A novel gene involved in the survival of *Streptococcus mutans* under stress conditions

Dan Li,† Yukie Shibata,† Toru Takeshita, Yoshihisa Yamashita

Section of Preventive and Public Health Dentistry, Division of Oral Health, Growth and Development, Kyushu University Faculty of Dental Science, Fukuoka, Japan

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Corresponding author. Yoshihisa Yamashita

Mailing address: Section of Preventive and Public Health Dentistry, Division of Oral Health, Growth and Development, Kyushu University Faculty of Dental Science, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-6350. Fax: 81-92-642-6354. Electric mail address: yoshi@dent.kyushu-u.ac.jp.

†Both authors contributed equally to this work.
A *Streptococcus mutans* mutant defective in aciduricity was constructed by random insertion mutagenesis. Sequence analysis of the mutant revealed a mutation in *gidA*, which is known to be involved in tRNA modification in *Streptococcus pyogenes*. Complementation of *gidA* by *S. pyogenes gidA* recovered the acid tolerance of *S. mutans*. Although the *gidA*-inactivated *S. pyogenes* mutant exhibited significantly reduced expression of multiple extracellular virulence proteins, the *S. mutans* mutant did not. On the other hand, the *gidA* mutant of *S. mutans* showed reduced ability to withstand exposure to other stress conditions (high osmotic pressure, high temperature, and bacitracin stress) besides an acidic environment. In addition, loss of GidA decreased the capacity for glucose-dependent biofilm formation by over 50%. This study revealed that *gidA* plays critical roles in the survival of *S. mutans* under stress conditions, including a lower pH.
Streptococcus mutans, the major etiological factor in human dental caries, is capable of forming the biofilm commonly known as "dental plaque" on the surfaces of teeth (1, 2). Within dental plaque, S. mutans synthesizes large amounts of acids from fermentable dietary carbohydrates. Acid accumulation can eventually dissolve the hard, crystalline structure of the teeth, resulting in the formation of carious lesions (3). S. mutans has the ability to tolerate the typically low pH of dental plaque and continue to produce acids, contributing to its cariogenicity. Acid tolerance is considered to be one of this microorganism’s most important virulence factors.

The most common mechanisms of acid tolerance among Gram-positive bacteria have been proposed to be i) proton pumps, ii) protection and/or repair of macromolecules, iii) cell membrane changes, iv) alkali production, v) regulation, vi) cell density and biofilms, and vii) alteration of metabolic pathways (4). A combination of these mechanisms can contribute to survival from acidic stress in Gram-positive bacteria. Many researchers have sought to explain the mechanisms of aciduricity in S. mutans, and various genes contributing to this property, such as htrA (protein repair) (5), uvrA (DNA repair) (6), dltC (cell membrane changes) (7), and ciaH (regulators) (8), have been examined. However, in a previous study (9), we compared their contributions to acid tolerance and found that inactivation of individual genes did not, with the exception of the diacylglycerol kinase (dgk) and glucose 1-phosphate uridylyltransferase (gluA) genes, cause a drastic decrease in acid resistance, suggesting that the aciduricity of this organism is regulated by mechanisms different from those of other Gram-positive bacteria. Considering the significantly reduced growth of the gluA mutant, even at neutral pH (9), dgk would seem to be the most specific
contributor to acid tolerance in *S. mutans* among the genes that were previously identified as being involved. However, the results of animal experiments showed that the *dgk* mutation drastically reduced smooth surface caries formation, but did not significantly affect fissure caries formation (10). Unknown factors involved in acid tolerance in addition to *Dgk* might contribute to the cariogenicity of *S. mutans*.

In this study, we used the random mutagenesis method, in combination with marker rescue techniques, to identify novel genes involved in the aciduricity of *S. mutans*. The data presented here demonstrate that the *gidA* gene of *S. mutans* is involved in survival in stress conditions, including pH reduction, and biofilm formation on solid surfaces.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Escherichia coli* strains and *S. mutans* strains were maintained and grown routinely as described previously (11). Antibiotics were used at the following concentrations: 200 µg/ml erythromycin and 50 µg/ml ampicillin for *E. coli* and 10 µg/ml erythromycin and 1000 µg/ml spectinomycin for *S. mutans*.

**DNA manipulation.** Standard recombinant DNA procedures such as DNA isolation, endonuclease restriction, ligation, and agarose gel electrophoresis were carried out as described by Sambrook and Russell (12). Transformations of *S. mutans* and *E. coli* were carried out as described previously (13, 14). Protein sequence similarity searches were performed with the BLAST program via the National Center for Biotechnology Information server.
**Random mutagenesis of S. mutans.** The random mutagenesis of *S. mutans* was carried out
as described previously (15). Briefly, we constructed a *S. mutans* UA159 genomic library by
inserting a complete Sau3AI digest of the *S. mutans* UA159 chromosome into
BamHI/BglII-digested pResEmBBN. pResEmBBN (15) can be used as an integration vector for
gene inactivation by a single crossover with the streptococcal chromosome because it has no
replicon in streptococcal species. *S. mutans* UA159 was randomly mutated by transformation with
the *S. mutans* genomic library. Transformants were spread on brain heart infusion (BHI; Difco,
Grand Island, NY) agar plates containing 10 µg/ml erythromycin. Transformants appearing on the
plates were replicated with a toothpick onto two BHI agar plates: one (plate A) was not modified
and the other (plate B) was adjusted to pH 5.5 using sodium acetate and acetic acid (total
concentration 50 mM). Colonies that grew on plate A but not on plate B were selected by visual
screening.

**Analysis of protein expression.** Overnight cultures of the strains were diluted in BHI broth
(pH 7.4) or BHI broth with the pH adjusted to 5.8 and incubated at 37°C in 5% CO₂ until the
late-exponential phase (an optical density at 550 nm [OD₅₅₀] of 0.3–0.5). These cultures were
precipitated with two volumes of acetone, and the pellets were resuspended in 4% sodium dodecyl
sulfate (SDS) solution. Following incubation at room temperature for 30 min, the bacteria were
centrifuged, and the supernatants were analyzed by SDS-polyacrylamide gel electrophoresis
(SDS-PAGE) and Western blotting using appropriate rabbit anti-virulence factor sera, as previously
described (16, 17), as a cell surface and secreted protein fraction. The centrifuged cells were washed
in Tris buffer (TB; 20 mM Tris, pH 7.5), resuspended in TB, and disrupted using glass beads and a
homogenizer (Mini-Beadbeater; Biospec Products, Bartlesville, OK). The cell extracts were
Evaluation of the acid sensitivity of the strains. The mutant strains and wild-type control UA159 strain cells were grown in BHI broth overnight at 37°C in 5% CO₂. The cultures were then diluted 1:10 into fresh BHI and grown to an OD₅₅₀ of ~0.5. Aliquots (20 µl) of cell suspensions with the same turbidity were inoculated into wells that contained 200 µl of fresh BHI medium whose pH had been adjusted to either 7.4 or 5.5 with 50 mM sodium acetate buffer. Growth was monitored by measuring OD₅₅₀ using a SpectraMax 340PC384 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Wells containing only BHI were used as controls. Growth curves were generated using means of the data obtained from three independent experiments.

Biofilm formation and quantification. To facilitate quantification, we used 96-well polystyrene microtiter plates for the growth of biofilms. Growth was initiated by inoculating individual wells of a 96-well microtiter plate with 5 µl of cell suspension in 200 µl of BHI broth supplemented with 1% (w/v) sucrose or glucose. The microtiter plates were incubated at 37°C in 5% CO₂ for 16 h without agitation. Next, the liquid medium was removed and the wells were rinsed once with sterile distilled water. The plates were air-dried, stained with 0.1% crystal violet (CV) solution for 15 min at room temperature, rinsed with distilled water to remove excess dye, and then air-dried. The CV on the abiotic surfaces was solubilized in 95% ethanol, and OD₆₀₀ was determined using a SpectraMax 340PC384 microplate spectrophotometer (Molecular Devices). The ability of each strain to form biofilms was estimated by means of data obtained from three independent experiments.

Metabolomic analysis. Metabolomic analysis was performed using a capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS)-based metabolomics technique by
Human Metabolome Technologies Inc. (Tsuruoka, Japan). *S. mutans* strains were grown at 37°C in 5% CO₂ until the culture reached an OD₅₅₀ of 0.5. The cultures were centrifuged and the resulting pellets were suspended in BHI medium (pH 5.5) and incubated at 37°C in 5% CO₂ for 30 min. The cultures were centrifuged and the cells were then washed twice with Milli-Q water (Millipore, Billerica, MA). Cold methanol containing internal standards (methionine sulfone and d-camphor-10-sulfonic acid, 5 µM) was added to the cell pellet. Then 2.0 ml of the methanol cell suspension was transferred to a Falcon Blue Max Jr. centrifuge tube (15 ml; 352097; Becton Dickinson & Co., Franklin Lakes, NJ) and mixed with 2 ml of chloroform and 800 ml of Milli-Q water. After thorough vortexing, the mixture was centrifuged at 2300 × g at 4°C for 5 min. The aqueous layer (1.6 ml) was distributed to four Amicon Ultrafree-MC ultrafilter tips (Millipore) and centrifuged at 9100 × g at 4°C for approximately 2 h. The filtrate was dried and dissolved in 25 µl of Milli-Q water. CE-TOFMS was performed as described previously (18–20). In the analysis, 108 metabolites in primary pathways were targeted and quantified.

**Statistical analysis.** The Bonferroni test was used to determine the significance of differences in multiple comparisons and Student’s *t*-test was used to determine significant differences between two groups. Differences were considered significant only for values of *P* < 0.01.

**RESULTS**
Isolation of an aciduricity-deficient mutant of *S. mutans*. *S. mutans* UA159 was randomly mutated by transformation with the *S. mutans* genomic library. This study aimed to identify novel genes other than *dgk* that are involved in the aciduricity of *S. mutans*. Therefore, to avoid the isolation of the *dgk* mutant, the *S. mutans* genomic library was constructed using UA159 genomic DNA digested with Sau3AI, for which no restriction site exists in the nucleotide sequence of the *dgk* gene. Eight transformants showing attenuated acid tolerance were selected from more than 10,000 transformants. These transformants were grown once again in BHI medium whose pH had been adjusted to pH 7.4 or 5.5 with 50 mM sodium acetate buffer. Five of eight transformants grew relatively well in broth medium, even that adjusted to pH 5.5, although they showed reduced growth on plate medium. Therefore, these five transformants were excluded from further analysis. We failed to recover genomic DNA flanking the insertion sites using marker rescue strategy from two of the remaining three transformants. The last remaining transformant was designated UA-AR. Southern blotting with a DIG-labeled PCR probe specific for the erythromycin resistance (*Em*') gene revealed that the probe hybridized with a 6.8-kb HindIII fragment of UA-AR, but did not hybridize with any fragments of wild-type UA159 strain (data not shown).

Cloning and sequencing of the region flanking the plasmid insertion site in the UA-AR strain. To recover the region flanking the integration plasmid (pResEmBBN) inserted in the chromosome of the UA-AR strain, we digested the UA-AR chromosome with HindIII and allowed it to self-ligate. *E. coli* DH5α was then transformed with this DNA. *Em*’ transformants were isolated on BHI agar plates containing erythromycin. Almost all of the plasmids isolated from transformants were 6.8 kb long, one of which was designated pAR-H. The nucleotide sequences of the flanking region in this plasmid were first determined using the primers designed inside the *Em*’ gene and
p15A replication origin of pResEmBBN, respectively, and the successive sequencing of the remainder was performed by primer walking. The results revealed that the inserted fragment of the integration plasmid was located within SMU.2141, which encodes a protein of 631 amino acids named glucose-inhibited division protein A (GidA). The amino acid sequence deduced from the gidA gene showed 88% identity with the tRNA (5-carboxymethylaminomethyl-2-thiouridylate) synthase subunit GidA of Streptococcus pyogenes MGAS10750.

**Acid sensitivity of the gidA deletion mutant.** To confirm the role of gidA in acid tolerance, we inactivated gidA by replacing the complete gidA gene with the Em' gene by double-crossover homologous recombination. A 658-bp fragment upstream and an 834-bp fragment downstream of the gidA gene were amplified from S. mutans UA159 genomic DNA, and inserted upstream and downstream, respectively, of the Em' gene in pBSSKII-Em'(21), in which the Em' fragment was cloned into HindIII- and EcoRV-digested pBluescript SK II (+). The resultant plasmid (pBSSKII-Em'-GidA-UD) was digested with BssHII, and the assembled fragment was transformed into S. mutans UA159. Then, we constructed growth curves for the parent strain UA159 and the mutant strain. As shown in Fig. 1, the gidA mutant showed extremely limited growth at pH 5.5, while it had a slightly reduced growth rate compared to that of the parent strain UA159 at pH 7.4.

Inactivation of the downstream gene (SMU.2140) or the second gidA gene (SMU.1003), which encodes a small GidA protein, did not result in loss of the acid tolerance (data not shown).

Complementation of the gidA gene was performed by insertion of the full gidA gene into the fructosyltransferase gene (which is not involved in the aciduricity of this organism) in the gidA deletion mutant. First, we constructed the spectinomycin-resistant (Spc') gidA mutant using pBSSKII-Spc', with the Spc' fragment cloned into SmaI-digested pBluescript SK II (+), as...
described above. Next, a 2001-bp fragment inside the ftf gene was amplified from S. mutans UA159 genomic DNA and inserted into pResEmBBN. A 1947-bp fragment including the full gidA gene and the ribosomal binding site was amplified from S. mutans UA159 genomic DNA and inserted downstream of the ftf fragment in pResEmBBN. The resultant plasmid was then transformed into the Spc\(\text{r}\)-gidA mutant using single-crossover homologous recombination. The obtained transformant showed the same level of acid resistance as UA159 (Fig. 2). In addition, complementation of S. mutans gidA by S. pyogenes gidA was performed in the same manner in an Spc\(\text{r}\)-gidA deletion mutant of S. mutans. A 1972-bp fragment including the full gidA gene and the ribosomal binding site of S. pyogenes was amplified from S. pyogenes MGAS8232 genomic DNA. This complementation recovered the aciduricity of S. mutans, indicating that S. pyogenes GidA can substitute for S. mutans GidA in S. mutans (Fig. 2).

**Production of virulence proteins.** The gidA inactivation in S. pyogenes was reported to lead to a significant reduction in the expression of multiple extracellular proteins involved in its virulence. Therefore, protein expression in the cytosolic, cell surface, and secreted fractions of the parent strain UA159 and the gidA mutant was analyzed by SDS-PAGE. When grown at pH 7.4, the protein profiles of the gidA mutant were similar to those of UA159 (Fig. S1A). In addition, examination of the expression levels of several secreted or cell surface virulence proteins, including glucosyltransferase-I (GTF-I) (22), glucosyltransferase-S (GTF-S) (22), surface protein antigen (PAc) (23), fructosyltransferase (FTF) (22), and autolysin (AtlA) (24), also demonstrated that the expression levels of these virulence proteins were similar between both strains (Fig. S1B). Nevertheless, some change in protein expression occurred between the wild-type UA159 and the gidA mutant when grown at pH 5.8, as shown in Fig. 3A. Western blotting showed that no
significant differences existed between UA159 and the mutant, as well as results at pH 7.4 (Fig. 3B).

Inactivation of gidA did not affect the expression levels of the virulence proteins in S. mutans. The gidA gene might regulate the expression of some proteins to respond to the pH reduction in S. mutans.

Responses of the gidA deletion mutant to bacitracin stress and environmental stressors other than low pH. The gidA deletion mutant was tested for its ability to grow at an elevated temperature (44°C) and high osmotic pressure (NaCl at a final concentration of 0.585 M). As shown in Fig. 4, this mutant had lost not only acid tolerance, but also the ability to survive at high osmotic pressure and high temperature. S. mutans is known to exhibit resistance to bacitracin; indeed, bacitracin is an essential component of isolation medium selective for this microorganism (25). Inactivation of the gidA gene significantly reduced growth in the presence of 5 U/ml bacitracin compared to that of the parent strain UA159 (Fig. 4C).

Characterization of the mnmE deletion mutant and the gidA-mnmE double mutant. In S. pyogenes, both GidA and MnmE contribute to the production of multiple secreted virulence proteins (26). Sequence homology analysis revealed the existence of an mnmE gene (SMU.1235) in S. mutans with similarity to the S. pyogenes mnmE gene. To examine whether the inactivation of mnmE, like that of the gidA gene, leads to the loss of aciduricity, a strain lacking mnmE was constructed in the same manner as the gidA mutant. Briefly, a 527-bp fragment upstream and 636-base-pair fragment downstream of the mnmE gene were amplified from S. mutans UA159 genomic DNA and inserted upstream and downstream, respectively, of the Em^r gene in pBSSKII-Em'. The resultant plasmid was digested with BssHII, and the assembled fragment was transformed into S. mutans UA159. As expected, the mnmE mutant hardly grew at pH 5.5 and the
mean OD_{550} value of this mutant after incubation for 16 h was 0.101, whereas that of the gidA mutant was 0.186 (Fig. 5A). The fact that the mnmE mutant had a reduced growth at pH 7.4 compared to the gidA mutant might be one of the causes of the difference in growth at pH 5.5 between these mutants. Next, a gidA-mnmE double deletion mutant was constructed and grown at pH 7.4 or 5.5. This double mutant showed a similar growth rate to that of the mnmE single mutant at pH 7.4 and 5.5. The responses of the mnmE mutant and the gidA-mnmE double mutant to high osmotic pressure, elevated temperature, and the presence of 5 U/ml bacitracin in liquid broth were similar to those of the gidA mutant (Fig. 5B–D).

**Role of the gidA and mnmE genes in biofilm formation.** We next determined whether any alterations in biofilm formation occurred as a result of inactivation of gidA and/or mnmE. The capacity of the wild-type and mutant strains to form biofilms in the wells of 96-well microtiter plates was evaluated. In the presence of sucrose, the gidA, mnmE, and gidA-mnmE mutants all formed biofilms similar to that formed by the wild-type strain (data not shown). In contrast, the biofilms formed by these three mutants in the presence of glucose alone (no sucrose) had over 50% less biomass than that of the wild-type strain (Fig. 6).

**Acid sensitivity of the gidA-dgk double mutant.** Our previous work demonstrated that the dgk gene of S. mutans responds specifically to pH reduction among many genes that have been reported to be involved in acid tolerance in S. mutans (9). Comparing the acid sensitivities of the GidA mutant and the Dgk mutant, the effect of Dgk deletion on the aciduricity of S. mutans was a little stronger than that of the GidA deletion (data not shown). We had great interest in examining whether inactivation of both the gidA and dgk genes leads to further loss of aciduricity in S. mutans.

A gidA-dgk double deletion mutant was constructed by double-crossover homologous...
recombination. Briefly, we first constructed the Spc\(^{-}\)-dgk mutant in the same manner as the Spc\(^{-}\)-gidA mutant. Next, the plasmid pBSSKII-Em\(^{-}\)-GidA-UD was digested with BssHII and the assembled fragment was transformed into the Spc\(^{-}\)-gidA mutant. At pH 7.4, the gidA-dgk double deletion mutant grew similarly to the gidA single mutant. In contrast, the growth rate of this double mutant at acidic pH was significantly weaker than those of the single mutants (Fig. 7).

**Metabolomic analysis.** Metabolomic analysis by CE-TOFMS was employed to investigate the effects of gidA inactivation on the metabolome of *S. mutans* exposed to acidic conditions (pH 5.5). Eighty-eight of 108 metabolites were identified and quantified (Table S1). Comparison of metabolome profile data for the gidA mutant and the wild-type showed little change in the metabolites in principal pathways such as the EMP pathway (glycolysis), the pentose phosphate pathway, the TCA cycle, and amino acid biosynthesis pathway. However, a few metabolites in the purine and pyrimidine biosynthesis pathways showed a significant difference between the wild-type strain and the gidA mutant (Table 1). Among metabolites of the purine biosynthesis pathway, levels of inosine monophosphate (IMP) were significantly lower (*P* < 0.001), and those of deoxyadenosine triphosphate (dATP) were significantly higher (*P* < 0.05), in the gidA mutant compared to the wild-type strain. Additionally, levels of cytosine, a metabolite of the pyrimidine biosynthesis pathway, were significantly higher (*P* < 0.01) in the gidA mutant compared to the wild-type strain. We tried to grow the gidA mutant at pH 5.5 in BHI supplemented with IMP disodium salt (1 and 10 mM). The addition of IMP did not result in the recovery of acid tolerance in this mutant (data not shown). These results, however, show that GidA might regulate proteins involved in the IMP biosynthesis pathway in *S. mutans*. 
DISCUSSION

This study showed that the deletion of GidA led to the significant loss of acid tolerance in *S. mutans*, which is considered to be one of the most important virulence factors of this microorganism. Complementation of GidA in the GidA mutant restored aciduricity to wild-type levels, indicating that GidA is certainly involved in the acid tolerance of *S. mutans*. Moreover, GidA played an important role in survival under stress conditions, such as high osmotic pressure, high temperature, and bacitracin stress. In addition, the GidA mutant formed sucrose-independent biofilms with reduced biomass in the presence of glucose (alone), whereas sucrose-dependent biofilm formation by this mutant was very similar to that of the wild-type strain. This ability to form biofilms stems from two distinct processes: initial sucrose-independent attachment and enhancement of the attachment by a sucrose-dependent mechanism (27). *S. mutans* GidA might be involved in initial attachment in the process of biofilm formation in this organism.

The results of homology searches showed that the amino acid sequence deduced from the *gidA* gene shows 88% identity with the tRNA (5-carboxymethylaminomethyl-2-thiouridylicate) synthase subunit GidA of *S. pyogenes*. The *S. pyogenes* GidA mutant exhibited significantly reduced levels of several secreted virulence proteins, including glycohydrolase, streptolysin O, surface M protein, and mitogenic factors, relative to those in the wild-type strain, resulting in the attenuation of pathogenicity (26). In addition, the *gidA* mutants of several bacteria, such as *Aeromonas hydrophila* (28), *E. coli* (29), *Pseudomonas aeruginosa* (30), *Pseudomonas syringae* (31), and *Salmonella enterica* (32, 33), have been shown to have reduced levels of virulence and/or
virulence factor production. Considering that *S. pyogenes* (which is in the same genus as *S. mutans*) and several other bacteria possess GidA proteins with similar functions, and that complementation of *S. mutans* gidA by *S. pyogenes* gidA restored the wild-type phenotype, we hypothesize that this gene regulates the expression of virulence proteins in *S. mutans*. However, the results of Western blotting using anti-GTF-I, anti-GTF-S, anti-PAc, anti-FTF, and anti-AtlA antibodies unexpectedly showed no distinct differences in the expression levels of these virulence proteins between the wild-type strain and the gidA mutant. Although the results of complementation showed that *S. pyogenes* GidA can substitute for *S. mutans* GidA in *S. mutans*, inactivation of the gidA gene had no effect on the production of extracellular virulence proteins in *S. mutans*. *S. mutans* GidA might regulate proteins other than those mentioned above or might have different roles from *S. pyogenes* GidA, which was able to complement the *S. mutans* gidA mutant.

MnmE, a GTPase, is also required for the production of secreted virulence proteins in *S. pyogenes* (26). In the present study, the mnmE mutant exhibited responses to various stressors similar to those of the gidA mutant, and the susceptibility of the gidA-mnmE double deletion mutant to the environmental changes was similar to that of the gidA mutant and the mnmE mutant. These results indicate that GidA and MnmE are involved in survive under stress conditions and biofilm formation in the same pathway in *S. mutans*.

Our previous study showed that the dgk gene responds highly specifically to pH reduction among many other genes reported previously to be involved in acid tolerance in *S. mutans* (9). This was still the case after we identified the gidA gene in this study. Like the GidA mutant, the Dgk mutant also exhibited reduced ability to survive under stress conditions. These two mutants showed similar responses to high osmotic pressure and bacitracin concentration, but growth of the GidA
mutant at high temperature was drastically reduced compared to that of the Dgk mutant (data not shown), indicating that the GidA mutant had a different phenotype relating to environmental stressors from that of the Dgk mutant. In further investigations, a dgk-gidA double mutant was constructed and grown at acidic pH. Notably, the acid tolerance of this mutant was significantly reduced compared to that of the dgk and gidA single mutants. Although, at present, how the dgk and gidA genes are associated with aciduricity in S. mutans is unknown, these results suggest that these two genes might regulate the different pathways associated with aciduricity in S. mutans.

In this study, metabolomic analysis was performed to elucidate how GidA is involved in the acid tolerance of S. mutans, and the difference in the response to the pH reduction between the gidA mutant and wild-type strain was revealed. The IMP concentration was significantly different between the two strains among 108 metabolites; the concentration in the gidA mutant reduced 5.2-fold compared with that of the wild-type strain. GidA might regulate the production of the proteins involved in IMP biosynthesis at low pH. IMP is an intermediate ribonucleoside monophosphate in purine metabolism and the endogenous source of both AMP and GMP. Previous studies (34–36) showed that the expression of the genes involved in purine metabolism changed in response to stress, such as heat and oxygen, in some bacteria. Serrano et al. (36) reported that the expression of the purN, purM, and purE genes involved in IMP biosynthesis increased 3.1-, 3.4-, and 1.8-fold, respectively, in response to oxidative stress in Lactobacillus plantarum. Considering these findings, IMP biosynthesis being involved in the aciduricity of S. mutans is very reasonable, although how GidA regulates IMP biosynthesis is unclear. In further studies, the comprehensive identification of proteins expressed differentially in the wild-type strain and gidA mutant under acidic pH condition is require to clarify the exact function of GidA in IMP biosynthesis.
Regardless, our study clearly shows that GidA plays an important role in aciduricity, a key virulence property of *S. mutans*.

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REFERENCES


33. **Shippy DC, Eakley NM, Lauhon CT, Bochsler PN, Fadl AA.** 2013. Virulence characteristics of *Salmonella* following deletion of genes encoding the tRNA modification enzymes GidA and MnmE. Microb. Pathog. **57**:1-9.


**FIGURE LEGENDS**

**FIG 1** Growth curves of the *gidA* mutant and the parent strain UA159 grown in BHI medium at pH 7.4 or 5.5. Growth was defined as the increase in OD_{550}, calculated by subtraction of OD_{550} at the initiation of growth from that at the indicated time points. The results are the means of three experiments.
FIG 2 Growth yield of the parent strain UA159, the gidA mutant, the *S. mutans* gidA complemented strain (Comp-SmgidA), and the *S. pyogenes* gidA complemented strain (Comp-SpgidA) at pH 7.4 or 5.5. The y-axis represents OD_{550} after incubation for 16 h. Data represent the mean ± standard deviation of three independent experiments. *a*, Significant difference against the OD_{550} value of the wild-type UA159 as determined using the Bonferroni test.

FIG 3 Protein expression in the gidA mutant and the parent strain UA159 grown at pH 5.8. (A) The cytosolic, cell surface, and secreted fractions were analyzed by SDS-PAGE (in 7.5% or 12.5% gels). The arrows indicate proteins exhibiting differential expression. (B) The proteins of the cell surface and secreted fractions were electrophoresed in a 7.5% gel, and subjected to Western blot analyses using rabbit anti-GTF-I, anti-GTF-S, anti-PAc, anti-FTF, and anti-AtlA sera.

FIG 4 Growth yield of the parent strain UA159 and the gidA mutant at 37°C and 44°C (A) in BHI broth supplemented with 0.5 M NaCl (B) and in BHI broth supplemented with 5 U/ml bacitracin (C). The y-axis represents OD_{550} after incubation for 16 h. Data represent the mean ± standard deviation of three independent experiments. *, Significant difference as determined using Student’s t-test.

FIG 5 Growth yield of the parent strain UA159, the gidA mutant, the mnmE mutant, and the gidA-mnmE double mutant at pH 7.4 and 5.5 (A) and 37°C and 44°C (B) in BHI broth.
supplemented with 0.5 M NaCl (C) and in BHI broth supplemented with 5 U/ml bacitracin (D). The
y-axis represents OD$_{550}$ after incubation for 16 h. Data represent the mean ± standard deviation of three independent experiments. $^a$, Significant difference against the OD$_{550}$ value of the wild-type UA159 as determined using the Bonferroni test; $^b$, significant difference against the OD$_{550}$ value of the gidA mutant as determined using the Bonferroni test.

**FIG 6** Biofilm formation of the parent strain UA159, the gidA mutant, the mnmE mutant, and the gidA-mnmE double mutant. Biofilms were cultivated in BHI broth containing 1% glucose and stained with 0.1% CV. OD$_{600}$ was then measured. Data represent the mean ± standard deviation of three independent experiments. $^a$, Significant difference against the OD$_{600}$ value of the wild-type UA159 as determined using the Bonferroni test.

**FIG 7** Growth yield of the parent strain UA159, the gidA mutant, the dgk mutant, and the gidA-dgk double mutant at neutral and acidic pH. The y-axis represents OD$_{550}$ after incubation for 16 h. Data represent the mean ± standard deviation of three independent experiments. $^a$, Significant difference against the OD$_{550}$ value of the wild-type UA159 as determined using the Bonferroni test; $^b$, significant difference against the OD$_{550}$ value of the gidA mutant as determined using the Bonferroni test; $^c$, significant difference against the OD$_{550}$ value of the dgk mutant as determined using the Bonferroni test.
FIG 3

(A) Cytosolic fractions

Cell-surface & secreted fractions

Western blotting

GTF-I
GTF-S
PAc
FTF
AtlA

UA159 ΔgalA
UA159 ΔgalA
UA159 ΔgalA
UA159 ΔgalA

FIG 3
FIG 4
FIG 7

Graph showing OD₅₅₀ at different pH levels (pH 7.4, pH 6.1, pH 5.8) for different strains: UA159, ∆gidA, ∆dgk, ∆gidA∆dgk. Bars indicate mean ± standard error. Letters (a, b, c) signify significant differences.
**TABLE 1** Comparison of metabolite levels in the wild-type UA159 and the gidA mutant exposed under acidic conditions

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pathway</th>
<th>Concentration (pmol/OD·ml)</th>
<th>P value&lt;sup&gt;b&lt;/sup&gt;</th>
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<td></td>
<td></td>
<td>UA159</td>
<td>∆gidA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inosine monophosphate</td>
<td>Purine biosynthesis</td>
<td>505.6</td>
<td>56.5</td>
</tr>
<tr>
<td>dATP</td>
<td>Purine biosynthesis</td>
<td>188.6</td>
<td>22.2</td>
</tr>
<tr>
<td>Cytosine</td>
<td>Pyrimidine biosynthesis</td>
<td>8.3</td>
<td>0.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Standard deviation (n = 3)

<sup>b</sup>Differences in relative quantity between UA159 and ∆gidA were evaluated for individual metabolites by Welch’s t-test.