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Molecular Characterization of a *Streptococcus mutans* Mutant Altered in Environmental Stress Responses

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A mutant defective in aciduricity, GS5Tn1, was constructed following mutagenesis of *Streptococcus mutans* GS5 with the conjugative transposon Tn*916*. The mutant grew poorly at acidic pH levels and was sensitive to high osmolarity and elevated temperatures. These properties resulted from a single insertion of Tn*916* into the GS5 chromosome, and the DNA fragment harboring the transposon was isolated in the cosmid vector, charomid 9-20. Spontaneous excision of Tn*916* from the cosmid revealed that Tn*916* inserted into a 8.6-kb *Eco*RI fragment. On the basis of the restriction analyses of insert fragments, it was found that Tn*916* inserted into a 0.9-kb *Eco*RI-*Xba*I fragment. Nucleotide sequence analysis of this fragment indicated the presence of two open reading frames, ORF1 and ORF2. By using a marker rescue strategy, a 6.0-kb *Hin*dIII fragment including the target site for Tn*916* insertion and the 5′ end of ORF1 was isolated and sequenced. The deduced amino acid sequences of ORF1 and ORF2 showed significant homology with the diacylglycerol kinase and Era proteins, respectively, from *Escherichia coli*. Nucleotide sequence analysis of the Tn*916* insertion junction region in the GS5Tn1 chromosome revealed that the transposon inserted near the 3′ terminus of ORF1. Restoration of ORF1 to its original sequence in mutant GS5Tn1 was carried out following transformation with integration vector pVA891 containing an intact ORF1. The resultant transformant showed wild-type levels of aciduricity as well as resistance to elevated temperatures and high osmolarity. These results suggest that the *S. mutans* homolog of diacylglycerol kinase is important for adaptation of the organism to several environmental stress signals.

Streptococcus mutans has been recognized as an important etiological agent in human dental caries (11, 20). These bacteria express two important characteristics which appear to be significant in cariogenicity. The first concerns the ability of these organisms to colonize tooth surfaces, while the other involves their strong acidogenicity, which leads to demineralization of enamel surfaces. The sucrose-dependent and -independent colonization factors concerned with the former property have been characterized by using genetic and biochemical approaches (22). Acid tolerance (aciduricity) has also been considered to be an important cariogenic property of *S. mutans*, and these organisms are known to be among the most acid tolerant of the oral bacteria (15). Consequently, *S. mutans* outgrows both *Streptococcus sanguis* and *Streptococcus mitior* during coculture at low pH (4). It is interesting that *S. mutans* failed to outgrow *Lactobacillus casei*, an oral bacterium, under conditions in which artificially low pH environments were rapidly generated, but *S. mutans* grown initially at pH 5.5 prior to mixing with *L. casei* was capable of growing competitively with the latter organism even at low pH (5). Proton permeability across the cytoplasmic membrane regulated by the proton-extruding ATPase has been reported to play an important role in the acid tolerance of *S. mutans* (3), and the reduction of environmental pH is known to induce not only the proton-extruding ATPase but also lactate dehydrogenase (17) and glycolytic activity (12) in *S. mutans* cells. These observations suggest that the acid tolerance of *S. mutans* is dependent on the adaptability of these organisms to the environmental stress caused by acidic pH levels. However, little information is presently available concerning environmental effects on the cariogenicity of *S. mutans*. Likewise, the physiological changes which result in acid tolerance are poorly understood at the molecular level.

To initiate the molecular characterization of environmental adaptability in *S. mutans*, a mutant defective in acid tolerance was constructed by using the conjugative transposon Tn*916*. Characterization of this mutant has led to the identification of a putative gene coding for diacylglycerol kinase (DGKase) activity which appears to play an important role in aciduricity as well as other environmental stress conditions.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains, plasmids, and cosmid vectors used are described in Table 1. *S. mutans* and *Escherichia coli* strains were maintained and grown routinely as previously described (2). The following antibiotic concentrations were used as indicated: for *E. coli*, 150 μg of erythromycin per ml, 10 μg of tetracycline per ml, or 50 μg of ampicillin per ml; and for *S. mutans*, 10 μg of erythromycin per ml or 4 μg of tetracycline per ml. Charomid 9-20 and pCH1 were maintained in *E. coli* DH1. All other vectors were propagated in *E. coli* JM109.

Transformation of *S. mutans* and *E. coli*. Transformation of *S. mutans* with chromosomal DNA was carried out by the method of Perry et al. (26). Transformation with transposon Tn*916* was performed as described by Caufield et al. (6). Transformation of *E. coli* with plasmids was routinely carried out by the calcium chloride procedure (2) or by using electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) as previously described (8). The Gigapack Plus in vitro packaging kit (Stratagene, La Jolla, Calif.) was used for cosmid cloning.

Screening for GS5 mutants defective in acid tolerance. GS5

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TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics[a]	Reference or source
E. coli		
JM109	F′ *recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ(*lac-proAB*) (*traD36 proAB*$^+$ *lacI*^{q} *lacZ* M15)	36
DH1	F$^-$ *recA1 endA1 gyrA96* (Nal^r) *thi-1 hsdR17 supE44 relA1*	14
S. mutans		
GS5	Wild type	18
GS5DD	Tc^r, *gtfD* gene inactivated	13
GS5Tn1	Tc^r, Tn*916* inserted in ORF1	This study
GS5Tn2	Tc^r, Tn*916* inserted into an unknown position, exhibits slow growth	This study
GS5Tn3	Tc^r, Tn*916* inserted into an unknown position, exhibits slow growth	This study
GS5Tn4	Tc^r, Tn*916* inserted into an unknown position, exhibits normal growth	This study
GS5Tn11	Tc^r Em^r, GS5Tn1 transformed with pES891	This study
GS5Em1	Em^r, GS5 transformed with *Xba*I- and *Kpn*I-digested pRK12	This study
Plasmids		
pAM620	Em^r Tc^r pVA891::pAD1*Eco*RI F′::Tn*916*	35
pBluescriptII KS+, SK±	Ap^r, phagemid cloning vector	32
pTSUT1	Em^r, constructed for marker rescue in streptococci, p15Aori (pResEmPvuII)	31a
pTSUT2	Km^r, p15Aori, MCS from pUC19 (pResKm10)	31a
pVA891	Em^r Cm^r, shuttle plasmid	23
pES891	Em^r, *Eco*RI-*Swa*I fragment from pHD1 was ligated into the *Eco*RI and *Pvu*II sites of pVA891	This study
Charomid 9-20	Ap^r, cosmid cloning vector	29
pCH1	Ap^r Tc^r, charomid 9-20::8.5-kb *Eco*RI-*Eco*RI fragment::Tn*916*	This study
pCH2	Ap^r, Tn*916* spontaneously excised from pCH1	This study
pCH21	Ap^r, pCH2 digested with *Sal*I and self-ligated	This study
pCH3	Ap^r, pCH21 digested with *Hin*dIII and self-ligated	This study
pRK11	Km^r, 2.8-kb *Eco*RI-*Hin*dIII fragment from pCH3 inserted into pTSUT2	This study
pRK12	Km^r Em^r, *Pvu*II-digested pTSUT1 ligated into the *Fsp*I site of pRK11	This study
pHD1	Em^r, constructed from *Hin*dIII-digested GS5Em1 chromosomal DNA by marker rescue	This study

[a] MCS, multicloning site; Ap, ampicillin; Em, erythromycin; Km, kanamycin; Tc, tetracycline.

was transformed with Tn*916* located on pAM620 as described above. The GS5 transformants were screened on tryptic soy (TS; Difco Laboratories, Detroit, Mich.) agar plates containing tetracycline. Transformants appearing on the plates were replicated onto two TS agar plates; one (plate A) was not modified, and the other (plate B) contained 50 mM sodium acetate (pH 4.4). More than 6,000 transformants were screened on both sets of plates to identify mutants defective in acid tolerance. After screening, three colonies which grew on plate A but not on plate B were isolated and designated GS5Tn1, GS5Tn2, and GS5Tn3. GS5Tn4 was randomly chosen from the transformants as a control able to grow on both plates.

Evaluation for environmental adaptability of Tn*916* transformants. Environmental adaptability of the transformants was evaluated by monitoring bacterial growth in Todd-Hewitt (TH) broth (Difco) containing 50 mM sodium acetate at appropriate pH values or the indicated concentrations of NaCl. Temperature sensitivity was determined from 37 to 46°C. To initiate the growth experiments, overnight cultures of each strain were adjusted to an optical density at 550 nm (OD_{550}) of 1.0 by adding fresh TH broth. The same volume of the resultant bacterial suspensions was inoculated into 3 ml of TH broth with or without the indicated modification. Bacterial growth was monitored by measurement of OD_{550}.

Evaluation of the stability of Tn*916* in GS5Tn1. Stationary-phase GS5Tn1 cells (approximately 10^8/ml) following overnight culture in TH broth in the presence of tetracycline were diluted 10^{-5} with fresh broth. Ten microliters of diluted bacterial suspension was inoculated into 2 ml of TH broth without tetracycline and incubated until the stationary phase (approximately 24 generations). The resulting cell culture was diluted and inoculated as described above and was again grown to the stationary phase. The same procedure was repeated twice more, and aliquots of the resulting culture sample were plated on TS agar plates containing no antibiotics. After incubation of the plates at 37°C (approximately 70 generations), colonies grown in the absence of tetracycline appeared on the plate. Eighty colonies were randomly selected from the plate and were replicated onto TS agar plates with or without tetracycline to evaluate tetracycline resistance.

DNA manipulations. DNA isolation, endonuclease restriction, and ligation were carried out as previously described (2). DNA fragments were routinely analyzed on 0.7 or 2.0% agarose (SeaKem GTG agarose; FMC BioProducts, Rockland, Maine) gels as previously described (24), and electrophoretic analysis of DNA fragments larger than 15 kb was carried out on 0.3% agarose (SeaKem Gold agarose; FMC BioProducts) gels at 15 V for 15 h. Southern blot analysis was performed as previously described (31), using biotin- or digoxigenin-labeled probes according to the instructions of the suppliers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; Boehringer Mannheim Biochemica, Mannheim, Germany).

DNA sequence analysis. Plasmid vectors pBluescriptII KS+, SK+, and SK− (Stratagene) were used to clone the insertion sequence fragments. A series of deleted plasmid clones was constructed essentially as described by Henikoff (16). The nucleotide sequences were determined by the dideoxy method (30) with a Sequenase kit (Stratagene) or fluorescent primer cycle sequencing kit (Applied Biosys-

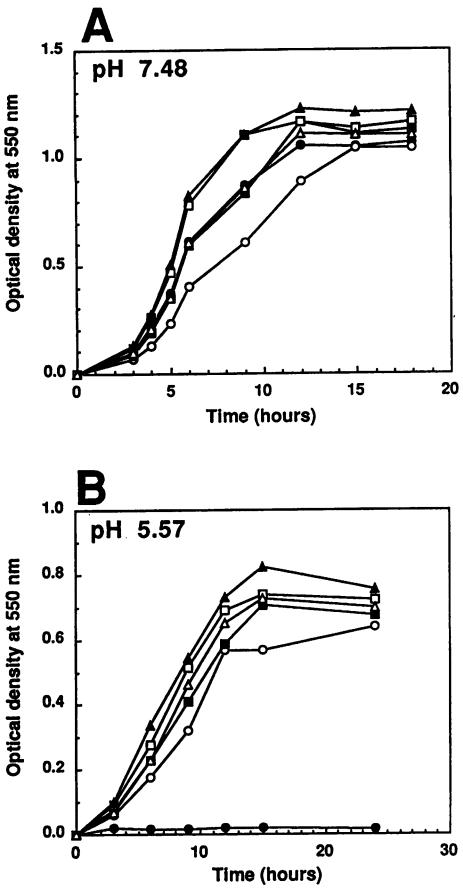

FIG. 1. Effect of pH on bacterial growth of various strains derived from *S. mutans* GS5, GS5Tn1 (●), GS5Tn2 (○), GS5Tn3 (■), GS5Tn4 (□), GS5DD (▲), and GS5Tn11 (△). Each strain was grown in TH broth containing 50 mM sodium acetate at pH 7.0 (A) or pH 4.6 (B); the actual pH value of each medium after addition of sodium acetate is indicated. Bacterial growth of each strain was defined as the increase in OD_{550}. The values are calculated by subtraction of OD_{550} at the initiation of growth from those at the times indicated and are shown as the means of three experiments.

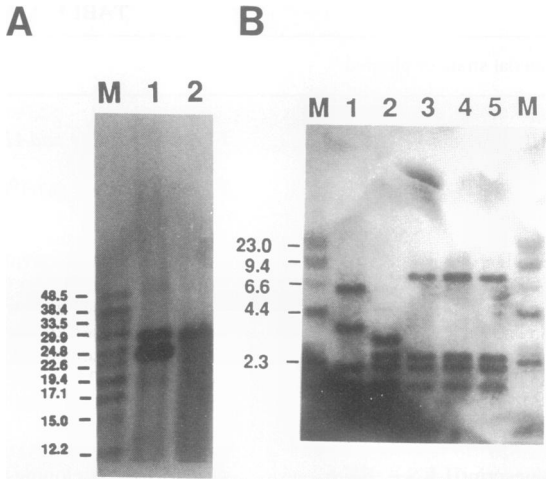

FIG. 2. Southern blot analyses of chromosomal DNA from GS5Tn1 and its derivatives. (A) DNA separation was carried out on 0.3% agarose gels at 15 V for 15 h. Lanes: 1, *Hin*dIII-digested lambda DNA; 2, *Eco*RI-digested chromosomal DNA from GS5Tn1; M, high-molecular-weight marker. Biotinylated pAM620 and biotinylated lambda DNA were used as probes. (B) DNA separation carried out on 0.7% agarose gels at 100 V for 40 min. Lanes: 1, *Hin*dIII-digested chromosomal DNA from GS5; 2, *Hin*dIII- and *Eco*RI-digested chromosomal DNA from GS5; 3, GS5Tn1 digested with *Hin*dIII and *Eco*RI; 4 and 5, GS5Tn1 derivatives digested with *Hin*dIII and *Eco*RI; M, *Hin*dIII-digested biotinylated lambda DNA. Biotinylated pCH21 was used as the probe. Numbers on the left are size markers (kilobases).

tems, Inc., Foster City, Calif.) and a model 381 automated sequencer (Applied Biosystems). The nucleotide sequences were analyzed with a Pustell sequence analysis program (International Biotechnologies, Inc., New Haven, Conn.).

Nucleotide sequence accession numbers. The nucleotide sequences of ORF1 and ORF2 have been deposited in the GenBank data base under accession numbers L12211 and L03428, respectively.

RESULTS

Acid sensitivity of GS5 Tn*916* mutants. The GS5Tn1, GS5Tn2, and GS5Tn3 mutants which exhibited acid sensitivity on TS agar plates were evaluated for acid sensitivity in TH broth. For this purpose, each strain, plus GS5Tn4 and GS5DD as controls, was grown in TH broth containing 50 mM sodium acetate (pH 7.48; TH broth A) and TH broth containing 50 mM sodium acetate (pH 5.57; TH broth B), and growth was monitored (Fig. 1). GS5DD was constructed by insertional inactivation of the *gtfD* gene with the tetracycline resistance gene from Tn*916* as previously reported (13). At neutral pH, all three candidates for aciduricity mutations, especially GS5Tn2, exhibited growth rates slightly lower than those of GS5Tn4 and GS5DD (Fig. 1A). However, only GS5Tn1 showed extremely limited growth in TH broth B (Fig. 1B). It was of interest that the growth rate of GS5Tn1 was faster than that of GS5Tn2 at neutral pH but that their growth relationship was reversed in acid environments. On the basis of these results, GS5Tn1 was selected for further characterization as a mutant defective in acid tolerance.

Determination of the number of insertions of Tn*916* and characterization of the inserted region. To determine the number of insertions of Tn*916* into the GS5Tn1 chromosome and to characterize the insertion site, Southern blot analysis of *Eco*RI-digested GS5Tn1 chromosomal DNA was carried out with biotinylated pAM620, which contains Tn*916* as a probe. *Eco*RI digestion was chosen because a restriction site for this enzyme does not exist within Tn*916* (10). The results clearly indicate a single insertion of Tn*916* into GS5Tn1 chromosomal DNA (Fig. 2A). On the basis of these results, the size of the *Eco*RI fragment into which Tn*916* was inserted was estimated to be around 25 kb. The size of Tn*916* was reported to be 16.4 kb (7), and the size of the *Eco*RI fragment prior to insertion of Tn*916* would therefore correspond to approximately 8.6 kb. While GS5Tn4 showed a single insertion of Tn*916* as did GS5Tn1, both GS5Tn2 and GS5Tn3 exhibited two or more insertions of Tn*916* following Southern blot analyses (data not shown). To confirm the genetic linkage of the Tn*916* insertion with the loss of acid tolerance in GS5Tn1, chromosomal DNA from GS5Tn1 was transformed into parental strain GS5. Twenty colonies were randomly chosen from the transformants newly constructed with GS5Tn1 chromosomal DNA. All of the 20 colonies exhibited a loss of acid tolerance; two colonies were randomly selected, and chromosomal DNA was prepared.

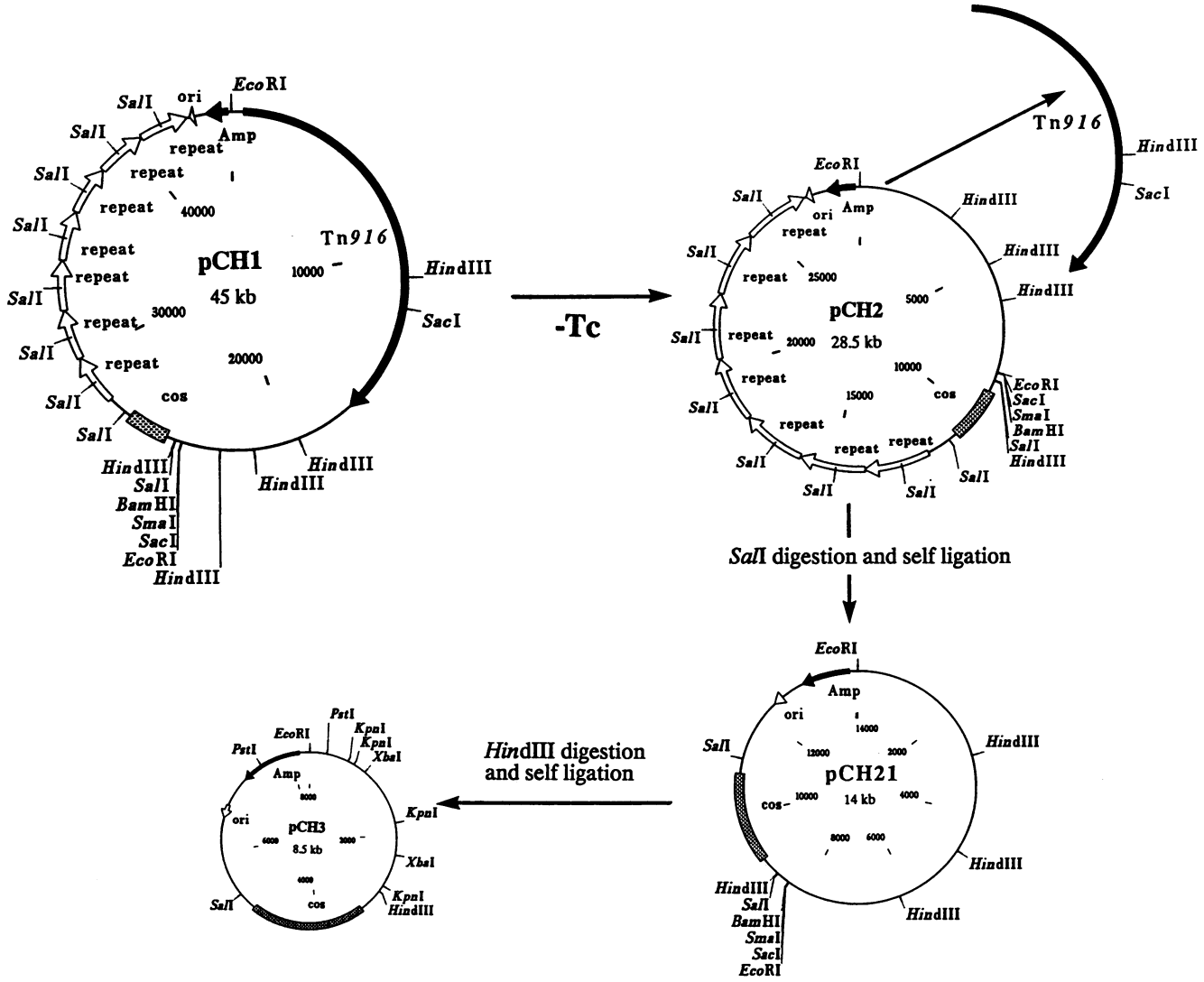

FIG. 3. Strategy for isolation of the DNA flanking Tn*916* insertion from GS5Tn1 chromosomal DNA. Tn*916* was spontaneously excised from pCH1 in the absence of tetracycline (−Tc) selection of pCH1-containing *E. coli*. Repeating spacers (open arrows) in pCH2 were removed by *Sal*I digestion and plasmid self-ligation. The stippled bar and hatched arrow represent the *cos* site and Tn*916*, respectively. Amp, ampicillin resistance gene.

Figure 2B shows a comparison of GS5 (lane 3) and its derivatives (lanes 4 and 5) by Southern blot analysis using pCH21 (described below) as a probe. The results clearly show identical patterns between GS5Tn1 and its derivatives (transformants utilizing GS5Tn1 chromosomal DNA). The results of GS5 analysis (lane 2) revealed that the 8.6-kb *Eco*RI fragment into which Tn*916* had inserted was divided into 2.8-, 2.0-, 1.5-, and 2.3-kb fragments by three *Hin*dIII sites within the insert.

Isolation of the Tn*916* inserted fragment in GS5Tn1 chromosomal DNA. Isolation of the DNA region on which Tn*916* resided is summarized in Fig. 3. The size of the *Eco*RI fragment including Tn*916* from GS5Tn1 was estimated to be around 25 kb (Fig. 4A), as described above. Charomid 9-20 is a 20-kb cosmid vector which packages DNA fragments ranging from 20 to 30 kb (29). GS5Tn1 chromosomal DNA was digested with *Eco*RI and ligated into the unique *Eco*RI site of charomid 9-20. The ligated DNA was packaged in vitro and transformed into strain DH1 as previously described (6). *E. coli* transformants exhibiting both ampicillin and tetracycline resistance were screened on Luria-Bertani agar plate containing both ampicillin and tetracycline. Cosmid DNA was prepared from one of the transformants and was designated pCH1. Although minor contamination of a 8.6-kb fragment was observed in the DNA when digested with *Eco*RI, two fragments with the predicted sizes of 20 and 25 kb predominated. The minor contamination was likely due to instability of Tn*916* in the *E. coli* background even in the presence of tetracycline as previously reported (6). Tn*916* was spontaneously excised from pCH1 in the absence of tetracycline, and pCH2 was obtained from a transformant which was ampicillin resistant and tetracycline sensitive. Restriction analyses of pCH1 and pCH2 revealed that Tn*916* inserted into 2.8-kb *Eco*RI-*Hin*dIII fragment. The charomid vector contains repeating spacer fragments (around 2 kb) that can be eliminated by *Sal*I digestion. The 8.6-kb insert

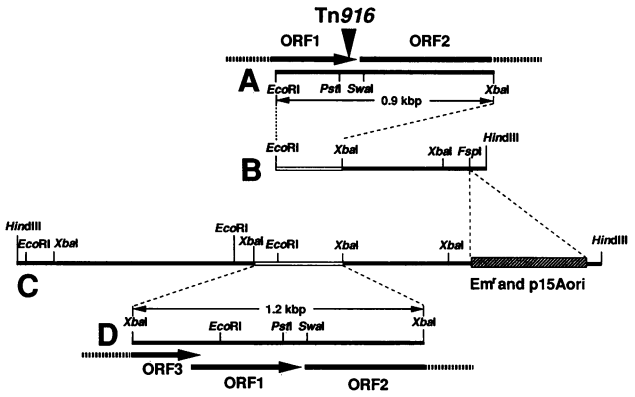

FIG. 4. (A) Location of Tn*916* in the chromosome of GS5Tn1 and the restriction map of the region shown as an open bar in panel B. Striped bars indicate continuation of the ORFs; a vertical arrow indicates the insertion site of Tn*916*. (B) Restriction map of the 2.8-kb *Eco*RI-*Hin*dIII fragment in pCH3 or pRK11. The open bar indicates the region which was sequenced. (C) The 8.0-kb *Hin*dIII fragment from pHD1 constructed by marker rescue. The erythromycin resistance gene (Em^r) and p15Aori from pTSUT1 (hatched bar) are located in the *Fsp*I site near the *Hin*dIII site. (D) Sequence of the *Xba*I fragment shown as an open bar in panel C.

fragment did not contain a *Sal*I site, and the repeating spacers were removed from pCH2 following *Sal*I digestion and self-ligation. The resulting plasmid was designated pCH21. Finally, *Hin*dIII digestion and self-ligation of pCH21 reduced the 8.6-kb insert fragment to a 2.8-kb *Eco*RI-*Hin*dIII fragment on which Tn*916* was inserted (the resulting plasmid was designated pCH3). To obtain a detailed restriction map of the 2.8-kb *Eco*RI-*Hin*dIII fragment, the fragment was subcloned into pTSUT2; the resultant plasmid was designated pRK11.

Nucleotide sequence of the Tn*916* insert region in GS5Tn1 chromosomal DNA. Comparison of the restriction maps of pCH1 and pRK11 revealed that the Tn*916* insertion occurred between the *Eco*RI-*Xba*I sites on the 2.8-kb *Eco*RI-*Hin*dIII fragment. The 0.9-kb *Eco*RI-*Xba*I fragment was then ligated into pBluescriptII SK+ and SK−, and the nucleotide sequence of the fragment was determined. Two open reading frames (ORFs) were found in this region (Fig. 4A). In addition, the Tn*916* insertion junction region was subcloned from pCH1 into pBluescriptII SK+, and the nucleotide sequences of the junction regions were determined (Fig. 5). Comparison of these sequences demonstrated that the precise insertion point of Tn*916* occurred within the 3′ terminus of ORF1 located between the *Pst*I and *Swa*I sites. To isolate the DNA fragment including the original target sequence, pTSUT1 (1.6 kb) was ligated to the *Fsp*I site located near the *Hin*dIII site in the 2.8-kb *Eco*RI-*Hin*dIII fragment on pRK11

	650			660			670			680		
Before insertion	ATT	ATT	TTT	ATA	CCA	AAA	ATT	TGG	TTT	TTG	CTT	TTT	CAT	TAA
	Ile	Ile	Phe	Ile	Pro	Lys	Ile	Trp	Phe	Leu	Leu	Phe	His	---
After insertion (Right end)	ATT	ATT	TTT	Tn916 TTA	TTA	AAA	ATA	GCA	TAA	AAA	TCT	AGT	TAT	CCG
	Ile	Ile	Phe	Leu	Leu	Lys	Ile	Ala	---					
(Left end)	Tn916 ACT	TTG	TTT	TTA	TTA	AAA	ATT	TGG	TTT	TTG	CTT	TTT	CAT	TAA

FIG. 5. Nucleotide sequences of the insertion junction regions at the right and left ends of Tn*916* in GS5Tn1. Subsequent changes of the amino acid sequence of ORF1 are shown in the middle. Numbers above the asterisks correspond to those in Fig. 6.

(Fig. 4B), and pRK12 was constructed. *Hin*dIII- and *Xba*I-digested pRK12 was transformed into GS5, and the erythromycin-resistant transformant, GS5Eml, was isolated. Southern blot analysis confirmed the structure of this construct (data not shown). The chromosomal DNA from GS5Eml was digested with *Hin*dIII, self-ligated, and transformed into *E. coli* JM109 following electroporation. Three transformants exhibiting erythromycin resistance appeared, and plasmid DNA was prepared from each transformant. Restriction analysis of the plasmids following *Hin*dIII digestion demonstrated the predicted 8.0-kb fragment corresponding to the 6.4-kb *Hin*dIII fragment containing the erythromycin resistance gene and p15Aori (plasmid designated pHD1). The 0.3-kb *Xba*I-*Eco*RI fragment which was contained in the 8.0-kb *Hin*dIII fragment from pHD1 and encodes the 5′ end of ORF1 (Fig. 4C) was subcloned into pBluescriptII SK+ and sequenced. The original target region and approximately 50 bp both upstream and downstream from the *Eco*RI site (Fig. 4D) were sequenced with pHD1, using a synthetic primer. The nucleotide sequence of the 1.2-kb *Xba*I fragment containing the target site prior to Tn*916* insertion is shown in Fig. 6; three putative ORFs were identified in this region. The intact sequence of ORF1 was apparently located in this region and contained 411 bp (initiating at base position 276) coding for a putative protein of 127 amino acids. Alternatively, initiation at base position 282 would yield a slightly smaller protein of 125 amino acids. A potential ribosome-binding site, AGGA, was identified starting at base position 261. Two additional ORFs were identified downstream (ORF2, initiating at base position 710) and upstream (ORF3) from ORF1. A potential ribosome-binding site for ORF2 was identified starting at base position 700.

Amino acid sequence comparison of the ORFs. The deduced amino acid sequences of the ORFs identified in the 1.2-kb *Xba*I fragment were compared with sequences of other proteins in the National Biomedical Research Foundation data base. The results showed significant homology between the ORF1 gene product and DGKase (19, 21) and between the ORF2 gene product and Era, a G protein from *E. coli* (1). No significant similarity between the putative ORF3 gene product and any other protein sequence was detected. As shown in Fig. 7, ORF1 showed 27.5% identical and 63.8% conservative amino acid sequence homology with the DGKase from *E. coli*, and the ORF2 gene product showed 44.3% identical and 82.8% conservative amino acid sequence homology with Era from *E. coli*.

Effect of Tn*916* insertion on sequences of ORF1 in GS5Tn1. Figure 5 depicts the changes of amino acid sequence of ORF1 following Tn*916* insertion. Tn*916* inserted in the 3′-terminal region of ORF1 and this ORF in GS5Tn1 lost five amino acids from its COOH terminus, and three amino acids at the right-end junction were also changed. The nucleotide changes at the left-end junction did not affect any of the ORFs.

Reconstruction of ORF1 in GS5Tn1 and characterization of GS5Tn1 revertant transformants. Figure 8 shows the strategy for reconstruction of incomplete ORF1 in GS5Tn1. The *Eco*RI-*Swa*I fragment from pHD1, which codes for the 3′ end of ORF1, was ligated into pVA891. The resultant plasmid, designated pES891, was transformed into GS5Tn1. Some of the erythromycin-resistant transformants obtained following allelic exchange were randomly picked and checked for appropriate incorporation of pES891 into chromosomal DNA by Southern blot analysis. The results with use of pCH3 as a probe demonstrated that GS5Tn1 showed a weak band at around 15 kb and a strong band at 8.0 kb (Fig.

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XbaI 10 20 30 40 50 60
TCTAGAATACAAACCAGAAAGCTCCCTTTCTTTTGATGGGGAAGACTTGGCCGACGATCC
 L E Y K P E S S L S F D G E D L A D D P

 70 80 90 100 110 120
AGATTTAGCCGAGGTGTTAACTGAATTTGATGCTTATATTGGTGAATTATTTATTTCTGT
 D L A E V L T E F D A Y I G E L F I S V

 130 140 150 160 170 180
CGATAAAGCTCGCGAGCAGGCGCAAGAATATGGTCACTCATTTGAGCGTGAGATGGGCTT
 D K A R E Q A Q E Y G H S F E R E M G F

 190 200 210 220 230 240
TTTGGCAGTACATGGCTTTTTACATATCAATGGTTATGACCACTACACTCCCCAAGAAGA
 L A V H G F L H I N G Y D H Y T P Q E E

 250 260 270 280 290 300
AAAAGAGATGTTTAGCTTACAGGAAGAGATATTAGATGCCTATGGACTTAAGAGATAATA
 K E M F S L Q E E I L D A Y G L K R

                ORF1 M P M D L R D N K
 310 320 330 340 EcoRI 360
AGCAAAGCCAAAAGAAATGGAAAAATAGAACTTTAACTTCCAGCCTTGAATTCGCTTTAA
 Q S Q K K W K N R T L T S S L E F A L T

 370 380 390 400 410 420
CGGGGATTTTTACGGCTTTTAAAGAAGAGCGTAATATGAAAAAACATGCAGTGTCAGCTC
 G I F T A F K E E R N M K K H A V S A L

 430 440 450 460 470 480
TTTTAGCTGTCATTGCTGGTTTGGTTTTTAAAGTATCAGTCATTGAGTGGCTTTTTCTTT
 L A V I A G L V F K V S V I E W L F L L

 490 500 510 520 530 540
TATTAAGTATTTTTTTGGTTATTACCTTTGAAATTGTCAATTCGGCTATTGAAAATGTGG
 L S I F L V I T F E I V N S A I E N V V

 MscI 560 570 580 590 PstI
TTGATTTGGCCAGTGACTATCATTTTTCCATGTTGGCTAAAAATGCTAAGGATATGGCTG
 D L A S D Y H F S M L A K N A K D M A A

 610 620 630 640 650 660
CAGGAGCTGTTCTTGTCATTTCAGGTTTTGCTGCCTTGACAGGCTTGATTATTTTTATAC
 G A V L V I S G F A A L T G L I I F I P

 670 680 690 700 710 SwaI
CAAAAATTTGGTTTTTGCTTTTTCATTAAAAATAGATAGAGGAGACATCATGTCATTTAA
 K I W F L L F H

                ORF2 M S F K
 730 740 750 760 770 780
ATCAGGATTTGTAGCTATTTTAGGTCGTCCCAATGTTGGGAAATCGACCTTTTTAAATCA
 S G F V A I L G R P N V G K S T F L N H

 790 800 810 820 830 840
TGTCATGGGGCAAAAAATTGCTATCATGTCTGATAAGGCACAAACAACCCGTAATAAAAT
 V M G Q K I A I M S D K A Q T T R N K I

 850 860 870 880 890 900
TATGGGAATTTATACTACAGATAAAGAGCAAATTGTTTTTATTGATACGCCTGGTATTCA
 M G I Y T T D K E Q I V F I D T P G I H

 910 920 930 940 950 960
TAAACCGAAAACTGCCTTAGGTGATTTTATGGTTGAGTCTGCCTATTCAACCCTGCGTGA
 K P K T A L G D F M V E S A Y S T L R E

 970 980 KpnI 990 1000 1010 1020
AGTGGACACAGTTCTTTTTATGGTACCTGCTGATGAAAAGCGGGGTAAAGGCGACAATAT
 V D T V L F M V P A D E K R G K G D N M

 1030 1040 KpnI 1060 1070 1080
GATTATAGAGCGTCTTAAAGCGGCCAAGGTACCTGTTATTTTGGTTATCAATAAGATTGA
 I I E R L K A A K V P V I L V I N K I D

 1090 1100 1110 1120 1130 1140
TAAGGTTCATCCGGATCAATTACTCGAACAGATTGATGATTTTAGAAATCAAATGGATTT
 K V H P D Q L L E Q I D D F R N Q M D F

 1150 1160 1170 1180 1190 BglII
TCAAGAAATTGTCCCCATTTCTGCCTTGCAAGGCAATAATGTCAGTCATCTGGTAGATCT
 Q E I V P I S A L Q G N N V S H L V D L

 1210 XbaI
TTTGGTAGATCATCTAGA
 L V D H L
```

FIG. 6. Nucleotide sequence of the intact 1,218-bp *Xba*I fragment disrupted by the Tn*916* insertion in GS5Tn1 and deduced amino acid sequences of the three ORFs located in this region, ORF1 (bases 276 to 686), ORF2 (bases 710 to 1218), and ORF3 (bases 1 to 295). The fragment corresponds to the open bar shown in Fig. 4C.

9, lane 1). In the correct allelic exchange, the lower band should not be altered, the larger band should move to a slightly lower position at approximately 12 kb, and a new third band of approximately 7.0 kb should appear. As shown in Fig. 9, three of four transformants yielded the expected pattern for correct allelic exchange of pES891 into chromosomal DNA; these were designated GS5Tn11.

```
A
ORF1   MPMDLRDNKQSQKKWKNRTLTSSLEFALTGIFTAFKEERNMKKHAVSALLAVIAGLVFKV  60
DGKase          MANNTTGFTRIIKAAGYSWKGLRAAWINEAAFRQEGVAVLLAVVIACWLDV  51

       SVIEWLFLLLSIFLVITFEIVNSAIENVVD-LASDYHFSMLAKNAKDMAAGAVLVISGFA 119
       DAITRVLLISSVMLVMIVEILNSAIEAVVDRIGSEYH--ELSGRAKDMGSAAVLIAIIVA 109

       ALTGLIIFIPKIWFLLFH
       VITWCILLWSHFG

B
ORF2   MSF-KS--GFVAILGRPNVGKSTFLNHVMGQKIAIMSDKAQTTRNKIMGIYTTDKEQIVF  57
Era    MSIDKSYCGFIAIVGRPNVGKSTLLNKLLGQKISITSRKAQTTRHRIVGIHTEGAYQAIY  60

       IDTPGIH-KPKTALGDFMVESAYSTLREVDTVLFMVPADEKRGKGDNMIIERLKAAKVPV 116
       VDTPGLHMEKKRAINRLLNNKAASSSIGDVELVIFVV-EGTRWTPDDEMVLNKLREGKAPV 119

       ILVINKIDKVH-PDQLLEQIDDFRNQMDFQEIVPISALQGNNVSHLVDLLVDHL 169
       ILAVNKVDNVQEKADLLPHLQFLASQMNFLDIVPISAETGLNVDTIAAIVRKHL 173
```

FIG. 7. Deduced amino acid sequence homology between the ORF1 gene product and DGKase from *E. coli* (A) and between the ORF2 gene product and Era from *E. coli* (B). Amino acids are numbered on the right. Asterisks and periods indicate identical and conservative amino acids, respectively.

To clarify whether ORF1 or ORF2 is important in acid stress adaptation, the properties of GS5Tn1 and GS5Tn11 (the ORF1 revertant transformant) were characterized. While GS5Tn1 failed to grow below pH 5.5 in TH broth, the acid tolerance of GS5Tn11 recovered to almost the same

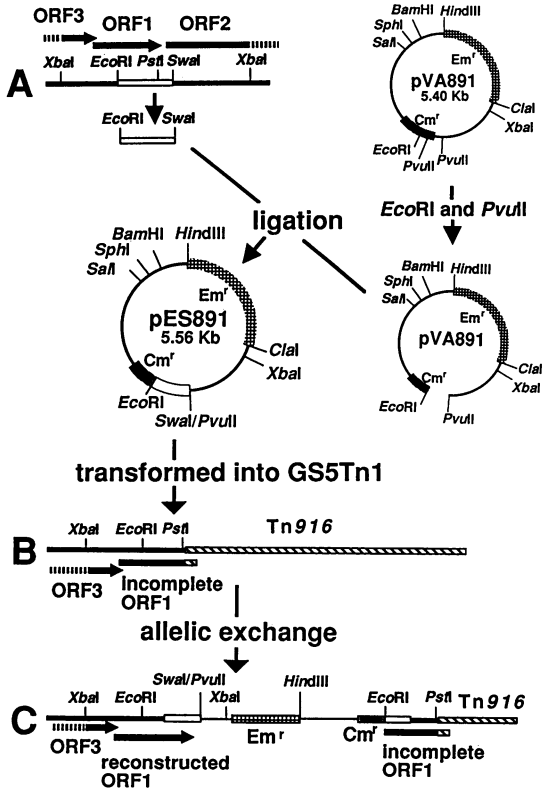

FIG. 8. Strategy for reconstruction of an incomplete ORF1 in GS5Tn1. (A) *Xba*I fragment containing the complete ORF1 from pHD1; (B) chromosomal DNA of GS5Tn1; (C) chromosomal DNA of GS5Tn11. Thick closed bars and those with arrows represent ORFs in *S. mutans*; striped bars indicate continuation of the amino acid sequences of ORF2 and ORF3.

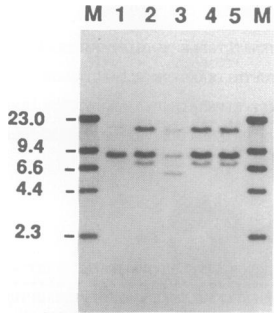

FIG. 9. Southern blot analysis of GS5Tn1 transformed by pES891, using digoxigenin-labeled pCH3 as a probe. Lanes: 1, *Hin*dIII-digested chromosomal DNA of GS5Tn1; 2 to 5, *Hin*dIII-digested chromosomal DNA of GS5Tn1 transformants (pES891); M, *Hin*dIII-digested and digoxigenin-labeled lambda DNA probe. Numbers on the left are size markers (kilobases).

level as did that of GS5DD (Fig. 1 and Table 2). It is of interest that GS5Tn1 had lost not only acid tolerance but also adaptability to environmental changes other than pH (Tables 3 and 4). GS5Tn1 lost its adaptability to high osmotic pressure as well as elevated temperatures relative to GS5DD; by contrast, GS5Tn11 recovered the same level of adaptability to high osmotic pressure and high temperature as did GS5DD (Tables 3 and 4).

DISCUSSION

The conjugative transposon Tn*916* was originally identified in *Enterococcus faecalis* DS16, and Gawron-Burke and Clewell proposed a strategy for its use in targeting gram-positive bacterial genes for cloning in *E. coli* (9). Recently, cloning of a mutacin-associated gene from *S. mutans* was accomplished by this strategy, and its utility for *S. mutans* was confirmed (6). In the present study, mutant GS5Tn1 defective in aciduricity was isolated following screening of 6,000 Tn*916* transformants. The mutant failed to grow below pH 5.5 in liquid broth (Table 2); interestingly, several physiological changes in *S. mutans* related to acid tolerance have been reported to occur at pH 5.5 (5, 12, 17). In addition, mutant GS5Tn1 exhibited decreased adaptability to high osmolarity and elevated temperatures (Tables 3 and 4) as a result of insertion of Tn*916* into the chromosome of GS5 (Fig. 2). Considering the properties of GS5Tn1, this mutant may be extremely useful in the characterization of environmental adaptability of *S. mutans*.

Sequence analysis of the 1.2-kb *Xba*I fragment including the original target site for Tn*916* (Fig. 6) demonstrated three putative ORFs (ORF1, ORF2, and ORF3) which appear to be transcribed in the same direction and may be part of a single operon. In mutant GS5Tn1, Tn*916* appeared to insert near the 3′ terminus of ORF1. It is likely, but not yet confirmed, that this ORF corresponds to the DGKase gene of *S. mutans*. There are several possible explanations for the properties of the mutant following Tn*916* insertion into ORF1. Since a potential promoter sequence exists at the right-end terminus of Tn*916* and hyperexpression of some downstream gene could occur as described previously (7), the properties of GS5Tn1 could result from induction of a GS5 protein. However, an appropriate ORF was not detected in the chromosome of GS5Tn1 immediately downstream from the Tn*916* right-end junction, and this possibility appears unlikely. Alternatively, the Tn*916* insertion may interrupt the transcription of an operon, and subsequently gene expression of ORFs locating downstream from the ORF1 may be repressed. To evaluate this hypothesis, insertional inactivation of ORF2 was attempted. Although linearized plasmid containing inactivated ORF2 constructed in *E. coli* was used for transforming GS5, all of the transformants isolated resulted from integration of contaminant intact plasmid DNA into the chromosome (data not shown). In addition, the *era* gene of *E. coli* was shown to be required for normal growth (25). These results suggest that the ORF2 gene product may be essential for the growth of *S. mutans*. Since no promoter-like sequences could be detected between ORF1 and ORF2 (Fig. 6), it is likely that ORF2 is expressed from a promoter located at the left-end terminus of Tn*916*. The fact that potential promoters exist at both right and left ends of the transposon needs to be considered when mutants constructed by this strategy are evaluated.

TABLE 2. Effect of pH on bacterial growth of GS5Tn1, GS5Tn11, and GS5DD

Final pH[a] (mean ± SD)	Bacterial growth[b] (mean ± SD)		
	GS5Tn1	GS5Tn11	GS5DD
7.48 ± 0.02	1.213 ± 0.096	1.279 ± 0.034	1.333 ± 0.048
6.85 ± 0.01	1.050 ± 0.186	1.241 ± 0.048	1.284 ± 0.039
6.57 ± 0.02	0.819 ± 0.299	1.180 ± 0.072	1.210 ± 0.083
6.27 ± 0.02	0.474 ± 0.324	1.133 ± 0.191	1.146 ± 0.078
5.92 ± 0.01	0.206 ± 0.177	1.073 ± 0.060	1.042 ± 0.103
5.74 ± 0.01	0.082 ± 0.075	0.995 ± 0.143	0.993 ± 0.190
5.57 ± 0.02	0.028 ± 0.006	0.914 ± 0.107	0.970 ± 0.068
5.45 ± 0.01	0.028 ± 0.005	0.862 ± 0.144	0.813 ± 0.170
5.34 ± 0.00	0.027 ± 0.003	0.735 ± 0.184	0.748 ± 0.257

[a] TH broth containing 50 mM sodium acetate buffer at the indicated pH values was prepared for measurement of the final pH of each preparation. Three preparations of TH broth were made and mean values were obtained from independent triplicates for each.

[b] Determined as the increase of OD_{550}. The values were obtained by subtraction of OD_{550} at initiation of culture from that at stationary phase.

TABLE 3. Effect of NaCl concentration on bacterial growth of GS5Tn1, GS5Tn11, and GS5DD

NaCl concn (mM) in TH broth	Bacterial growth[a] (mean ± SD)		
	GS5Tn1	GS5Tn11	GS5DD
0	1.199 ± 0.008	1.317 ± 0.033	1.411 ± 0.018
0.2	0.676 ± 0.002	1.037 ± 0.028	1.129 ± 0.012
0.3	0.328 ± 0.054	0.811 ± 0.021	0.995 ± 0.041
0.4	0.049 ± 0.004	0.683 ± 0.008	0.779 ± 0.002
0.5	0.015 ± 0.002	0.553 ± 0.023	0.640 ± 0.015

[a] Determined as described in Table 2, footnote *b*.

TABLE 4. Effect of temperature on bacterial growth of GS5Tn1, GS5Tn11, and GS5DD

Temp (°C) of incubation in TH broth	Bacterial growth[a] (mean ± SD)		
	GS5Tn1	GS5Tn11	GS5DD
37	1.253 ± 0.062	1.340 ± 0.026	1.463 ± 0.033
43	0.675 ± 0.013	1.150 ± 0.008	1.021 ± 0.013
44	0.206 ± 0.003	0.781 ± 0.009	0.624 ± 0.026
45	0.084 ± 0.015	0.379 ± 0.029	0.310 ± 0.023
46	0.036 ± 0.036	0.129 ± 0.006	0.101 ± 0.005

[a] Determined as described in Table 2, footnote *b*.

The most likely explanation for the behavior of mutant GS5Tn1 was based on the results of the complementation experiment (Fig. 8). The recovery of environmental adaptability observed in GS5Tn11 clearly demonstrated the role of the ORF1 gene product in this process. The ORF1 gene product has significant homology with the DGKase from *E. coli* (Fig. 7A). DGKase catalyzes the ATP-dependent phosphorylation of *sn*-1,2-diacylglycerols and generates phosphatidic acid. This pathway represents a minor source of phosphatidic acid in *E. coli* (27); the physiological function of the enzyme de novo has not yet been elucidated. Rotering and Raetz reported that diacylglycerol was involved in minor neutral lipid metabolism in *E. coli* (28), and more recent data suggest that diacylglycerol may play a role in signal transduction in *E. coli* as well as in eukaryotic cells (33, 34). Although no information concerning diacylglycerol metabolism in *S. mutans* is available, our current findings suggest that ORF1 encodes an enzyme similar to DGKase and that the diacylglycerol-phosphatidic acid system may be involved in signal transduction of extracellular environmental signals in *S. mutans*. Furthermore, it is of interest that ORF1 and ORF2 may be part of the same operon and that the latter gene appears to code for a G protein similar to the *E. coli era* gene product (25). It will be of interest to examine the possible interaction of the two gene products as a potential mechanism for environmental adaptability. Alternatively, a defect in ORF1 may lead to abnormal membrane function. Such an alteration could also affect the response of *S. mutans* to environmental stress. However, little information is currently available regarding phospholipid metabolism in *S. mutans*. Therefore, additional work will be necessary to decide between these interesting regulatory possibilities. As discussed above, GS5Tn1 appears to provide a desirable model for characterization of the molecular mechanism of environmental adaptability in bacteria. Characterization of the putative operon in which ORF1 and ORF2 are found and further evaluation of GS5Tn1 are now in progress.

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