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Recombination between *gtfB* and *gtfC* Is Required for Survival of a dTDP-Rhamnose Synthesis-Deficient Mutant of *Streptococcus mutans* in the Presence of Sucrose

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The *rml* genes are involved in dTDP-rhamnose synthesis in *Streptococcus mutans*. A gene fusion between *gtfB* and *gtfC*, which both encode extracellular water-insoluble glucan-synthesizing enzymes, accompanied by inactivation of the *rml* genes was observed for cells grown in the presence of sucrose. The survival rates of *rml* mutants isolated in the absence of sucrose were drastically reduced in the presence of sucrose. The rates were consistent with the frequency of spontaneous gene fusions between *gtfB* and *gtfC*, suggesting that the spontaneous recombinant organisms were selected in the presence of sucrose. The *rml* mutants with a *gtfB-gtfC* fusion gene had markedly reduced water-insoluble glucan synthetic activity and lost the ability to colonize glass surfaces in the presence of sucrose. These results suggest that the *rml* mutants of *S. mutans*, which are defective in dTDP-rhamnose synthesis, can survive only in the absence of water-insoluble glucan synthesis.

Water-insoluble glucan, which is primarily composed of α1,3-linked glucose residues, plays an especially important role in the cariogenicity of *Streptococcus mutans* (4, 9). Two genes coding for glucosyltransferase (GTF), which is responsible for water-insoluble glucan synthesis, *gtfB* (coding for GTF-I) and *gtfC* (coding for GTF-SI), have been isolated from *S. mutans* (1, 5). In experiments using specific-pathogen-free rats, it has been shown that the expression of both genes in addition to production of the water-soluble glucan-synthesizing enzyme (GTF-S) encoded by *gtfD* is required for maximal in vivo virulence of the organism (20, 21).

On the other hand, rhamnose-containing cell wall polysaccharides on the *S. mutans* cell surface are major cell surface antigens and determine the organism's serological properties. In vitro stimulation of human monocytes with the serotype f-specific polysaccharide antigen induces the release of inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β (15). Furthermore, the antigen provokes nitric oxide production in the rat aorta (10). Recently, we isolated four genes (*rmlA*, *rmlB*, *rmlC*, and *rmlD*) involved in dTDP-L-rhamnose synthesis and subsequently determined their roles in cell wall polysaccharide synthesis, since dTDP-L-rhamnose is an immediate precursor of the poly-L-rhamnose backbone of the polysaccharides (17, 18). Inactivation of any of these four genes totally prevented cell wall polysaccharide synthesis.

In this study, we found that fusions between the *gtfB* and *gtfC* genes were observed in each of the *rml* mutants isolated previously. Further characterization of these mutants revealed that *gtfB-gtfC* gene fusions regularly accompanied *rml* gene inactivation only in the presence of sucrose. These results are discussed in relation to the simultaneous recombination of genes located in a locus distant from the inactivated gene.

We previously constructed *rml* mutants of the serotype c *S. mutans* strain Xc (Xc23 [*rmlA*], Xc24 [*rmlB*], Xc21 [*rmlC*], and Xc26 [*rmlD*]) which were isolated on mitis salivarius agar plates (17, 18) (Table 1). All of these *rml* mutants showed similar colony morphology on mitis salivarius agar plates and were easily distinguished from the parental strain, Xc, by colony morphology. The mutant colonies were smaller than the colonies of the parental strain and were circular and convex with a dull surface which was smooth even in the presence of sucrose. When the mutants were propagated in liquid broth, their doubling times in the logarithmic growth phase were one-third of that of the wild-type strain, and this was reflected in the colony size. The morphological changes of the mutant colonies on sucrose-containing agar plates suggested changes in the production of extracellular polysaccharides or cell surface proteins. Therefore, we compared the expression of polysaccharide-synthesizing enzymes and the cell surface protein antigen with a molecular mass of 190 kDa (PAc) by the *rml* mutants with that by the parental strain, Xc, using Western blotting of the whole culture broth, which was precipitated with acetone and included cells and extracellular components as described previously (16, 23). For all of the *rml* mutants, the GTF-I and GTF-SI bands were not detected and a single band at a position intermediate between GTF-I and GTF-SI reacted with anti-GTF-I antiserum (Fig. 1A). On the other hand, production of GTF-S (Fig. 1B), fructosyltransferase (FTF) (Fig. 1C), and PAc (Fig. 1D) did not differ greatly from that for Xc, except that an additional band that reacted weakly with anti-GTF-S serum was seen at a position 20 kDa smaller than GTF-S for the *rml* mutants. Rabbit anti-GTF-I serum and anti-GTF-S serum were kindly provided by K. Fukushima, Nihon University, Matsudo Dental School, Matsudo, Japan. Rabbit anti-PAc serum was prepared as described previously (13). FTF was purified from 5 liters of Xc100L (Table 1) culture supernatant. The FTF protein in the culture supernatant was purified by using a preparative electrophoresis apparatus (Nippon Eido Co., Tokyo, Japan) according to the procedure used for purification of the *gtfB-gtfC* fusion gene product (21). The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis for protein (8) and water-soluble polysaccharide synthetic activity with raffinose used as a substrate. Rabbit anti-FTF serum was raised by subcutaneous injection of the purified FTF protein. The pro-

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TABLE 1. *S. mutans* strains used in this study

Strain	Genotype or relevant characteristics[a]	Reference
Xc	c wild-type strain	7
Xc21	Em^r; Xc carrying P15A replicon and the gene encoding Em^r inserted into *rmlC*	18
Xc23	Em^r; Xc carrying P15A replicon and the gene encoding Em^r inserted into *rmlA*	18
Xc24	Em^r; Xc carrying P15A replicon and the gene encoding Em^r inserted into *rmlB*	18
Xc26	Em^r; Xc carrying P15A replicon and the gene encoding Em^r inserted into *rmlD*	17
Xc21R	Em^r; *rmlC* mutant strain transformed with chromosome of Xc21, which was isolated on tryptic soy agar plate	This study
Xc23R	Em^r; *rmlA* mutant strain transformed with chromosome of Xc23, which was isolated on tryptic soy agar plate	This study
Xc24R	Em^r; *rmlB* mutant strain transformed with chromosome of Xc23, which was isolated on tryptic soy agar plate	This study
Xc26R	Em^r; *rmlD* mutant strain transformed with chromosome of Xc23, which was isolated on tryptic soy agar plate	This study
Xc21RS	Em^r; *rmlC* mutant strain transformed with chromosome of Xc21, which was isolated on tryptic soy agar plate containing 5% sucrose	This study
Xc23RS	Em^r; *rmlA* mutant strain transformed with chromosome of Xc23, which was isolated on tryptic soy agar plate containing 5% sucrose	This study
Xc24RS	Em^r; *rmlB* mutant strain transformed with chromosome of Xc23, which was isolated on tryptic soy agar plate containing 5% sucrose	This study
Xc26RS	Em^r; *rmlD* mutant strain transformed with chromosome of Xc23, which was isolated on tryptic soy agar plate containing 5% sucrose	This study
Xc100L	Subculture variant of Xc showing decreased levels of PAc, GTF-I, and GTF-SI production and increased levels of GTF-S and FTF production	23

[a] Em^r, erythromycin resistance. To obtain and maintain erythromycin-resistant mutants, 10 μg of erythromycin/ml was used.

duction of GTF-I and GTF-SI in the *rml* mutants is similar to that in UA101 and SP2 (19, 21), which both contain a spontaneous *gtfB-gtfC* fused gene as the result of an event of homologous recombination between the *gtfB* and *gtfC* genes. To evaluate the status of *gtfB* and *gtfC* in the *rml* mutants, *Eco*RI-digested chromosomal DNA was analyzed by Southern blotting with a digoxigenin (DIG)-labeled PCR probe (probe I) corresponding to the 1.6-kb *Bam*HI fragment of *gtfB* as described previously (21, 23). While *Eco*RI digests of strain Xc chromosomal DNA exhibited two positive bands of 4.7 and 7.6 kb, a single 7.6-kb band was observed in *Eco*RI digests of chromosomal DNA from all the *rml* mutants (Fig. 2). These results indicate that the *gtfB* and *gtfC* genes were combined into a single fusion gene in the *rml* mutants, as seen in UA101 and SP2 previously (19, 21).

The original *rml* mutants were isolated on mitis salivarius agar containing 5% sucrose. It is possible that the sucrose content of the agar used for transformation might be related to the occurrence of fusions between the *gtfB* and *gtfC* genes in the transformants, because the *gtfB* and *gtfC* gene products are enzymes involved in synthesizing water-insoluble glucan from sucrose. The *rml* mutants were reconstructed by using tryptic soy agar plates in the absence of sucrose with chromosomal DNA from the original *rml* mutants (Xc23, Xc24, Xc21, and Xc26) isolated on mitis salivarius agar plates (17, 18). Chromosomal DNA was prepared from the original *rml* mutant strains and the wild-type strain (Xc) was transformed with the chromosomal DNA according to the methods described previously (14, 22). Ten transformants were isolated in each case, and Western blot analyses using antisera against PAc, GTF-I,

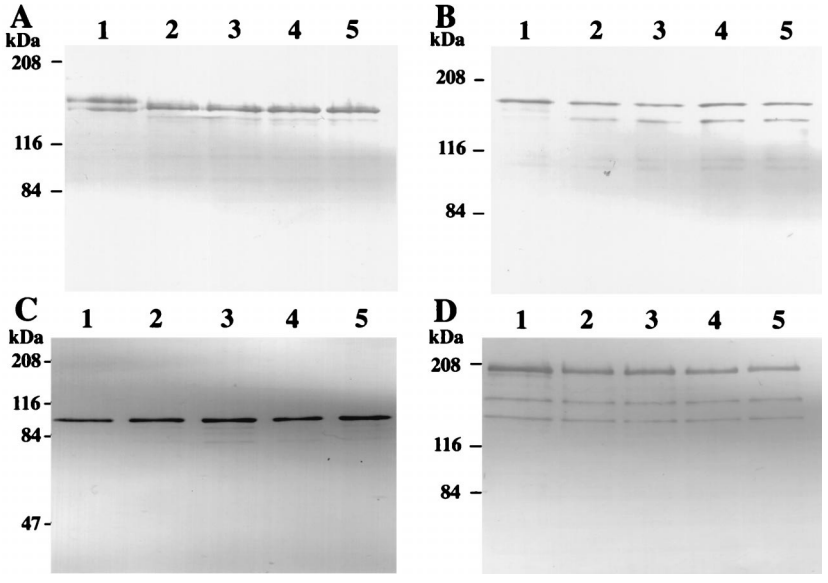

FIG. 1. Western blot analyses of GTF-I, GTF-SI, GTF-S, FTF, and PAc in acetone-precipitated whole culture broth of *rml* mutants. (A to D) Results obtained with rabbit anti-GTF-I, anti-GTF-S, anti-FTF, and anti-PAc sera, respectively. Lanes 1 to 5 are for *S. mutans* Xc, Xc23, Xc24, Xc21, and Xc26, respectively. The molecular mass standards (expressed in kilodaltons) are shown on the left.

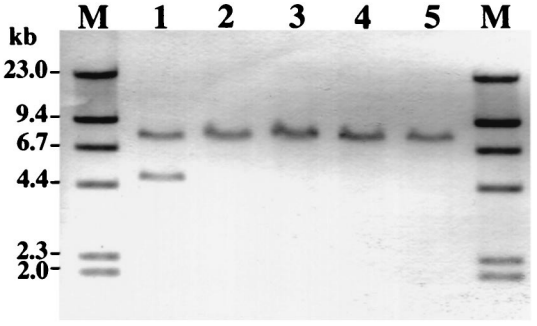

FIG. 2. Southern blot analysis of chromosomal DNA from the *rml* mutants. *Eco*RI-digested chromosomal DNA was hybridized with DIG-labeled probe I. Lanes 1 to 5 contain chromosomal DNA of Xc, Xc23, Xc24, Xc21, and Xc26, respectively. M: *Hin*dIII-digested and DIG-labeled lambda DNA. The target DNA was hybridized with the probe overnight at 42°C in the presence of 50% formamide. The numbers on the left are size markers.

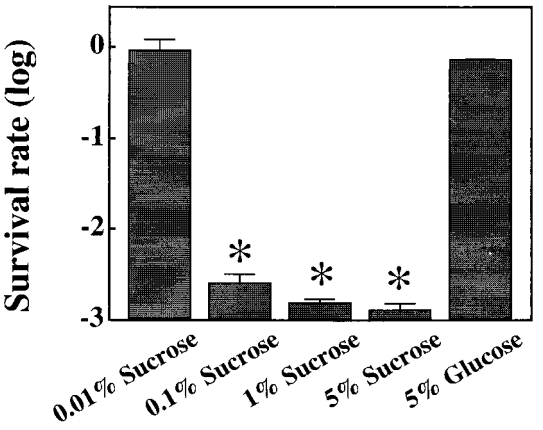

FIG. 3. Survival rates of *S. mutans* Xc24R cells grown in the presence of sucrose or glucose. *S. mutans* cells were grown in brain heart infusion broth until an optical density at 550 nm of about 0.4 was attained. The broth was sonicated three times for 10 s at 20% pulse power with a sonicator (Cell Disrupter model W-225R; Heat Systems, Inc., Farmingdale, N.Y.) and spread on tryptic soy agar supplemented with 0 to 5% (wt/vol) sucrose or 5% (wt/vol) glucose. The survival rates for cells grown at each concentration of added sucrose or glucose were calculated by dividing the number of CFU by the number of CFU for cells grown on medium without additional glucose or sucrose. Each column and bar represents the mean percent survival ± standard deviation for four different experiments. The statistical differences in survival rates were analyzed by using the Mann-Whitney U test. *, $P < 0.05$ (compared to survival rate of cells grown in the presence of 5% glucose).

GTF-S, and FTF were performed. All of the transformants showed similar results in the Western blot analyses, and 1 transformant in each set of 10 transformants was randomly selected. The resulting four transformants were designated Xc23R, Xc24R, Xc21R, and Xc26R, according to the origin of the chromosomal DNA used for transformation as described in Table 1. Similarly, the reconstructed mutant strains that were obtained on tryptic soy agar plates containing 5% sucrose were designated Xc23RS, Xc24RS, Xc21RS, and Xc26RS (Table 1). The reconstructed *rml* mutants were confirmed to have lost serotype c-specific antigenicity accompanied by a drastic decrease of the amounts of rhamnose and glucose in the cell wall. These were detected by immunodiffusion analysis with serotype c-specific antiserum and high-pressure liquid chromatography as described previously (18).

The colony morphology of each of the mutants isolated on agar plates in the presence or absence of 5% sucrose was identical to that of the original *rml* mutants grown on tryptic soy agar and mitis salivarius agar plates. Western blot analyses of the polysaccharide-synthesizing enzymes of the *rml* mutants reconstructed on sucrose-containing agar plates produced results completely identical to those (shown in Fig. 1) obtained for the original *rml* mutants (data not shown). The *rml* mutants selected on sucrose-free agar plates also showed the same results as those observed with the original *rml* mutants (data not shown), except for the result obtained with anti-GTF-I serum. Western blot analysis showed that Xc23R, Xc24R, Xc21R, and Xc26R produced the same levels of both GTF-I and GTF-SI proteins as the wild-type strain (data not shown). Southern blot analysis confirmed that these mutants had intact *gtfB* and *gtfC* genes (data not shown).

To elucidate the mechanism of the *gtfB-gtfC* gene fusion, the survival rates of the *rml* mutants were examined in the presence of 0 to 5% sucrose or 5% glucose. The survival rate of Xc24 was not greatly affected, even in the presence of 5% glucrose. Compared with the survival rate of cells grown in the presence of 5% glucose, the survival rate ($1.2 \times 10^{-3} \pm 0.7 \times 10^{-3}$) of Xc24R cells was decreased significantly when they were grown in the presence of 0.1% or more sucrose but not in the presence of 0.01% sucrose (Fig. 3). The survival rates of the other *rml* mutants (Xc21R, Xc23R, and Xc26R) did not decrease greatly even in the presence of 0.1% sucrose. However, the majority of colonies were very tiny and rough with irregular margins, and a few colonies exhibited the typical *rml* mutant colony morphology. In addition, these tiny background colonies were not observed when cells were grown in the presence of 1% or more sucrose, and the survival rates of these mutants decreased to the same level (1.1×10^{-3} to 2.0×10^{-3}) as observed for Xc24R. We previously observed that Xc24 is sensitive to osmolarity stress (24). Sucrose osmolarity stress may cause the low survival rate of Xc24R in the presence of sucrose observed in this study. Therefore, we examined the effects of 5% glucose on the survival rates of strain Xc and the *rml* mutants. Glucose, however, had barely any effect on the survival rate of any of the *rml* mutant strains, including Xc24R (Fig. 3), suggesting that the reduced survival of the *rml* mutants reconstructed in the absence of sucrose on sucrose-containing plates is not due to osmolarity stress.

The water-insoluble glucan production activity of the *rml* mutants was visually analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels as described previously (21). Xc23R, Xc24R, Xc21R, and Xc26R produced the same level of water-insoluble glucan as the wild-type strain (Xc) (data not shown). On the other hand, Xc23RS, Xc24RS, Xc21RS, and Xc26RS produced barely detectable levels of water-insoluble glucan (data not shown). Furthermore, the in vitro sucrose-dependent adherence ability of the *rml* mutants was examined. In glass tubes, *S. mutans* cells were grown to stationary phase in brain heart infusion broth in the absence of sucrose. At the stationary phase, the cells were ultrasonicated with a sonicator and sucrose was added to the broth at a final concentration of 1% (wt/vol). After incubation at 37°C for 12 h, cells adhering to the glass surfaces were stained with Coomassie brilliant blue R-250. Xc24R and Xc26R could colonize glass surfaces in the presence of 1% sucrose like the wild-type strain, while Xc24RS and Xc26RS could not (data not shown).

All of the *rml* mutants reconstructed in the presence of 5% sucrose exhibited recombination between *gtfB* and *gtfC*, whereas no *rml* mutant with the *gtfB-gtfC* fusion gene was isolated in the absence of sucrose. This suggests that sucrose in the selection medium has an obvious effect on the status of *gtfB*

and *gtfC* in *rml* mutants. Moreover, the cells of the Xc24RS strain and of the other *rml* mutant strains surviving in the presence of ≥0.1% or ≥1% sucrose, respectively, possessed the *gtfB-gtfC* fusion gene (data not shown), indicating that gene fusion between *gtfB* and *gtfC* is necessary for the *rml* mutants to survive in the presence of sucrose. In spite of the fact that strains Xc21R, Xc23R, and Xc26R seemed to be slightly more resistant to the presence of sucrose than Xc24R, their survival rates in the presence of 1% or more sucrose were similar to that of Xc24R. It was previously reported that in vitro spontaneous recombination between *gtfB* and *gtfC* occurs at a frequency ranging from 1×10^{-3} to 3×10^{-3} (14, 19), which is in agreement with the survival rates of the *rml* mutants grown in the presence of sucrose. It is reasonable to speculate that fusion between the *gtfB* and *gtfC* genes in the *rml* mutants in the presence of sucrose results from the selection of the spontaneous recombinant organisms and not from an increased frequency of recombination.

In addition to the *gtfB* and *gtfC* gene products, *S. mutans* produces GTF-S and FTF extracellularly. These enzymes catalyze production of extracellular water-soluble glucan and fructan, respectively, from sucrose. No remarkable change in GTF-S or FTF production was observed in any *rml* mutants (Fig. 1B and C), suggesting that water-soluble polysaccharides may not be related to the viability of the *rml* mutants. Since *S. mutans* strains with the *gtfB-gtfC* fusion gene are less able to produce water-insoluble glucan than strains without the fusion gene, while water-soluble glucan production is not affected (19, 21), water-insoluble glucan synthesis may be the determining factor interrupting the growth of the *rml* mutants that have intact *gtfB* and *gtfC* genes. For Xc24R, the difference between 0.01 and 0.1% sucrose had a dramatic effect on the survival rate, and 0.01% sucrose did not affect the survival rate of the *rml* mutant greatly. These results are consistent with the K_m values (around 3 to 10 mM) reported for GTF-I and GTF-SI (2, 3, 11, 12), because 0.01% sucrose is much lower than the K_m values of these enzymes for sucrose. With such a low concentration of the substrate, neither GTF-I nor GTF-SI is likely to produce enough water-insoluble glucan to suppress the growth of Xc24R.

The reason for the difference in viability between Xc24R and the other *rml* mutants (Xc21R, Xc23R, and Xc26R) in the presence of 0.1% sucrose remains to be elucidated. Although the other *rml* mutants do survive in the presence of 0.1% sucrose, even if they have intact *gtfB* and *gtfC* genes, their growth is severely inhibited. It seems that 0.1% is close to the critical sucrose concentration for the survival of these *rml* mutants. The suggested survival mechanism for Xc24 seems to be applicable to the survival of strains Xc21R, Xc23R, and Xc26R.

We concluded that dTDP-rhamnose synthesis-deficient mutants of *S. mutans* cannot grow in the presence of sucrose unless the ability to produce water-insoluble glucan is reduced by a spontaneous recombination between *gtfB* and *gtfC*. However, we could not determine whether dTDP-rhamnose itself or a glucose-rhamnose polysaccharide is required for the survival of the *rml* mutants in the presence of sucrose. Recently, Hazlett et al. (6) reported that inactivation of the *gbpA* gene encoding an *S. mutans* glucan-binding protein promotes the in vivo recombination between *gtfB* and *gtfC*. Although they did not determine the mechanism for the accumulation of the *gbpA* mutants with a *gtfB-gtfC* fusion gene, the cell surface structure seems to be important for maintaining a steady state of intact *gtfB* and *gtfC* genes.

S. mutans strains with the *gtfB-gtfC* fusion gene have a reduced sucrose-dependent ability to adhere to glass surfaces, and the *rml* mutants with the *gtfB-gtfC* fusion gene failed to adhere to glass surfaces even in the presence of 1% sucrose. It is interesting that the dTDP-rhamnose synthesis pathway is necessary for *S. mutans* to survive in the presence of water-insoluble glucan synthesis, which is implicated in the cariogenicity of the organism. Disruption of the dTDP-rhamnose synthesis pathway triggers a virulence-attenuating gene recombination in *S. mutans*. In the future, this pathway could become the target of a novel class of antimicrobial agents for caries preventive therapy.

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