

## Trigger Factor in *Streptococcus mutans* Is Involved in Stress Tolerance, Competence Development, and Biofilm Formation

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**Trigger factor is a ribosome-associated peptidyl-prolyl *cis/trans* isomerase that is highly conserved in most bacteria. A gene, designated *ropA*, encoding an apparent trigger factor homologue, was identified in *Streptococcus mutans*, the primary etiological agent of human dental caries. Inactivation of *ropA* had no major impact on growth rate in planktonic cultures under the conditions tested, although the RopA-deficient mutant formed long chains in broth. Deficiency of RopA decreased tolerance to acid killing and to oxidative stresses induced by hydrogen peroxide and paraquat, and it reduced transformation efficiency about 200-fold. Addition of synthetic competence-stimulating peptide to the culture medium enhanced transformability of both the mutant and wild-type strains, although the *ropA* strain did not attain levels of competence observed for the parent. Loss of RopA decreased the capacity of *S. mutans* to form biofilms by over 80% when cultivated in glucose, but it increased biofilm formation by over 50% when sucrose was provided as the carbohydrate source. Western blot analysis revealed that the expression of glucosyltransferases B and D was lower in the RopA-deficient mutant. These results suggest that RopA is a key regulator of acid and oxidative stress tolerance, genetic competence, and biofilm formation, all critical virulence properties of *S. mutans*.**

Trigger factor is a ribosome-associated peptidyl-prolyl *cis-trans* isomerase which is a molecular chaperone that is highly conserved in most bacteria (13, 30). It is generally believed that trigger factor is central to protein biogenesis and bacterial survival of environmental insult, although the full spectrum of activities of this protein in bacterial physiology and virulence expression is not yet fully appreciated. In *Escherichia coli*, trigger factor, which is associated with the 50S ribosomal subunit, binds polypeptides as they emerge from the ribosome and functions in cooperation with the DnaK chaperone complex in folding of newly synthesized proteins (40). Trigger factor also interacts with GroEL, greatly promoting the affinity and binding capacity of GroEL for various denatured proteins (16–18). In *E. coli*, trigger factor is a protein that protects cells against low temperature (15), while DnaK is required for growth at temperatures above 37°C and below 15°C (40). The deficiency of both trigger factor and DnaK results in massive aggregation of cytosolic proteins and is lethal above 30°C, although the lethality can be overcome by overexpression of GroEL/ES (40).

In the gram-positive pathogen *Streptococcus pyogenes*, an apparent trigger factor homologue, RopA, has been shown to be essential for secretion and maturation of the secreted cysteine protease SpeB (29, 30). Neither the precursor nor the processed form of SpeB was readily detectable in the supernate of a RopA-deficient mutant that was generated via transposon insertion (30). In mutants that were lacking the peptidyl-prolyl isomerase activity as a result of site-specific mutations in the central domain of RopA, which is critical for substrate

binding, the SpeB precursor was expressed and secreted but it took over 8 h longer for the mutants to convert the SpeB precursor to the processed form than the wild-type strain (29). The SpeB protein produced by these site-specific *ropA* mutants was found to have only a small fraction of the proteolytic activity of the SpeB produced by the parent, suggesting that the peptidyl-prolyl isomerase activity is essential for the establishment of an active conformation of this protease (29, 30).

*Streptococcus mutans*, the primary etiological agent of human dental caries, lives in biofilms on the tooth surface. *S. mutans* has the ability to utilize a wide variety of sugars, and the sustained production of lactic acid from glycolysis carried out by this bacterium is directly linked to demineralization of tooth enamel and caries formation (4). Therefore, the abilities to adhere to and form biofilms on tooth surfaces, to catabolize carbohydrates and generate acids, and to survive low pH and other environmental stresses are critical to the cariogenicity of this pathogen (4). Several surface-associated proteins have been shown to function as high-affinity adhesins and play a central role in initiation of biofilm formation by *S. mutans* (3, 41). SpaP (also called P1), a surface protein of the antigen I/II family, is critical in *S. mutans* for sucrose-independent adherence to the tooth (3). A gene designated *brpA* (for biofilm regulatory protein) has recently been identified to encode a putative surface-associated polypeptide, and loss of BrpA in *S. mutans* causes major defects in biofilm formation on abiotic surfaces (41). *S. mutans* produces three glucosyltransferases (GtfB, -C, and -D) which synthesize adhesive extracellular glucans from sucrose. Gtfs are of central importance in dental plaque formation and development of caries (33, 39). Extracellular glucans, especially the  $\alpha(1,3)$ -linked, water-insoluble forms, facilitate adherence of *S. mutans* to the tooth surface and modulate cell-cell interaction by serving as binding sites for Gtf proteins and glucan-binding proteins, a group of pro-

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teins with no known enzymatic activities that contribute to sucrose-dependent adherence and biofilm cohesiveness (11, 12, 31). As a major constituent of the biofilm matrix, glucans can further influence the development and structure of oral biofilms by modulating permeability to water and nutrients and by serving as an extracellular carbon and energy source (37). In addition to specific adherence-promoting gene products, recent studies have revealed regulatory networks in *S. mutans* that are required for biofilm formation and stress tolerance. The cell density-dependent Com system, which is known to control genetic competence development in *S. mutans* and other naturally competent streptococci, is involved in biofilm formation and acid tolerance in *S. mutans* (25–27). Likewise, *luxS* and *relA* homologues, and components of the general stress response pathway, strongly influence biofilm development and architecture and resistance to acid and oxidative insult (22–24, 42).

Trigger factor of *S. mutans* was found to be up-regulated in response to deficiency of LuxS (Z. T. Wen and R. A. Burne, unpublished data), which was shown to affect acid and oxidative stress tolerance and biofilm formation (42). The expression of trigger factor was also increased in cells stimulated by the synthetic competence-stimulating peptide (CSP) (G. Svensater, personal communication) and in populations that were grown in biofilms (38). In this study, we examined an apparent trigger factor homologue in *S. mutans* for its role in stress tolerance, competence development, and biofilm formation.

#### MATERIALS AND METHODS

**Bacterial cultivation.** *S. mutans* UA159 and its derivatives were maintained on brain heart infusion (BHI) medium, with or without addition of erythromycin (Em; 10 µg/ml). Preparation of competent cells and transformation of *S. mutans* were done as previously described (5, 27). For biofilm formation, *S. mutans* strains were grown in a semidefined biofilm medium (BM) with either 0.8% (wt/vol) glucose (BMG) or 0.5% sucrose (BMS) as the carbohydrate source (28). For growth studies, TV medium (5) or Todd-Hewitt broth (BBL; Becton Dickinson, Cockeysville, Md.) supplemented with 0.3% yeast extract (THYE) (25) was also used. All agar media were prepared similarly, with agar (Difco Laboratories, Detroit, Mich.) added at a concentration of 1.5% (wt/vol).

**DNA manipulations.** Unless otherwise stated, standard recombinant DNA techniques were performed as described by Sambrook et al. (34). All restriction and modifying enzymes and reagents were purchased from Invitrogen and used as described by the supplier. For Southern hybridizations, 12 µg of genomic DNAs were digested with EcoRI and transferred to NewBond nylon membranes (NEN Life Science Products, Inc.). Probes were labeled with [ $\alpha$ -<sup>32</sup>P]dATP using a random primers DNA labeling system from Invitrogen. Hybridizations and washes were carried out under high-stringency conditions.

**Acid killing and hydrogen peroxide challenge.** The ability of cells to withstand acid challenge was determined by acid killing using the method of Belli and Marquis (1). Briefly, *S. mutans* strains were grown in BHI that had been adjusted to pH 7.0 with HCl. Cultures were harvested at mid-exponential phase (optical density at 600 nm [OD<sub>600</sub>],  $\approx$ 0.3) by centrifugation at 3,800  $\times$  g at 4°C for 10 min, washed once with 0.1 M glycine buffer (pH 7.0), and then subjected to acid killing by incubating the cells in 0.1 M glycine buffer, pH 2.8, for 45 min. Surviving cells were appropriately diluted, plated on BHI plates in triplicate, and incubated in a 5% CO<sub>2</sub> aerobic atmosphere at 37°C for 24 to 48 h. To evaluate oxidative stress tolerance, *S. mutans* strains were challenged with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; Fisher Scientific, Fair Lawn, N.J.) or paraquat (methyl viologen; catalog no. M2254; Sigma). For paraquat challenge, aliquots from a sterile paraquat solution were added directly to bacterial cultures to give final concentrations of 25 or 50 mM, and the impact on bacterial growth was monitored using a Bioscreen C reader (Helsinki, Finland) (27). For H<sub>2</sub>O<sub>2</sub> challenge, mid-exponential-phase cells were harvested by centrifugation and resuspended in 5 ml of 0.1 M glycine buffer, pH 7.0. Hydrogen peroxide was then added to the cell suspension to give a final concentration of 0.2% (vol/vol). A 100-µl sample was obtained right before, and 2 h after, the addition of H<sub>2</sub>O<sub>2</sub>. To inactivate hydrogen peroxide,

catalase (5 mg/ml; catalog no. C-9322; Sigma) was added to samples immediately after collection. The survival rate was determined by plating in triplicate on BHI plates.

**Biofilm formation assay.** Biofilm assays were done as previously described (41). Briefly, overnight cultures of *S. mutans* UA159 and its derivatives were transferred to prewarmed BHI and grown at 37°C in a 5% CO<sub>2</sub> aerobic atmosphere to late exponential phase (OD<sub>600</sub>  $\approx$  0.5). The cultures were then diluted 1:100 in prewarmed BM, and 200 µl of the cell suspension was inoculated into the wells of 96-well (flat-bottom) cell culture clusters (Costar 3595; Corning Inc., Corning, N.Y.). Plates were incubated at 37°C, in a 5% CO<sub>2</sub> atmosphere for 24 h. The culture medium was then decanted, and the plates were washed twice with 200 µl of sterile distilled water to remove the planktonic and loosely bound cells. The adherent bacteria were stained with 50 µl of 0.1% crystal violet for 15 min. After rinsing twice with 200 µl of water, the bound dye was extracted from the stained cells using 200 µl of 99% ethanol, and the plates were sealed in a plastic bag and set on a shaker (Reliable Scientific) overnight to allow full release of the dye. Biofilm formation was then quantified by measuring the absorbency of the solution at 600 nm in an enzyme-linked immunosorbent assay reader (Bio-Rad).

**SEM.** For scanning electron microscopy (SEM), biofilms were grown on hydroxylapatite (HA) disks that were deposited in the wells of 24-well culture clusters (Corning Inc.) (41). Briefly, late-exponential-phase cultures were grown in BHI as described above and diluted 1:100 in BMG or BMS, and 2 ml of the diluted culture was added to each well. After incubating for 24 h at 37°C in a 5% CO<sub>2</sub> aerobic atmosphere, the HA disks were carefully rinsed by dipping twice in phosphate-buffered saline buffer (50 mM Na<sub>2</sub>PO<sub>4</sub> [pH 7.0], 0.85% NaCl) and fixed overnight with Trump fixative solution. Following dehydration through a series of ethanol rinses, the disks were mounted and coated with gold, and the biofilms were then analyzed by SEM in the University of Florida EM core facility.

**Western blot analysis.** To prepare cell extracts, mid-exponential-phase cultures were harvested by centrifugation. The cell pellets were resuspended in 10 mM potassium phosphate buffer, pH 7.0, containing phenylmethylsulfonyl fluoride and sodium azide at final concentrations of 1 mM and 0.02%, respectively. Cells were homogenized using a Bead Beater (Biospec, Bartlesville, Okla.) with glass beads (0.1 mm), as previously described (6). For extracellular enzyme analysis, cell-free supernates were recovered from late-exponential-phase cultures (OD<sub>600</sub>  $\approx$  0.5) and buffered with 10 mM potassium phosphate, pH 7.0, containing phenylmethylsulfonyl fluoride and sodium azide. The supernates were then concentrated approximately 20-fold by passage through a Centricon filter with a 30-kDa molecular mass cutoff (catalog no. 4209; Millipore Co., Bedford, Mass.). Proteins were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE), blotted to polyvinylidene difluoride membrane, and probed for GtfB and -C, which share extensive homology, and GtfD using polyclonal antibodies induced against *S. mutans* GtfB or GtfD, respectively (43).

**2-D gel analysis.** For two-dimensional (2-D) gel analysis, exponential-phase cultures (OD<sub>600</sub>  $\approx$  0.5) grown in BHI were harvested by centrifugation, washed, and homogenized using a Bead Beater as previously described (42). Cell extracts were sent to Kendrick Labs, Inc. (Madison, Wis.), where 200 µg of protein was subjected to 2-D gel electrophoresis using the methods of O'Farrell (32). The first-dimensional isoelectric focusing step was carried out using 2% pH 4-to-8 ampholines, and the second dimension was carried out in an SDS-10% polyacrylamide gel. Gels were stained with Coomassie blue, and protein profiles were analyzed by densitometry using software from Alpha Innotech (San Leandro, Calif.).

#### RESULTS AND DISCUSSION

**Construction of a RopA-deficient mutant.** The *ropA* gene was identified by a BLAST search of the *S. mutans* genome database ([www.genome.ou.edu/smutans](http://www.genome.ou.edu/smutans)) with the *S. pyogenes* RopA amino acid sequence. *S. mutans ropA* (Smu0082 in the Oral Pathogens database [<http://www.stdgen.lanl.gov/oragen/>]) is a 1,281-bp open reading frame encoding a polypeptide of 427 amino acid residues with 74% identity to RopA of *S. pyogenes* (accession no. AAC82391), an apparent homologue of trigger factor (13, 30, 36). Analysis of the flanking region revealed that the *ropA* gene of *S. mutans* is immediately upstream of genes that are predicted to code for transposase fragments and genes for nitrite transport and hypothetical proteins upstream, but

TABLE 1. Primers used for construction of mutants

Primer	DNA sequence (5' → 3') <sup>a</sup>	Application	Amplicon (bp)
ropA55'	ACTATGTCGTGACACCAGCATC	5' fragment for TW90	415
ropA53'	TAGAGCTCTTCTTTCCACCAGATATGCTC	5' fragment for TW90	
ropA35'	ATAAGCTTATCGTAAATGTAACAACACCAC	3' fragment for TW90	512
ropA33'	AGCAAACATGGAACGCAGAGC	3' fragment for TW90	
TF-P1	AAAGAAGTTTTTGCCAATCGC	5' fragment for SMUTig	781
TF-P2	GGCCGCCTCGTAAATGTAACAACACCACG	5' fragment for SMUTig	
TF-P3	GGCCGCCCATGGAAGCTGAGCAAGTGCG	3' fragment for SMUTig	718
TF-P4	ATTTATCAAGGCATGACGGGG	3' fragment for SMUTig	
TF5'	ATGGATCCGGCTGCAACTACACCGACTGC	Internal fragment for TW90A	595
TF3'	GAGTCGACCCGATTCTACCGAAGAGCTCGGTATC	Internal fragment for TW90A	

<sup>a</sup> The incorporated restriction sites are underlined.

they are all transcribed in the opposite orientation to that of *ropA*.

To analyze the role of RopA in *S. mutans*, a *ropA* mutant was constructed by replacing nucleotide 56 to nucleotide 1205 of the coding sequence with an erythromycin resistance (*erm*) gene (7) using a PCR-ligation-mutation strategy (20). Specifically, a 415-bp 5' fragment and a 512-bp 3' fragment were amplified by PCR using gene-specific primers (Table 1) to incorporate an SstI site at the 3' end of the 5' fragment and a HindIII site at the 5' end of the 3' fragment. The fragments were then digested with restriction enzymes and ligated with the *erm* gene that was released with the same enzymes (41). The resulting ligation mixture was then used to transform *S. mutans* UA159. A mutant with an inactivated *ropA* gene, strain TW90, was isolated on BHI plates with erythromycin (10 µg/ml) and confirmed by PCR and Southern blot analysis (data not shown). Another deletion mutant, strain SMUTig, which has nucleotides 34 to 1177 of the coding sequence replaced with an erythromycin resistance element, was generated from *S. mutans* UA159 using a similar strategy. An insertional mutant, TW90A, was generated by single-crossover insertion using a suicide vector, pSU20Erm:ropA, which contains an internal fragment of *ropA* amplified by PCR (Table 1) to transform *S. mutans* UA159. Strain TW90A was used during the early stage of this study but was later replaced by the double-crossover mutant TW90 for acid and oxidative stress tolerance and biofilm formation. Similar experiments were also run with strain SMUTig and yielded similar results. In these cases, only the data generated from strain TW90 are presented. For transformation efficiency assays, only strain SMUTig was used.

**RopA deficiency diminishes tolerance to stresses.** Strain TW90 formed mucoid, smooth, and shiny colonies on BHI agar plates, in contrast to the rough, dry colonies formed by the parent strain, UA159. When grown in BHI, BM, THYE, or TV broth, strain TW90 displayed no major differences in growth rates compared to UA159 (data not shown). However, TW90 did have a tendency to form longer chains in THYE, BHI, and BMG broth cultures, with an average of  $26 \pm 7$  cells per chain for the mutant versus  $11 \pm 4$  for strain UA159.

Compared to UA159, TW90 took significantly longer to form visible colonies on agar plates that were adjusted to pH 5.0 (data not shown) or to obtain similar optical densities in broth media that had been adjusted to pH 5.5. In this acidified

medium, strain UA159 had a doubling time of  $84.4 \pm 1.2$  min compared with  $107.9 \pm 2.5$  for the mutant, indicative of defects in acid tolerance. To further analyze the impact of RopA deficiency on acid tolerance, mid-exponential-phase cultures of TW90 that were grown in BHI were subjected to acid killing (42). When incubated in buffer at pH 2.8 for 45 min, TW90 demonstrated a decrease of more than 45-fold in survival rate compared to UA159 (Fig. 1). When incubated in medium containing 25 mM paraquat, strain TW90 also displayed a lag phase that was more than 2 h longer than that of the wild type. Growth was further inhibited when the concentration of paraquat was increased to 50 mM, with a doubling time of  $484.2 \pm 67.3$  min for TW90 compared to  $199.2 \pm 19.6$  min for UA159 (data not shown). Similarly, when mid-exponential-phase cultures were challenged with 0.2% (vol/vol) hydrogen peroxide for 2 h, TW90 showed more than a 28-fold decrease in survival rate compared to UA159 (Fig. 1).

There are a variety of reasons that the RopA-deficient mutants may be more sensitive to low pH and oxidative stress. In *S. mutans* and other mutans streptococci, ATP-dependent pro-

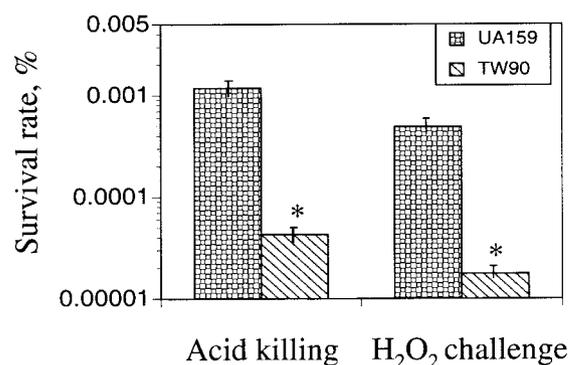


FIG. 1. Acid killing and hydrogen peroxide challenge assays. For acid tolerance, all *S. mutans* strains were grown in BHI until mid-exponential phase ( $OD_{600} \approx 0.3$ ), harvested by centrifugation, washed once with 0.1 M glycine buffer, pH 7.0, and then subjected to acid killing at pH 2.8 for 45 min. For hydrogen peroxide challenge, the mid-exponential-phase cultures were incubated in buffer containing 0.2% (vol/vol) hydrogen peroxide for 2 h. The surviving cells were plated on BHI agar plates in triplicate, and results are expressed as survival rate over time. See text for more details. Data presented are means  $\pm$  standard deviations (error bars) of three independent experiments. \*,  $P < 0.01$ .

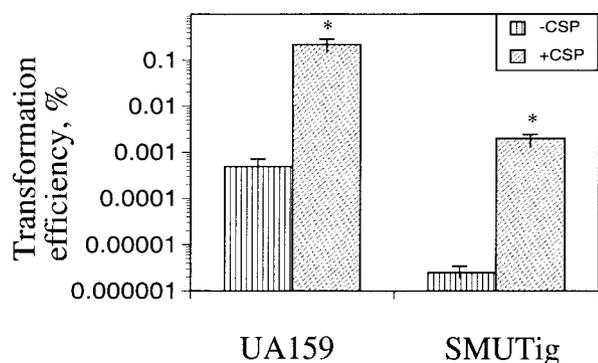


FIG. 2. Transformation efficiency. *S. mutans* strains were evaluated for their ability to transform foreign DNA (plasmid) with or without inclusion of the synthetic CSP ( $500 \text{ ng } \mu\text{l}^{-1}$ ). The bar graph represents the average of at least three independent experiments, with standard deviations denoted by the error bars. \*,  $P < 0.01$ .

ton extrusion mediated by the membrane-bound F-ATPase is the primary mechanism that these bacteria employ to maintain intracellular pH homeostasis (2). Ffh, a homologue of eukaryotic signal recognition particle protein, has also been recently reported as a major determinant in acid tolerance in *S. mutans* (8, 10). Considering the fact that trigger factor in *E. coli* and *S. pyogenes* is critical to folding of nascent proteins and establishment of active conformation of functional proteins (13, 29, 30, 40), RopA deficiency may cause defects in membrane integrity and alter expression or distribution of membrane-associated proteins, such as F-ATPase and Ffh. As detailed in the following sections, defects in the regulatory Com system could also contribute to the impaired acid tolerance of the RopA-deficient mutants.

**The *ropA* mutant is less competent for genetic transformation.** In *S. mutans* and other naturally competent streptococci, the ability to transport and incorporate foreign DNA, genetic competence, is primarily controlled by ComCDE, with ComC being the genetic competence factor (21, 26). The competence factor peptide, a quorum-sensing pheromone, is processed and secreted into the environment (21, 27). When the concentration of the processed peptide reaches a certain threshold, it is detected by ComD, a transmembrane histidine kinase, which then phosphorylates ComE, a response regulator, triggering transcription of a set of genes that are required for DNA uptake and the related processes. Consistent with the findings in 2-D gel analyses of *S. mutans* (G. Svensater, personal communication), inclusion of synthetic CSP ( $1 \mu\text{g}/\mu\text{l}$ ) in the culture medium of an *S. mutans comC*-defective strain was also found to increase *ropA* transcription by 1.4-fold, suggesting RopA is involved in the ComC-mediated regulatory functions. To assess the impact of RopA deficiency on competence development, transformation efficiency was measured in the RopA-deficient strain using a procedure described elsewhere (27). It was found that the transformation efficiency was approximately 200-fold lower for the *ropA* mutant than for the wild-type strain (Fig. 2). However, addition of CSP at a level of  $500 \text{ ng } \mu\text{l}^{-1}$  was able to enhance the transformation efficiency of strain SMUTig by about 3 logs. Still, the transformation efficiency of the mutant could not be restored to the level of the parental strain that had been treated with CSP.

One interpretation of the observed defects in transformation of SMUTig is that RopA is involved in processing or secretion of the competence factor peptide and that lack of RopA causes defects in production of competence factor. An inability to fully restore competence by addition of CSP suggests that detection of the mature peptide by the cells may also be affected by the loss of RopA. Given the established role of trigger factor as a critical factor in processing, secretion, and presentation of membrane-associated and extracellular proteins (13, 29, 30, 40), deficiency of RopA in *S. mutans* could cause defects in extracellular and membrane-associated proteins that control competence factor sensing or DNA uptake. Similar to the *S. mutans* strains that are defective in either production or detection of competence factor (27), the RopA-deficient mutant also formed longer chains that were about three times the cell number of the parent, and the mutant cells appeared to be a little shorter and fatter than the parental cells under SEM (data not shown), further suggesting that there may be defects in cell division or membrane biogenesis from RopA deficiency.

**Deficiency of RopA causes alterations in biofilm formation.** As measured by the amount of crystal violet-stained, adherent cells on a polystyrene surface, the capacity of TW90 to form biofilms was decreased by over 80% when glucose was the carbohydrate source, compared to the wild-type strain grown under the same conditions. The biofilm formation defect could be overcome by growth in sucrose-containing medium. In fact, when grown in BM with sucrose, the RopA-deficient strain displayed a markedly better adherence to the sides of culture tubes (data not shown) and actually formed over 50% more biofilms than did the parent. Similar trends were observed under SEM of biofilms formed on HA surfaces (Fig. 3). When grown on HA disks with glucose as the carbohydrate source, TW90 was able to bind to the HA surfaces, but in a diminished capacity compared to UA159. On HA disks, it also formed longer chains than did the wild type, but failed to develop into 3-D biofilms (Fig. 3). In contrast, when sucrose was present, TW90 accumulated and formed mature biofilms with multicellular clusters, and the biofilms formed by TW90 appeared to have larger, more compact cell clusters surrounded by relatively smaller gaps than those formed by strain UA159. As supported by counting of CFU (data not shown), the enhanced biofilm formation by TW90 in sucrose appears to be mainly due to increases in cell number.

The basis for the decrease in biofilm formation is yet to be established, but a likely factor is that the *ropA* strains have altered expression or presentation of surface proteins. Relatively little is known about the role of specific surface structures in the formation of *S. mutans* biofilms, and so further characterization of the surface-associated proteins in the RopA-deficient strains is under way to identify critical surface-anchored and secreted gene products required for biofilm maturation. Formation of longer chains by the *ropA* mutant may also make it difficult for the bacterial cells to remain attached by the relatively weak forces that mediate adhesion to the polystyrene when cells are grown in glucose.

Another explanation for the decrease in biofilm formation may be related to the defects in the competence regulon. Recent studies in *S. mutans* by Li et al. have shown that the Com system is involved in acid tolerance and biofilm formation

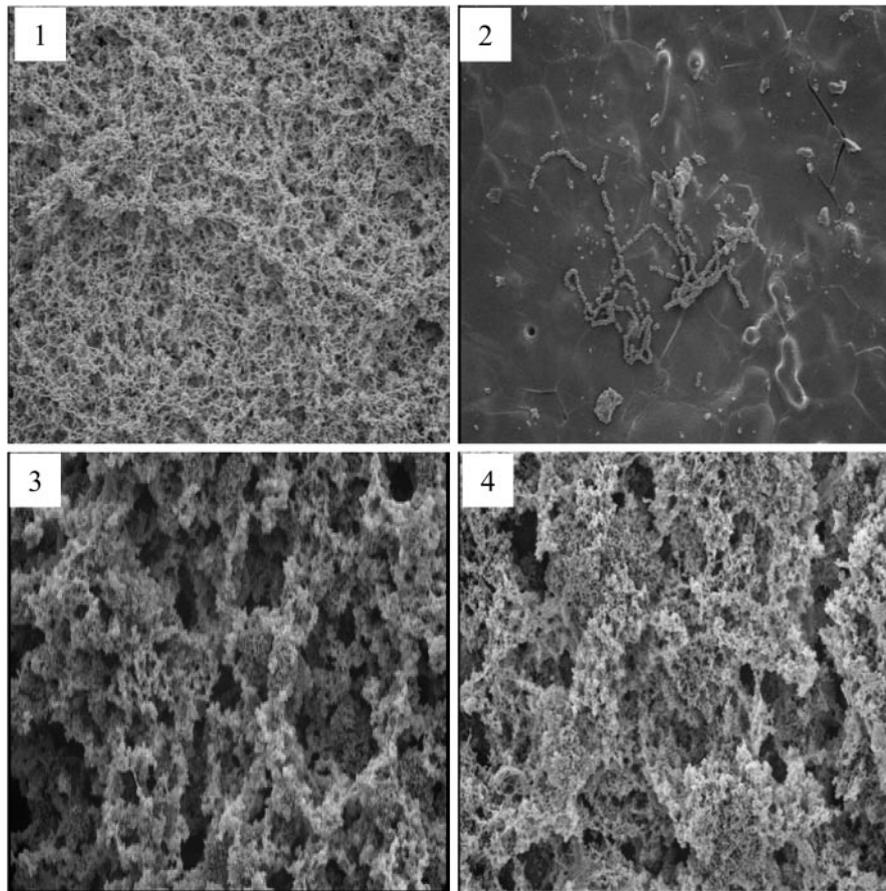


FIG. 3. SEM analysis of biofilms. The 24-h biofilms of *S. mutans* strains UA159 (panels 1 and 3) and TW90 (panels 2 and 4) were grown on HA disks that were deposited in 24-well cell culture clusters in semidefined BM medium with glucose (panels 1 and 2) or sucrose (panels 3 and 4) as the carbohydrate source. Magnification in all images,  $\times 1,000$ .

(25, 27). *S. mutans* strains that were defective in the *comC*, *comD*, or *comE* genes, which encode the major components of the cell density-dependent regulatory system, demonstrated a diminished acid tolerance, and such defects could be overcome by addition of synthetic CSP to the culture medium (25). Compared to the parental strain, these *com*-deficient strains formed biofilms with less biomass and abnormal architecture. Inclusion of synthetic CSP in the culture medium or complementation with a wild-type *comC* gene in a shuttle vector partially restored the wild-type biofilm architecture (27). As demonstrated by transformation efficiency (Fig. 2), RopA deficiency causes defects in genetic competence. Therefore, the observed

impairments in biofilm formation as well as acid tolerance by TW90 could be, at least in part, attributed to the defects in expression and/or function of the Com system.

**RopA deficiency affects Gtf expression.** To examine whether changes in expression of the Gtfs could be responsible for the changes in sucrose-dependent biofilm formation, cell extracts and cell-free supernates of UA159 and TW90 grown in BHI were analyzed by Western blotting using polyclonal antisera induced against GtfB and GtfD, respectively (43). In *S. mutans*, GtfB and GtfC show extensive homology (9), so the polyclonal anti-GtfB antibody reacts strongly with both GtfB and GtfC. As shown in Fig. 4, the bands reactive with anti-GtfB

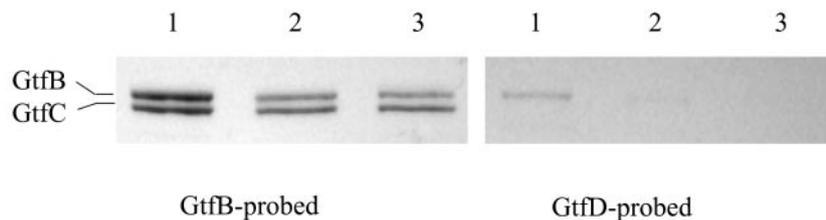


FIG. 4. Western blot analysis. Whole-cell extracts (20  $\mu$ g of total protein) of UA159 (lanes 1), TW90A (lanes 2), and TW90 (lane 3) were separated by SDS-10% PAGE. The separated proteins were then blotted to a polyvinylidene difluoride membrane and probed with anti-GtfB (A) and anti-GtfD (B) polyclonal antibodies.

antibody in samples prepared from strain TW90 were significantly less intense than those prepared from UA159. Such changes were even more apparent in the cell-free supernates. Interestingly, in contrast to GtfB, the bands corresponding to GtfC in TW90 were just slightly decreased. When probed with anti-GtfD antibody, the band reactive with anti-GtfD was virtually undetectable in cell extracts of TW90.

One of the major contributors to sucrose-dependent bacterial adherence by *S. mutans* is the production of glucans through the cooperative functions of the glucosyltransferases (GtfB, -C, and -D). Although more complex than presented, GtfB generally assembles glucans rich in  $\alpha(1,3)$  linkages, GtfD synthesizes  $\alpha(1,6)$ -linked glucans, and GtfC makes both  $\alpha(1,3)$ - and  $\alpha(1,6)$ -linked glucans. Both  $\alpha(1,3)$ -linked water-insoluble and  $\alpha(1,6)$ -linked water-soluble glucans are important in cell-cell and cell-surface adhesive interactions, while the  $\alpha(1,3)$ -rich, water-insoluble glucans have been shown to be the major contributor to bacterial adherence to hard surfaces (19, 35, 39). A recent study by Ooshima et al. (33) has shown that the presence of all three enzymes in an optimal ratio is necessary for efficient sucrose-dependent adherence. Down-regulation of Gtf enzymes, especially GtfB and GtfD in the RopA-deficient strain, would likely alter the ratio of these enzymes and, consequently, the composition of the glucans synthesized, which in turn would alter biofilm accumulation and architecture. Most likely, with very low levels of GtfD, the polymers made by the RopA-deficient mutant could be predominantly the water-insoluble,  $\alpha(1,3)$ -linked glucans, which better support adherence, potentially enhancing biofilm formation. Other possible reasons for the enhancement of sucrose-dependent biofilm formation could include altered expression or activity of glucan-binding proteins, including the glucan-binding protein activity of the Gtf enzymes.

**Further evidence of the relationship between stress tolerance and biofilm formation.** In an attempt to uncover the factors that are involved in altered stress tolerance and biofilm formation as a result of RopA deficiency, whole-cell extracts were analyzed in 2-D gels. Comparison of the protein profiles stained with Coomassie blue revealed that loss of RopA altered the expression of at least 33 proteins, 22 of which were up-regulated and 11 were down-regulated (data not shown), with the role and identity of the majority of the altered proteins being unknown. Still, among the up-regulated proteins that could be identified were DnaK, GroEL, and GroES, molecular chaperones that are inducible by heat shock and acid stress and are believed to play a central role in the ability of oral bacteria to cope with frequent stresses encountered in the oral cavity (14, 24). Given the fact that trigger factor in *E. coli* is associated with GroEL/ES in promoting the affinity of these chaperones and binding capacity to various stress-damaged proteins (16, 17, 40), deficiency of RopA would likely diminish the capacity of the organism to deal with stresses imposed by low pH, oxygen, and other detrimental conditions. The up-regulation of molecular chaperones DnaK, GroEL, and GroES in the *ropA* mutant is therefore consistent with the weakened tolerance to acid and oxidative stresses by TW90 (Fig. 1).

**Summary.** *S. mutans*, an obligate biofilm-forming bacterium and the primary etiological agent of human dental caries, possesses a gene that is predicted to encode a homologue of bacterial trigger factor. The role for RopA in *S. mutans* viru-

lence expression appears to be substantial. The results suggest that RopA in *S. mutans* may be involved in adherence to, and formation of biofilms on, tooth surfaces and that the protein is essential for tolerance of low pH, toxic oxygen radicals, and other adverse conditions in the oral cavity. The underlying molecular basis for the observed defects in the RopA-deficient mutant is under investigation.

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