Oral Microbiology Immunology 2009: 24: 211–217
Printed in Singapore. All rights reserved

ORAL MICROBIOLOGY AND IMMUNOLOGY

Molecular and biological characterization of *gtf* regulation-associated genes in *Streptococcus mutans*

Y. Terao[1], R. Isoda[1], J. Murakami[2], S. Hamada[3], S. Kawabata[1]

[1]Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan, [2]Division of Special Care Dentistry, Osaka University Dental Hospital, Suita, Osaka, Japan, [3]Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan

Terao Y, Isoda R, Murakami J, Hamada S, Kawabata S. Molecular and biological characterization of gtf *regulation-associated genes in* Streptococcus mutans*. Oral Microbiol Immunol 2009: 24: 211–217.* © 2009 John Wiley & Sons A/S.

Introduction: Surface protein antigen (PAc) and glucosyltransferases (GTF) are major adhesive molecules of *Streptococcus mutans*, though the mechanism of their regulation has not been fully elucidated.
Methods: To investigate the regulation mechanism, we determined a nucleotide sequence in the upstream region of the *pac* locus in *S. mutans* and identified two open reading frames (ORF), designated as *orf1* and *orf2*. Each ORF was inactivated and functional analyses were performed.
Results: Western blot analyses revealed that the expression level of PAc was unaffected, while that of cell-associated GTF was diminished in both mutant strains. Furthermore, they showed higher hydrophobicity levels and an impaired sucrose-dependent adherence to smooth surfaces. RNA dot blot analysis demonstrated that transcriptions of the *gtfB* and the *gtfC* genes, which encode GTF-I and GTF-SI, respectively, were downregulated, while that of *pac* was comparable to the wild-type strain. In addition, the GTF activities of the mutant strains were significantly lower than those of the wild-type, though a greater amount of total glucan produced by the mutants was noted in culture supernatants.
Conclusion: These findings suggest that *orf1* and *orf2* are associated with positive regulation of the *gtfB* and *gtfC* genes.

Key words: *Streptococcus mutans*; glucosyltransferase; gene regulation

Shigetada Kawabata, Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan
Tel.: +81 6 6879 2896;
fax: +81 6 6879 2180;
e-mail: kawabata@dent.osaka-u.ac.jp

Accepted for publication November 3, 2008

Streptococcus mutans is a major causative agent of human dental caries and its ability to adhere to tooth surfaces is considered to be central to the pathogenic capability of this organism (6). In addition, a number of functional molecules on the cell surface of *S. mutans* have been identified, including cell-associated glucosyltransferase (GTF) and the adhesin PAc.

GTFs catalyze the synthesis of adhesive glucans from sucrose and *S. mutans* is known to harbor at least three different types of GTFs; GTF-I, GTF-SI and GTF-S, which are encoded by the *gtfB*, *gtfC* and *gtfD* genes, respectively. Cell-associated GTF-I is responsible for the generation of water-insoluble glucans and GTF-SI, which is also cell-associated, catalyzes the synthesis of both water-soluble and water-insoluble glucans, while GTF-S is secreted into culture supernatant and synthesizes water-soluble glucans (4, 24). It remains unknown how *S. mutans* uses the different GTFs; however, glucan-mediated binding is considered to provide a strong adherence to tooth surfaces for *S. mutans*. In addition, another important role of glucan is the development of biofilm, which is often referred to as dental plaque.

PAc is a 190-kDa surface protein of *S. mutans* and similar functional proteins are also expressed by other oral streptococci (14, 16). Independent research groups have identified this molecule so it is also referred to as P1 (12), B (18) and antigen I/II (19). PAc binds to the salivary agglutinin contained in the pellicle that often coats teeth in the oral cavity, thereby enabling *S. mutans* to adhere to tooth surfaces. Although PAc has drawn the interest of many researchers since being

identified, little is known regarding how its expression is regulated by *S. mutans*. In *Streptococcus sobrinus*, the negative regulatory gene *par* exists upstream of the *pag* operon and the *pag* gene shows a high homology to *S. mutans pac* (23). Therefore, we speculated that *pac* regulatory genes exist upstream of the *pac* operon in *S. mutans*. In the present study, we determined the nucleotide sequence of the region and identified two open reading frames (ORFs), then investigated the functions of these putative regulatory genes by constructing gene-targeted mutant strains.

Materials and methods

Bacterial cells and culture conditions

S. mutans strain MT8148 (serotype *c*) was isolated from a Japanese patient and used as a wild-type control strain (4, 24). Unless specified elsewhere, brain–heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) was used as the base culture medium. For selection of transformed *S. mutans* strains, kanamycin was added to a final concentration of 30 μg/ml. To prepare intermediate plasmid constructs, *Escherichia coli* strain XL-10 Gold (Stratagene, La Jolla, CA) was used and grown in Luria–Bertani broth (Sigma, St Louis, MO) or on Luria–Bertani agar plates supplemented with 100 μg/ml of ampicillin.

Manipulation of DNA and RNA

Genomic DNA from *S. mutans* was purified using a Puregene DNA isolation kit (Qiagen, Düsseldorf, Germany) and transformation of *S. mutans* was performed as described previously (8). Plasmid DNA preparation, transformation of *E. coli*, and polymerase chain reaction (PCR) were performed as previously described (20). DNA sequencing was carried out using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 DNA sequencer (Applied Biosystems). RNA preparations from *S. mutans* and RNA dot blot analysis were performed as described previously (23). The probes used were amplified by PCR with the primers listed in Table 1.

Determination of nucleotide sequence upstream of the *pac* gene

Genomic DNA was digested with either *Hin*dIII or *Eco*RI. The resultant fragments were then subcloned into a pUC19 cloning vector that had been digested with either *Hin*dIII or *Eco*RI to construct genomic DNA libraries. These libraries were screened with a *pac*-specific probe to select positive clones. The resultant clones that possessed the upstream region of the *pac* gene were sequenced as described previously (20).

ORF analysis

Potential ORFs were initially identified using GeneWorks version 2.4 (IntelliGenetics, Mountain View, CA). The ORF data were then analyzed using a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/), with the resultant ORFs designated as *orf1* and *orf2*. Sequence data for these ORFs were submitted to DDBJ/EMBL/GenBank under accession no. AB040534, during which time we renamed *orf2* as *rgtB*.

Construction of inactivated mutants

Coding regions of the *orf1* and *orf2* genes were amplified by PCR using appropriate primer sets, which are listed in Table 1. Each of the PCR products was purified by electrophoresis on agarose gel and subcloned into a pGEM-T vector (Promega, Madison, WI) to give rise to the circular plasmids pYT1071 and pYT1072 for *orf1* and *orf2*, respectively, based on the manufacturer's instructions. To construct targeting plasmids for inactivation of the *orf1* and *orf2* genes, pYT1071 and pYT1072 were digested with *Hin*dIII and ligated with a kanamycin-resistant gene, *aphA3*, with the new constructs designated as pYT1082 and pYT1079, respectively. These plasmids were then linearized by *Apa*I digestion and introduced to *S. mutans* strain MT8148, to inactivate genomic *orf1* and *orf2* through homologous recombination.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blot analyses

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting were carried out as described previously (5). Blotted membranes were reacted with rabbit anti-GTF-I/SI immunoglobulin G (IgG), which recognized both GTF-I and GTF-SI or anti-GTF-S IgG, followed by swine antirabbit IgG conjugated with alkaline phosphatase (Dakopatts, Glostrup, Denmark).

Hydrophobicity

Surface hydrophobicity was determined as described previously (8). Briefly, lyophilized bacterial cells were suspended in PUM buffer (100 mM sodium phosphate buffer, pH 7.1; 30 mM urea; 0.8 mM $MgSO_4$) at a final concentration of 0.6 mg/ml and mixed with *n*-hexadecane. Following vigorous shaking, the optical densities of the aqueous phases were measured at 550 nm (OD_{550}).

Adherence to a smooth surface

A sucrose-dependent adhesion assay was performed as described by Kawabata and Hamada (8). Briefly, 25 μl of precultured

Table 1. Primers used in this study

Gene	Primer	Sequence(5′ to 3′)
orf1	orf1/F1	GGAGCTGCATCTGTTTTAATTGG
	orf1/R1	CGACGAATACCACTGTAGGAACG
orf2	orf2/F3	CGACGAATACCACTGTAGGAACG
	orf2/R1	ATGAATTTTAGTATGAATAAC
Pac	pac/F1	CACTGTGTGGTGCTGTTCTAGG
	pac/R1	CAGCCTGATAAGGCAAGGGC
gtfB	gtfB/F1	CGACAGCAATTAGACTGT
	gtfB/R1	TTAGTACGAACTTTGCCG
gtfC	gtfC/F1	GCAACATCTCAACCAACC
	gtfC/R1	ATCTGTACTATAGACCTG
gtfD	gtfD/F1	AGAACAGCAGACCTCAGA
	gtfD/R1	TTATGACAGGCTCAAGTC
gbp	gbp/F1	CTCTGAAGAGCACCATCC
	gbp/R1	TATAGAACCAGTCTGTCG
ftf	ftf/F1	GTACACCGGAAGTAGGTC
	ftf/R1	CTTCCATCTTCATTTACGG
dexA	dexA/F1	GCGTTATGCTGCTATTGG
	dexA/R1	CTCTGCCATAGATACACC
aga	aga/F1	CCTGCCAGCCATTATGATGTG
	aga/R1	CGTTGCGACCGCCACCTCCTG
msmR	msmR/F1	ACTGCCATACTGCGAATG
	msmR/R1	TTGATCACTACGGTTCAG
recA	recA/F1	CCGGAATCTTCTGGTAAG
	recA/R1	CTAATTCACCTGTACGAG
aphA3	aphA3/R2	GGGATGAAGCCTGATTGGGAG

suspension was inoculated into 5 ml BHI broth supplemented with 1% sucrose and cultured for 18 h at 37°C at a 30° angle, after which the cell suspension was transferred to another glass tube (fraction A). A 5-ml quantity of sterile saline was added to the tube and then it was subjected to vortexing for 3 s, after which the mixture was transferred to a new glass tube (fraction B). Finally, to dissociate bacterial cells that had tightly adhered to the glass tube, another 5-ml quantity of sterile saline was added to the tube and adherent cells were dissociated completely using a sonicator (fraction C). The turbidity of each fraction was determined at OD_{550} and per cent adhesion of the test bacteria was defined by the result of C/(A+B+C) (with A, B and C representing the turbidity of fractions A, B and C, respectively). Each of the turbidity values was justified before calculation by subtracting the appropriate background (OD_{550} of BHI medium or saline without bacterial cells), which was measured beforehand.

GTF activities

GTF activities on *S. mutans* cell surfaces and in culture supernatants were determined as described previously (9, 10). Test strains were cultured in 5 ml BHI broth for about 18 h at 37°C until the OD_{550} value reached 0.8. The culture supernatant and cellular portion were separated by centrifugation, and then the cellular portion was washed twice and suspended in 5 ml phosphate-buffered saline. Ten-microliter samples from the supernatant and the cell suspension were each reacted with 10 μl 0.2 M potassium phosphate buffer (pH 6.8) containing 20 mM [^{14}C-glucose]sucrose (1.85 GBq/mol; MP Biomedicals, Solon, OH) for 1 h at 37°C, after which the reaction mixture was adsorbed onto a piece of filter paper. The sample was then dehydrated and washed three times with methanol, and used as a total glucan sample. Another set of samples was similarly prepared, but was washed with distilled water in place of methanol. These samples served as insoluble glucan samples. Synthesized [^{14}C]glucan was measured using a liquid scintillation counter (Aloka Co. Ltd, Mitaka, Japan).

Statistical analysis

Values are shown as the mean ± standard error. Significant differences were estimated with a Mann–Whitney *U*-test. For this study, findings of $P < 0.05$ were considered significant.

Results

Analysis of genomic region neighboring *pac* gene

We determined the full DNA sequence of the upstream region of the *pac* gene on the chromosome of *S. mutans* strain MT8148 and investigated further for the presence of putative *pac* regulatory genes. As a result, two ORFs, designated *orf1* and *orf2*, were identified, as shown in Fig. 1A. Next, we attempted to define the functions of these genes through analysis of the deficient mutant strains of one of the two genes. The genes were inactivated by inserting a kanamycin-resistance gene, *aphA3*, into the target gene, and the resulting inactivated mutants of *orf1* and *orf2* were designated as TR-15.1 and TR-15.2, respectively. Insertion of the *aphA3* gene was confirmed by PCR, as shown in Fig. 1B. PCR assays with different genomic DNA preparations provided a single specific band only when the proper primer set was used, i.e. *aphA3*/R2 and *orf1*/R1 for the TR-15.1 genomic template, and *aphA3*/R2 and *orf1*/R2 for that of TR-15.2 (Fig. 1C). Template DNA from the wild-type strain MT8148 did not yield any PCR products, indicating that our PCR system was reliable for detecting the *aphA3* insert.

Expression levels of PAc, GTF-I/SI and GTF-S

We speculated that *orf1* and *orf2* are regulatory genes for PAc expression so the protein expression levels of PAc in TR-15.1 and TR-15.2 were examined by Western blotting using a PAc-specific primary antibody (Fig. 2A, B). There were no significant differences between the wild-type and deficient mutant strains with regard to PAc expression level in the 8 M urea extracts of the cells or culture supernatants. The method of 8 M urea extraction used is able to isolate various streptococcal surface proteins, such as GTF-I, GTF-SI, FbaA, FbaB and Lbp (5, 26–28). In the Western blotting analyses, the densities of specific bands became lower in lanes that represented cellular samples from strains TR-15.1 and TR-15.2, as compared to the wild-type strain, when the anti-GTF-I/SI

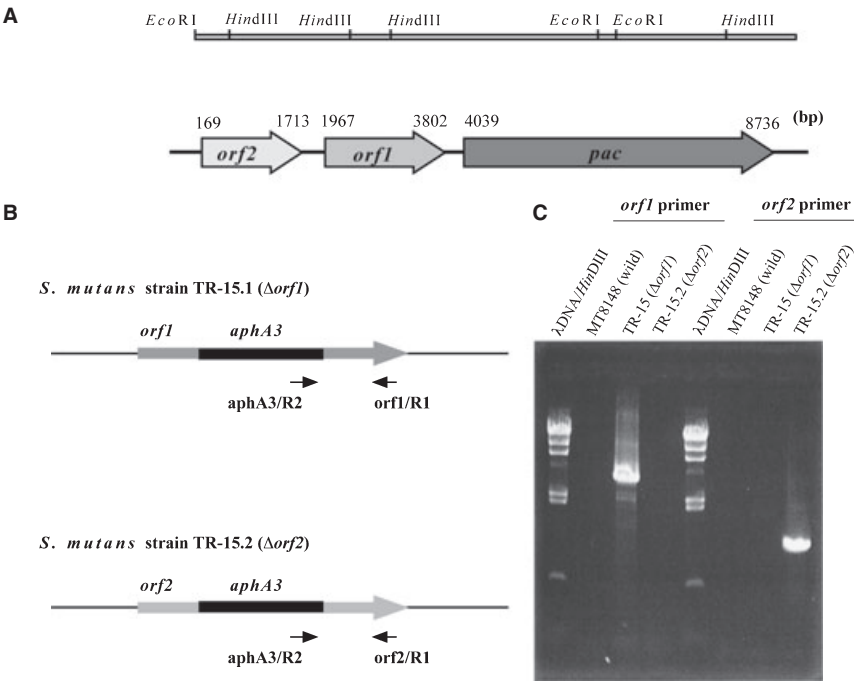

Fig. 1. Identification of putative regulatory genes upstream of the *pac* region in *Streptococcus mutans* strain MT8148 and inactivation of those genes. (A) Physical map of the *S. mutans* strain MT8148 chromosome neighboring the *pac* gene (top). The nucleotide sequence upstream of the *pac* gene was determined as described in the Materials and methods section. Two open reading frames (ORFs), *orf1* and *orf2*, were found located upstream of the *pac* gene by ORF analysis (bottom). (B) Each ORF was inactivated by insertion of the *aphA3* gene, which was confirmed by polymerase chain reaction (PCR) assay results. The forward primer *aphA3*/R2 was constructed within the inserted sequence and the reverse primers, *orf1*/R1 and *orf2*/R1, were designed in proximity of the C-terminus of *orf1* and *orf2*, respectively. (C) Analysis of PCR products by agarose gel electrophoresis. Specific bands corresponding to the PCR products were obtained from the mutant strains only when the appropriate specific primers were used.

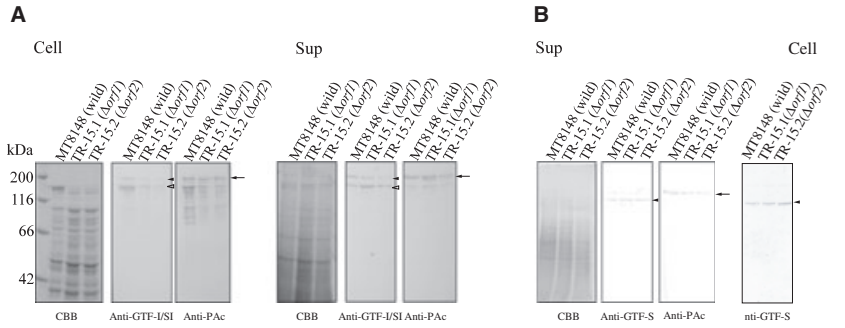

Fig. 2. Phenotypic analyses of *orf1*- and *orf2*-inactivated strains. (A) Protein expressions of GTF-I/SI and PAc were examined by Western blotting using anti-GTF-I/SI and anti-PAc specific antibodies. 'Cell' indicates the cellular portion that was extracted with 8 M urea from the bacterial cells. 'Sup' represents the culture supernatant from the *Streptococcus mutans* strains. (B) Western blot analyses of culture supernatants with specific antibodies against GTF-S and PAc.

primary antibody was used in place of the anti-PAc primary antibody. These findings indicated that the expression level of GTF-I/SI was decreased in both mutant strains. We also examined the expression level of GTF-S protein in culture supernatants and cellular samples from these strains, however, failed to find any significant differences.

Surface hydrophobicity and sucrose-dependent adhesion to smooth surface

Cell-surface hydrophobicity analysis clearly revealed that the *orf1*- and *orf2*-deficient mutant strains had higher levels of hydrophobicity in contrast to the parental wild-type strain (Fig. 3A). Furthermore, sucrose-dependent adhesion was significantly ($P < 0.01$) lower for the *orf1*- and *orf2*-deficient strains as compared to the wild type strain (Fig. 3B).

RNA dot blotting

The effects of *orf1* and *orf2* inactivation on transcriptions of various genes including adhesion molecules were examined by RNA dot blotting. The results revealed that the transcriptional levels of *pac* were apparently the same in the wild-type and the mutants. On the other hand, transcriptions of the *gtfB* and *gtfC* genes to messenger RNA appeared to be suppressed, whereas that with the *gtfD* gene was not. It is important to note that inactivation of *orf1* did not change the transcriptional levels of neighboring *orf2* and the *pac* genes, and that *orf1* and the *pac* genes in the *orf2*-inactivated mutant strain were also not affected, indicating that inactivation of either *orf1* or *orf2* did not have polar effects on the neighboring genes. A panel of probes available in our laboratory was used to examine as many genes as possible; the examined genes and their coding proteins are listed in Fig. 4. The *recA* and *gbp* genes were downregulated only in the *orf2*-inactivated strain, while the remaining genes were not affected.

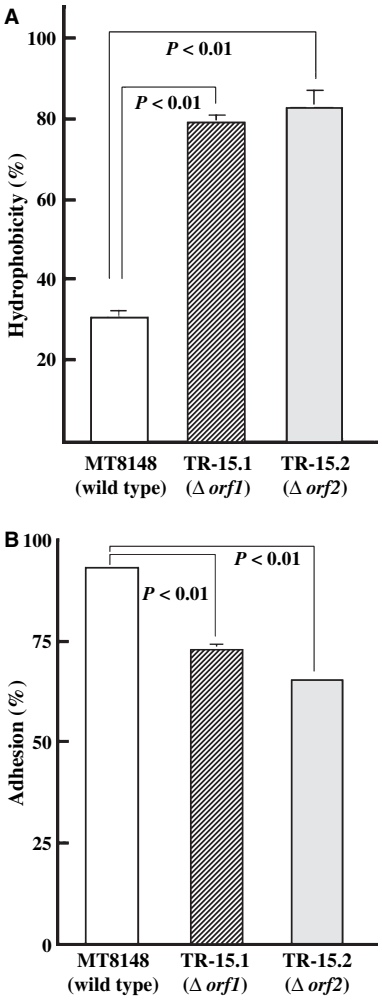

Fig. 3. Additional phenotypic analyses of the *orf1*- and *orf2*-inactivated strains. (A) Hydrophobicity and (B) sucrose-dependent adhesion to a smooth surface were examined as described in the Materials and methods section.

GTF activity

Finally, the comprehensive GTF activities of the mutant and wild-type strains were evaluated. Overall, inactivation of *orf1* and *orf2* led to lower GTF activities for synthesizing insoluble glucan. In contrast, the GTF activities for total glucan synthesis were higher in the supernatants of the inactivated mutant strains as compared to the wild-type (Table 2).

Discussion

S. mutans is considered to be an important etiological agent of dental caries and has been intensively investigated in studies of various strains. From our work with strain MT8148, we have accumulated a vast amount of data from both *in vivo* and *in vitro* experiments. In 2002, the entire genome sequence of *S. mutans* strain UA159 was published (1) and has now become the standard strain for *S. mutans* investigations. When compared with SMU_609 of UA159, ORF1 of strain MT8148 exhibited a 97% homology at the nucleotide level and 96% homology at the amino acid level. Since *orf2* in MT8148 shares a 96% homology at the nucleotide level and 97% homology at the amino acid level with SMU_608 in UA159, we consider that the present findings are applicable to *S. mutans* strain UA159, which is the standard strain of *S. mutans*.

In the GenBank database, the SMU_608 locus of UA159 is registered as a 'putative peptide release factor' based on its homology to other streptococcal genes. Consequently, it is possible that *orf2* does not regulate *gtf* genes directly, but rather is involved in the maturation of *gtf*-regulatory genes such as peptide elongation. Furthermore, these *gtf*-regulatory genes may be global regulatory genes that control a number of different genes, which may also explain the finding in this study showing that the *recA* and the *gbp* genes appeared to be downregulated in the *orf2*-inactivated strain.

SMU_609 of UA159 is identical to the 40-kDa cell wall protein of *S. mutans* OMZ175, which was identified by Ogier et. al. (15) SMU_609 is currently annotated as a 'putative 40-kDa cell wall protein' based on their work; however, its function has not been specified. Recently, Catt et. al. (3) showed that the repeat area

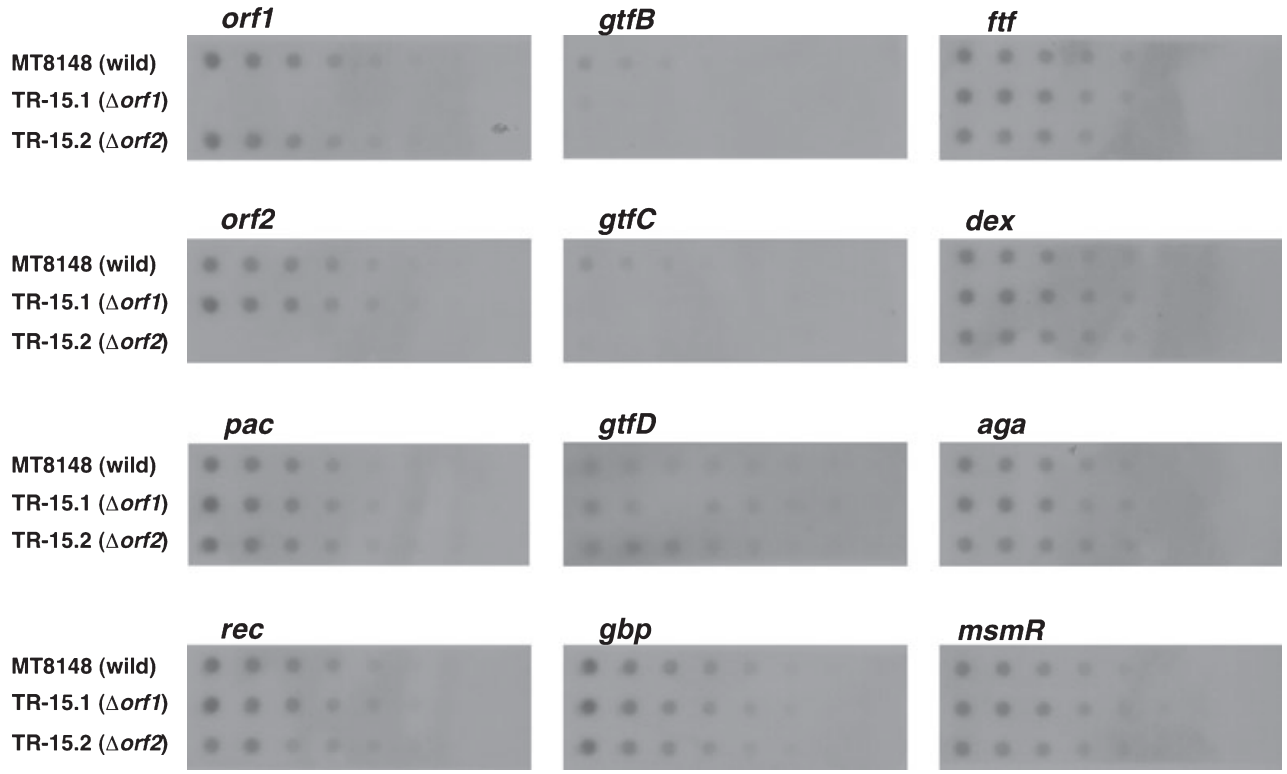

Fig. 4. Messenger RNA expression levels of various genes in *orf1*- and *orf2*-inactivated mutants as well as the wild-type were examined by RNA dot blotting. The genes examined are listed, with their coding proteins described in parentheses: *recA* (recombination protein RecA protein), *gtfB* [glucosyltransferase (GTF) –I], *gtfC* (GTF-SI), *gtfD* (GTF-S), *gbp* (glucan-binding protein), *ftf* (fructosyltransferase), *dexA* (dextranase precursor), *aga* (alpha-galactosidase) and *msmR* (MSM operon regulatory protein).

Table 2. Effects of *orf1* and *orf2* inactivation on glucosyltransferase activities

Strain	Total glucan	Insoluble glucan
Cell		
MT8148 (wild)	7469 ± 125	1069 ± 101
TR-15.1(*Δorf1*)	1757 ± 55	92 ± 6
TR-15.2(*Δorf2*)	1255 ± 13	86 ± 9
Sup.		
MT8148 (wild)	1703 ± 68	192 ± 6
TR-15.1(*Δorf1*)	2892 ± 189	137 ± 12
TR-15.2(*Δorf2*)	2516 ± 427	167 ± 19

Values are given as means ± SE in counts per minute, $n = 3$. Numbers shown represent the relative amount of radiolabeled glucans synthesized by either cell-associated GTFs (Cell) or free GTFs in the culture supernatant (Sup), with [^{14}C-glucose]sucrose used as the substrate. Samples used for measurements of total and insoluble glucans were prepared as described in the Materials and methods section.

of SMU_609 was translated to a 67-kDa cell surface protein SmaA, which possesses a YSIRK-signal sequence and LPXTG-cell anchoring motif. SmaA has a high homology to the *Streptococcus agalactiae* murein hydrolase Bsp, though it is still unclear if enzymatic activity is involved in gene regulation or whether Bsp is a multifunctional molecule (3). Since inactivation of *orf1* functioned similarly to *orf2* activation with regard to *gtf* regulation, *orf1* may also operate through the same intermediate regulator(s) as *gtf2*.

In the present inactivated mutant strains (TR-15.1 and TR-15.2), nearly all GTF-related phenotypic changes occurred parallel to the changes in transcriptions of the *gtfB* and the *gtfC* genes, i.e. the protein expression levels of GTF-I and GTF-SI were depressed, sucrose-dependent adherence was downregulated, and the amount of synthesized insoluble glucan was decreased, except for the activities of total glucan synthesis in the culture supernatant. That exception is difficult to explain, because the expression of GTF-S and transcription from the *gtfD* gene were unchanged in both mutant strains. A plausible explanation is an increase of GTF-S activity. Absence of the catalytic products of GTF-I and GTF-SI in the same system may confer advantages on GTF-S, such as better access to substrates or improved product clearance. Previously reported results support this possibility. Ooshima et al. suggested that the ratio of all three GTFs has effects on the sucrose-dependent adherence of *S. mutans* and the production of insoluble glucan (17). Furthermore, Tamesada et al. showed that GTF-SI has a strong effect on sucrose-dependent adhesion, even though insoluble glucan is considered to be a major constituent of adhesive glucan (25). These findings indicate that three GTFs can interact with each other and that the ratio of different GTFs affects the nature of the final glucan product.

Another intriguing result obtained from inactivation of *orf1* and *orf2* is the effect on surface hydrophobicity (Fig. 3A). Although the surface molecules that contribute to the hydrophobicity of *S. mutans* have not been completely identified (13), Koga et al. reported that PAc is a major factor in surface hydrophobicity (11). The *orf*-inactivated strains in this study carried the same levels of PAc as the wild-type, however, the mutants showed significantly higher levels of hydrophobicity. This may indicate that molecules other than PAc significantly contribute to the hydrophobicity of *S. mutans*, and those molecules are affected by the inactivation of *orf1* and *orf2*. As noted above, ORF1 has a high homology to SMU_609 of UA159 and it is

almost certain that SMU_609 is a cell surface protein, because of the existence of a YSIRK signal sequence and an LPXTG cell-anchoring motif. Therefore it is possible that ORF1 itself can be the molecule that contributes to the hydrophobicity in MT8148. It is also possible that PAc was not affected in quantity but was changed in quality: for example, the lack of a post-translational modification may be able to make PAc more hydrophobic.

Additional findings have been reported concerning the molecular mechanisms involved with regulation of *gtf* genes. Biswas recently found that CovR negatively regulates the expression of the *gtfB* and *gtfC* genes by directly binding to the promoter region of *S. mutans* strain UA159 (2). CovR is part of the CovRS two-component regulatory system in group A *Streptococcus*, whereas *S. mutans* CovR is reported to work as an orphan response regulator and is referred to in various ways, such as *GcrR* (21) or *TarC* (7). Furthermore, Senadheera et al. reported that the VicRK signal transduction system in *S. mutans* UA159 negatively regulated the *gtfBCD*, *gbp* and *ftf* genes (22). In contrast, scant information is available regarding the molecular mechanisms of *pac* regulation. The present results suggest that the *pac* regulatory system in *S. mutans* is more complicated than that in *S. sobrinus*, despite the high homology between *pac* and *pag* at the nucleotide sequence level (23). Although *S. sobrinus* possesses the negative regulatory gene *par* upstream of the *pag* gene, complete sequence determination of the upstream region of the *pac* gene did not provide ORFs other than *orf1* and *orf2*. Homologies between Par in *S. sobrinus* and ORF1/ORF2 at the amino acid level are low (11% for each protein), and the sequenced 2850-base area upstream of *pag* has a low homology (41%) to the nucleotide sequence in the corresponding area upstream of *pac*. Hence, there may not be any *cis* regulatory proteins for the *pac* gene. Nevertheless, Yamashita et al. reported a spontaneous variant of *S. mutans* strain Xc, in which expression of the *pac* gene was found to be downregulated (29). Therefore, a downregulating mechanism of *pac* in *S. mutans* must exist, though that study did not identify which genes were altered in the variant strain.

For *S. mutans*, the surface adhesin PAc and adhesive glucans are indispensable for competing against other oral bacteria for a niche, i.e. the tooth surface, in the oral cavity. It is quite reasonable for *S. mutans* to have redundant regulatory mechanisms to maintain high expression level of GTFs, as far as the energy supply allows. Further investigation is needed to identify how *S. mutans* uses PAc and GTFs for adherence to the tooth surface.

In conclusion, the present findings indicate that *orf1* and *orf2* are novel components involved in the regulation of GTFs in *S. mutans*. Furthermore, we also propose that *orf1* and *orf2* do not regulate *gtf* genes directly, but rather may be associated with some intermediate regulators of those genes. The present findings may contribute to a better understanding of the *gtf*-regulatory mechanism.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science, and a grant from the Naito Foundation.

References

1. Ajdic D, McShan WM, McLaughlin RE et al. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. Proc Natl Acad Sci USA 2002: **99**: 14434–14439.
2. Biswas S, Biswas I. Regulation of the glucosyltransferase (*gtfBC*) operon by CovR in *Streptococcus mutans*. J Bacteriol 2006: **188**: 988–998.
3. Catt DM, Gregory RL. *Streptococcus mutans* murein hydrolase. J Bacteriol 2005: **187**: 7863–7865.
4. Fujiwara T, Tamesada M, Bian Z, Kawabata S, Kimura S, Hamada S. Deletion and reintroduction of glucosyltransferase genes of *Streptococcus mutans* and role of their gene products in sucrose dependent cellular adherence. Microb Pathog 1996: **20**: 225–233.
5. Hamada S, Horikoshi T, Minami T et al. Oral passive immunization against dental caries in rats by use of hen egg yolk antibodies specific for cell-associated glucosyltransferase of *Streptococcus mutans*. Infect Immun 1991: **59**: 4161–4167.
6. Hamada S, Slade HD. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol Rev 1980: **44**: 331–384.
7. Idone V, Brendtro S, Gillespie R et al. Effect of an orphan response regulator on *Streptococcus mutans* sucrose-dependent adherence and cariogenesis. Infect Immun 2003: **71**: 4351–4360.
8. Kawabata S, Hamada S. Studying biofilm formation of mutans streptococci. Methods Enzymol 1999: **310**: 513–523.
9. Kawabata S, Torii M, Minami T, Fujiwara T, Hamada S. Effects of selected surfactants on purified glucosyltransferases from mutans streptococci and cellular adherence to smooth surfaces. J Med Microbiol 1993: **38**: 54–60.
10. Koga T, Asakawa H, Okahashi N, Hamada S. Sucrose-dependent cell adherence and cariogenicity of serotype *c Streptococcus mutans*. J Gen Microbiol 1986: **132**: 2873–2883.
11. Koga T, Okahashi N, Takahashi I, Kanamoto T, Asakawa H, Iwaki M. Surface hydrophobicity, adherence, and aggregation of cell surface protein antigen mutants of *Streptococcus mutans* serotype *c*. Infect Immun 1990: **58**: 289–296.
12. Lee SF, Progulske-Fox A, Bleiweis AS. Molecular cloning and expression of a *Streptococcus mutan*s major surface protein antigen, P1 (I/II), in *Escherichia coli*. Infect Immun 1988: **56**: 2114–2119.
13. McBride BC, Song M, Krasse B, Olsson J. Biochemical and immunological differences between hydrophobic and hydrophilic strains of *Streptococcus mutans*. Infect Immun 1984: **44**: 68–75.
14. Navarre WW, Schneewind O. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. Microbiol Mol Biol Rev 1999: **63**: 174–229.
15. Ogier JA, Scholler M, Lepoivre Y, Gangloff S, M'Zoughi R, Klein JP. A 40-kilodalton cell wall protein-coding sequence upstream of the *sr* gene of *Streptococcus mutans* OMZ175 (serotype f). Infect Immun 1991: **59**: 1620–1626.
16. Okahashi N, Sasakawa C, Yoshikawa M, Hamada S, Koga T. Cloning of a surface protein antigen gene from serotype *c Streptococcus mutans*. Mol Microbiol 1989: **3**: 221–228.
17. Ooshima T, Matsumura M, Hoshino T, Kawabata S, Sobue S, Fujiwara T. Contributions of three glycosyltransferases to sucrose-dependent adherence of *Streptococcus mutans*. J Dent Res 2001: **80**: 1672–1677.
18. Russell RR. Wall-associated protein antigens of *Streptococcus mutans*. J Gen Microbiol 1979: **114**: 109–115.
19. Russell MW, Lehner T. Characterisation of antigens extracted from cells and culture fluids of *Streptococcus mutans* serotype *c*. Arch Oral Biol 1978: **23**: 7–15.
20. Sambrook J, Russell D. Molecular Cloning: A Laboratory Manual. 3rd edn. Woodbury, NY: Cold Spring Harbor Laboratory Press, 2001: Chapter 1 and Chapter 8.
21. Sato Y, Yamamoto Y, Kizaki H. Construction of region-specific partial duplication mutants (merodiploid mutants) to identify the regulatory gene for the glucan-binding protein C gene *in vivo* in *Streptococcus mutans*. FEMS Microbiol Lett 2000: **186**: 187–191.
22. Senadheera MD, Guggenheim B, Spatafora GA et al. A VicRK signal transduction system in *Streptococcus mutans* affects *gtfBCD*, *gbpB*, and *ftf* expression, biofilm formation, and genetic competence development. J Bacteriol 2005: **187**: 4064–4076.
23. Takahashi I, Okahashi N, Hamada S. Molecular characterization of a negative regulator of *Streptococcus sobrinus* surface

protein antigen gene. J Bacteriol 1993: **175**: 4345–4353.
24. Tamesada M, Kawabata S, Bian Z, Fujiwara T, Kimura S, Hamada S. Modification of sucrose dependent cell adherence by deletion and reintroduction of the *gtf* genes in *Streptococcus mutans*. Adv Exp Med Biol 1997: **418**: 665–668.
25. Tamesada M, Kawabata S, Fujiwara T, Hamada S. Synergistic effects of streptococcal glucosyltransferases on adhesive biofilm formation. J Dent Res 2004: **83**: 874–879.
26. Terao Y, Kawabata S, Kunitomo E, Murakami J, Nakagawa I, Hamada S. Fba, a novel fibronectin-binding protein from *Streptococcus pyogenes*, promotes bacterial entry into epithelial cells, and the *fba* gene is positively transcribed under the Mga regulator. Mol Microbiol 2001: **42**: 75–86.
27. Terao Y, Kawabata S, Kunitomo E, Nakagawa I, Hamada S. Novel laminin-binding protein of *Streptococcus pyogenes*, Lbp, is involved in adhesion to epithelial cells. Infect Immun 2002: **70**: 993–997.
28. Terao Y, Kawabata S, Nakata M, Nakagawa I, Hamada S. Molecular characterization of a novel fibronectin-binding protein of *Streptococcus pyogenes* strains isolated from toxic shock-like syndrome patients. J Biol Chem 2002: **277**: 47428–47435.
29. Yamashita Y, Tsukioka Y, Nakano Y, Shibata Y, Koga T. Molecular and genetic analysis of multiple changes in the levels of production of virulence factors in a subcultured variant of *Streptococcus mutans*. FEMS Microbiol Lett 1996: **144**: 81–87.