ (<i>orf1orf2orf3</i>)	44.16 ± 0.26	79.27 ± 0.16 ^{¶¶}	94.58 ± 5.83*	47.89 ± 0.93
AH325	Δ (<i>orf1orf2orf3ftsY</i>)	62.16 ± 1.25 [†]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	65.11 ± 0.42 [‡]
AH351	Δ <i>ffh</i> Δ <i>ftsY</i>	63.84 ± 1.24 [†]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	76.01 ± 0.04 ^{§¶}
AH307(pDCerm-FtsY)	Δ <i>ftsY</i> +pDCerm- <i>ftsY</i>	46.08 ± 0.76	77.90 ± 0.91*	110.82 ± 3.10* [‡]	49.33 ± 0.29
AH312	Δ <i>scRNA</i>	72.91 ± 1.98 [†]	0 ± 0* [†]	0 ± 0* [†]	73.45 ± 0.91 [§]
AH341	Δ <i>ffh</i> Δ <i>scRNA</i>	76.57 ± 1.35 [†]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	88.10 ± 0.78 ^{§¶}
AH374	Δ <i>yidC1</i>	50.71 ± 0.40 [§]	99.25 ± 1.00 ^{¶¶}	116.43 ± 5.68*	53.49 ± 2.34
AH386	Δ <i>yidC1</i> Δ (<i>ylxM</i> - <i>ffh</i> - <i>satC</i>)	91.30 ± 1.43 [†]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	89.41 ± 0.05 [§]
AH390	Δ <i>yidC1</i> Δ <i>ftsY</i>	64.93 ± 1.65 [§]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	68.88 ± 0.86 [§]
AH394	Δ <i>yidC1</i> Δ <i>scRNA</i>	67.75 ± 0.82 [§]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	72.94 ± 0.37 ^{§¶}
AH398	Δ <i>yidC2</i>	89.86 ± 0.98 [†]	0 ± 0 ^{¶¶}	0 ± 0 ^{¶¶}	92.81 ± 0.08 [†]
AH402	Δ <i>yidC2</i> Δ (<i>ylxM</i> - <i>ffh</i> - <i>satC</i>)	ND	NT	NT	NT
AH405	Δ <i>yidC2</i> Δ <i>ftsY</i>	ND	NT	NT	NT

Results are expressed as the mean ± standard error (in minutes). Statistical significance compared to wild-type strain NG8 by using Student's *t* test is indicated as follows: †, *P* < 0.001; §, *P* < 0.01; ‡, *P* < 0.05. Statistical significance compared to nonstress conditions by using Student's *t* test is indicated as follows: ¶, *P* < 0.001; *, *P* < 0.01; ¶¶, *P* < 0.05. ND, not determined. Growth was only detectable after ≥48 h of culture in liquid media. NT, not tested.

Results and Discussion

Elimination of *ffh* Is Not Lethal. We previously identified *S. mutans* mutants unable to initiate growth at acidic pH (14). One transposon (Tn917)-insertion-generated mutation mapped to the *ylxM*-*ffh* intergenic region of the *sat* operon (15). Mutations of individual genes within the *sat* operon (Fig. 4) were generated by insertional inactivation and, with the exception of the acid-sensitive *ffh* mutant MK4, were indistinguishable from wild-type *S. mutans* (16). The *S. mutans* UA159 genomic sequence (40) demonstrates no *ffh* homologs outside of the *sat* operon. Because MK4 possessed DNA encoding the N-terminal 209 aa of Ffh, the question remained whether the truncated protein retained residual functional activity; therefore, a mutant strain was generated in which the entire *ffh* gene was deleted and growth was assessed (Table 1). The cellular growth yield of AH329 (Δ *ffh*) was similar to the wild-type under nonstress conditions (not shown), but the Td of the mutant was increased relative to the parent (*P* < 0.001). Importantly, strain AH329 demonstrated a similar phenotype as MK4, indicating that the N-terminal region of Ffh did not contribute to MK4's viability. The effect of disruption of *ffh* on tolerance to several environmental stressors, including acid, salt, and hydrogen peroxide, was evaluated in parallel for mutants MK4 and AH329. As expected, growth of both was severely impaired upon acid shock at pH 5.0 (Table 1). In addition, these mutant strains were unable to initiate growth when transferred into 3.5% salt-containing medium, a defining characteristic of *S. mutans*. Although addition of H₂O₂ to the culture medium did not have as pronounced an effect on the *ffh* mutants as did acid or salt stress, the Td of the complete *ffh* deletion mutant strain AH329 was significantly increased (*P* < 0.05) relative to the nonstress condition. There was no effect of high (42°C) or low temperature (30°C) on growth of either mutant strain (not shown). Taken together, the results indicate that complete removal of Ffh from *S. mutans* is not lethal, but does render the organism less able to contend with several environmental stressors. Interestingly, mutant MK4 was shown previously to adapt to acid stress and grow when pH was allowed to drop gradually from normal metabolism, indicating that given

time compensatory mechanisms are engaged upon environmental challenge in the absence of an intact SRP pathway (39).

Elimination of FtsY Is Similar to Elimination of Ffh. We hypothesized that elimination of FtsY, the "docking protein" for Ffh, would result in a similar phenotype as elimination of Ffh itself. A single copy of *ftsY* was identified in the *S. mutans* genome with the *B. subtilis* and *E. coli* *ftsY* genes as queries. In *S. mutans*, *ftsY* appears to be part of a putative four-gene operon, where it is the most downstream gene (Fig. 4b). Analysis of the deduced amino acid sequences of *orfs1–3* suggests that the first protein (276 aa) is a conserved hypothetical protein, whereas the second and third (273 aa and 494 aa) have significant levels of sequence similarity (47–81%) to conserved hypothetical proteins in the haloacid dehydrogenase-like family. *S. mutans* FtsY demonstrates high homology (44% identity, 65% similarity) with *E. coli* FtsY that consists of two distinct domains; the highly conserved NG domain containing the GTP binding site and the N-terminal acidic (A) domain (41). The A domain has been reported as essential for proper targeting of membrane proteins (42); however, more recent *in vivo* analyses showed that, in *E. coli*, the N-terminal A-domain was not required for SRP-mediated protein targeting and membrane assembly (43). In contrast, in the halophilic Archaea *Haloferax volcanii*, the full-length FtsY is essential for the association of Ffh protein with the cell membrane (44). Therefore, the mode of action and requirement of the A domain appears to differ among species.

Similar to the Δ *ffh* mutant, the viability of the *S. mutans* Δ *ftsY* mutant AH307 was in contrast to what is found in *E. coli* and *B. subtilis* (45, 46). The Td of strain AH307 was increased compared to the wild-type NG8 (*P* < 0.001), and a longer lag phase (not shown) was observed when AH307 was cultured without stress. As with the *ffh* mutant strains, AH307 failed to grow when transferred to pH 5.0, or to 3.5% NaCl in either liquid (Table 1) or solid (data not shown) media. The Td of AH307 was not significantly different in the presence of 0.3 mM H₂O₂ in contrast to that of the *ffh* mutants, and temperature had no effect on cell growth (data not shown). To establish further the dispensability of the more complete SRP pathway, the double mutant strain

Fig. 2. H^+ /ATPase-specific activities in membrane fractions from *S. mutans* wild-type and SRP and YidC mutant strains grown under nonstress or pH 5.0 acid shock conditions. Statistical significance (Student's *t* test) compared to the pH 5.0 value for the same strain is indicated: *, $P < 0.05$; †, $P < 0.005$; ‡, $P < 0.0001$. Statistical significance (Student's *t* test) compared to wild-type at the same pH is also indicated: §, $P < 0.005$; ¶, $P < 0.0001$.

derived membrane fractions prepared from mutant strains lacking Ffh, scRNA, or FtsY compared to the wild-type when analyzed by two-dimensional electrophoresis (data not shown) as described (62).

ATPase activity of membrane fractions from *S. mutans* mutant strains was assayed from cells cultured at pH 7.0 or pH 5.0, and levels of ATPase activity compared to the wild-type (Fig. 2). The ATPase specific activities from membrane fractions of all SRP-pathway mutant strains grown and maintained at pH 7.0 were reduced significantly, with a similar or even greater reduction observed after acid shock. As expected, the enzymatic activity of membrane fractions from acid-shocked (pH 5.0) wild-type cells was significantly increased relative to the pH 7.0-maintained culture and reflects the ability of *S. mutans* to rapidly adapt to this environmental challenge; this was not the case for the mutant strains in which the specific activities remained unchanged or decreased significantly in response to acid-shock. Expression of the enzymatically active F_1 component was not likely affected by the *ffh* mutation because we showed that ATPase activity from whole deacylated cells was identical in chemostat-grown wild-type and *ffh*-mutant MK4 cultures (39). However, differences in activities of membrane fractions were similar to those shown here. Taken together, these results indicate that, although an enzymatically active proton ATPase can be incorporated into *S. mutans* membranes in the absence of a functional SRP pathway, the efficiency of the process is impaired, particularly under stress conditions, and suggest that multiple pathways are involved in membrane incorporation of ATPase subunits to maintain pH homeostasis in this highly aciduric organism.

Comparison of *S. mutans* Homologs With Known Secretion Machinery Components; Potential Functional Overlap of the SRP Pathway with YidC2. Much of what is known about membrane biogenesis and protein secretion in Eubacteria has been gleaned from studies in *E. coli* and *B. subtilis*. That *S. mutans* can survive extensive genetic disruption of the SRP cotranslational protein translocation pathway indicates that important biological differences must exist between it and more widely studied organisms. The *S. mutans* genome contains single copies of genes encoding the SecA ATPase and the SecY, SecE, and SecG translocase components; however, no homologs encoding SecD or SecF are apparent. As expected for a Gram-positive organism, no homologs of SecB are present, nor is a reported functional substi-

tute of SecB, CsaA, from *B. subtilis* (63). A single gene encoding a homolog of YajC was identified, although it is present in *S. mutans* in the absence of genes encoding SecD and SecF, which together with YajC comprise an operon in *E. coli* (22).

The genomes of *S. mutans* and other streptococcal species harbor two genes encoding YidC/Oxa-family homologs. They are referred to in this report as *yidC1* (SMU.337) and *yidC2* (SMU.1727) and are assigned by BLAST analysis as homologs of *oxa1* and *oxa2*, respectively. However, as is the case between *E. coli* YidC and mitochondrial Oxa1 and Oxa2, the overall level of primary sequence identity between the *S. mutans* YidC homologs and other known YidC/Oxa1/Oxa2 proteins is low (<30%). Despite limited homology, complementation experiments indicate a degree of conserved function between the bacterial and mitochondrial proteins (30, 64), but a correlation between *S. mutans* YidC1 and YidC2 compared to YidC/Oxa1/Oxa2 function remains to be determined. Real-time PCR indicates that both *yidC1* and *yidC2* are expressed in *S. mutans* (data not shown). *B. subtilis* also harbors two YidC/Oxa-family homologs, designated SpoIIJ and YqjG (65). SpoIIJ, but not YqjG, is required for sporulation. Both are reportedly involved in membrane protein biogenesis and protein secretion and their simultaneous presence is required for survival.

Recent evidence suggests a previously unrecognized level of versatility of inner membrane protein biogenesis in *E. coli* in that its single YidC is involved in both Sec-translocase-dependent and -independent assembly (66). Mitochondria of higher eukaryotes lack the Sec machinery and an SRP pathway (67), but integrate hydrophobic proteins, including F_0F_1 -ATPase subunits using Oxa1. Therefore, it is possible that one of the YidC homologs of *S. mutans* can function in cotranslational insertion of integral membrane proteins and may serve to compensate for a lack of SRP components in this organism. To evaluate this question, genes encoding YidC1 and YidC2 were deleted singly and from mutant strains lacking integral SRP components. Elimination of *yidC1* did not result in a discernible phenotype compared to the wild-type under nonstress or acid, salt or oxidative stress conditions (Table 1). In addition, superimposing a *yidC1* deletion onto deletions of genes encoding SRP pathway components did not result in any additional effects beyond those observed in the SRP mutants alone. On the other hand, elimination of *yidC2* resulted in a stress-sensitive phenotype and increased doubling time under nonstress conditions comparable to SRP pathway mutants. As expected, similar to the SRP mutant strains, the ATPase-specific activities from the membrane fractions of the acid-sensitive *yidC2* mutant strain grown and maintained at pH 7.0 and 5.0 were significantly reduced compared to the wild type (Fig. 2). This was not the case for the stress tolerant *yidC1* mutant strain, in which ATPase levels were comparable to the wild-type. Growth of *S. mutans* was profoundly diminished in the absence of genes encoding both YidC2 and SRP pathway components even without stress. After transformation, colonies did not appear before 3 days, and when subcultured into broth, growth was not apparent for at least 48 h. Simultaneous deletion of *yidC1* and *yidC2* has not been achieved to date and may be lethal, although this conclusion will require evaluation using a conditional-lethal system.

Although compromised with respect to stress tolerance, the finding that *S. mutans* survives simultaneous elimination of multiple key components of the SRP pathway highlights the observation that at present there are "more questions than answers" in the membrane protein assembly field (reviewed in ref. 22). YidC2 also plays a role in the ability of *S. mutans* to contend with rapid environmental challenge and appears to function in concert with the SRP pathway in a global stress response in this organism. Overlapping and compensatory pathways clearly enable microorganisms to adapt and maintain membrane function in the face of fluctuating environmental conditions.

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