Transcriptional Repressor Rex Is Involved in Regulation of Oxidative Stress Response and Biofilm Formation by *Streptococcus mutans*

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Abstract

The transcriptional repressor Rex has been implicated in regulation of energy metabolism and fermentative growth in response to redox potential. *Streptococcus mutans*, the primary causative agent of human dental caries, possesses a gene that encodes a protein with high similarity to members of the Rex family of proteins. In this study, we showed that Rex-deficiency compromised the ability of *S. mutans* to cope with oxidative stress and to form biofilms. The Rex-deficient mutant also accumulated less biofilm after 3-days than the wild-type strain, especially when grown in sucrose-containing medium, but produced more extracellular glucans than the parental strain. Rex-deficiency caused substantial alterations in gene transcription, including those involved in heterofermentative metabolism, NAD⁺ regeneration and oxidative stress. Among the up-regulated genes was *gtfC*, which encodes glucosyltransferase C, an enzyme primarily responsible for synthesis of water-insoluble glucans. These results reveal that Rex plays an important role in oxidative stress responses and biofilm formation by *S. mutans*.

Keywords

Redox sensing; oxidative stress; biofilm formation; *Streptococcus mutans*

INTRODUCTION

*Streptococcus mutans* lives almost exclusively in biofilms on the tooth surface, an environment that experiences dramatic fluctuations in nutrient availability, pH, and oxygen tension. As the primary etiological agent of human dental caries, the ability to survive various harsh challenges in the oral cavity is known to be critical to its pathogenicity (Burne, 1998). While the molecular mechanisms that govern carbohydrate utilization, acid production and low pH adaptation by this microorganism are well-studied (Abranches, *et al.*, 2008, Lemos & Burne, 2008, Zeng & Burne, 2008), limited information is available.

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concerning oxygen metabolism and oxidative stress and their impact on the expression of virulence traits by *S. mutans*.

*S. mutans* lacks a complete respiratory chain and does not normally carry out oxidative phosphorylation, but the organism has a high capacity to metabolize oxygen (Marquis, 1995). When grown on the tooth surface, *S. mutans* must cope with various oxidative stress conditions, including damaging reactive oxygen species (ROS) and unfavorable cellular redox potential (Marquis, 1995). ROS, such as \( \cdot O_2^- \), \( H_2O_2 \), and \( HO^- \) are produced inside the bacterial cells when growing in an aerobic environment. ROS are toxic as they are highly reactive and can cleave RNA/DNA and oxidize essential proteins and lipids. It was recently shown that aeration significantly decreased the ability of *S. mutans* to form biofilms (Ahn & Burne, 2007, Ahn, et al., 2007). Notably, growth in the presence of oxygen dramatically altered the cell surface, affecting hydrophobicity and the localization of glucosyltransferases B and C (Ahn, et al., 2007). The presence of oxygen also induced complex changes in the transcriptome of *S. mutans*, including genes affecting cell envelope biogenesis, energy metabolism and stress tolerance.

Bacteria can sense oxygen tension through monitoring the accumulation of metabolites or the altered redox state of specific compounds as a result of changes in cellular homeostasis (Wang, et al., 2008). Recent studies in *Streptomyces coelicolor* and *Bacillus subtilis* identified a new type of regulator, termed Rex (for redox repressor) that directly responds to changes in the cytoplasmic NADH/NAD\(^+\) ratio (Brekasis & Paget, 2003, Wang, et al., 2008, Pagels, et al., 2010). In *B. subtilis*, the transcription of Rex-repressed genes is activated in response to oxygen limitation, which leads to production of cytochrome *bd* and NADH-linked lactate dehydrogenase, ensuring efficient oxygen utilization and recycling the excess of NADH (Larsson, et al., 2005, Gyan, et al., 2006). In *Staphylococcus aureus*, Rex regulates pathways for anaerobic fermentation and NAD\(^+\) regeneration (Pagels, et al., 2010). *S. mutans* possesses a rex gene (SMU.1053) that encodes a protein with high similarity to the Rex family of proteins. In this study, we constructed a deletional mutant and characterization of this Rex-deficient mutant revealed that Rex plays an important role in regulation of central metabolism, oxidative stress and biofilm formation by *S. mutans*.

**MATERIALS AND METHODS**

**Plasmids, bacterial strains, and growth conditions**

*Streptococcus mutans* UA159 and its derivatives were maintained in brain heart infusion (BHI) medium. Solid media were prepared similarly, but agar (Difco Laboratories) was added at a concentration of 1.5% (w/v). When needed, kanamycin (1 mg/ml), erythromycin (10 µg/ml) or spectinomycin (1 mg/ml) was added to the growth medium. Unless stated otherwise, all cultures were grown aerobically in a 37°C chamber containing 5% CO\(_2\) under static conditions. For growth studies, a Bioscreen C (Oy Growth Curves AB Ltd, Finland) was used to culture cells at 37°C, aerobically and the optical densities were monitored every 30 minutes following shaking for 10 seconds (Zeng, et al., 2006).

**Construction of mutant strains**

Strains deficient in rex were generated using a PCR-ligation-mutation strategy described elsewhere (Lau, et al., 2002, Wen & Burne, 2004) (Table 1). The resulting mutants were further analyzed by PCR and DNA sequencing to verify the deficiency and sequence accuracy. For mutant complementation, the gene of interest plus its putative promoter region were directly cloned into shuttle vector pDL278 (LeBanc & Lee, 1991). Following sequence confirmation, the resulting construct was transformed into the mutant, and transformants

*FEMS Microbiol Lett. Author manuscript; available in PMC 2012 July 1.*
carrying with the wild-type copy of rex were isolated from plates containing the appropriate antibiotics.

**Analysis of biofilm formation**

For biofilm formation, *S. mutans* strains were cultivated using a modified semi-defined biofilm medium (BM) (Loo, *et al.*, 2000) with glucose (20 mM, BMG), sucrose (10 mM, BMS), or glucose (18 mM) and sucrose (2 mM) (BMGS) as the supplemental carbohydrate sources (Wen, *et al.*, 2006). For scanning electron microscopy (SEM), biofilms were grown on hydroxylapatite (HA) discs on BMG and BMS for 24 hours, and SEM analysis was carried out as described previously (Wen, *et al.*, 2005, Wen, *et al.*, 2006). For confocal analysis, biofilms were grown under similar conditions for 24- and 72-hours, and were treated with either *Live/Dead BacLight* fluorescent dye (Invitrogen) or concanavalin A lectin conjugated with Alexa Fluor 488 and SYTO 59 (Invitrogen) prior to optical dissections using an Olympus Fluoview BX61 confocal laser scanning microscope (CLSM) (Olympus). Simulated XYZ three-dimensional images were generated using Slidebook 5.0 (Olympus).

**Phenol-sulfuric acid assay**

To measure the extracellular glucose polymers in biofilms, a phenol-sulfuric acid assay was used with known concentrations of glucose as the standards (Mukasa, *et al.*, 1985, Kumada, *et al.*, 1987, Ausubel, *et al.*, 1992, Werning, *et al.*, 2008). Briefly, 3-day biofilms were grown in BMGS on glass slides in 50 ml tubes as described elsewhere (Phan, *et al.*, 2000, Wen, *et al.*, 2010, Wen, *et al.*, 2010). Following brief sonication, bacterial cells were removed by centrifugation (4,000×g, 4°C for 15 minutes). EPS in the supernatant fluid was precipitated with 2 volumes of ethanol overnight at −20°C, and was washed twice with 80% ethanol before the optical density at 490 nm was measured (Ausubel, *et al.*, 1992, Werning, *et al.*, 2008).

**Hydrogen peroxide killing assays**

To evaluate the ability of *S. mutans* strains to withstand oxidative stress, 3-day biofilms were prepared using glass slides as described above, and hydrogen peroxide challenge assays were carried out as detailed elsewhere (Wen & Burne, 2004, Wen, *et al.*, 2006, Wen, *et al.*, 2010).

**RNA extraction, DNA microarray analysis and RealTime-PCR assays**

For transcriptional profiling, *S. mutans* strains were grown in 50 ml of BHI broth, and following brief treatment with RNAProtect as suggested by the manufacturer, total RNAs were isolated using hot phenol as previously described (Wen & Burne, 2004, Wen, *et al.*, 2005, Wen, *et al.*, 2006). To remove all DNA, RNA preps were treated with DNaseI (Ambion, Inc.) and retrieved with the RNeasy purification kit (QIAGEN, Inc.). Array analysis was performed by using the whole-genome *S. mutans* microarrays that were obtained from The J. Craig Venter Institute (JCVI, http://pfgrc.jcvi.org) by following the protocols recommended by JCVI as described elsewhere (Abranches, *et al.*, 2006, Wen, *et al.*, 2006, Wen, *et al.*, 2010). Array data were normalized with the TIGR Microarray Data Analysis System (http://www.jcvi.org/software) and further analyzed using BRB Array Tools 3.0.1 (developed by Dr. Richard Simon and Amy Peng Lam, National Cancer Institute, MD, http://linus.nci.nih.gov/BRB-ArrayTools.html) as described elsewhere (Abranches, *et al.*, 2006, Wen, *et al.*, 2006). Genes that were differentially expressed by a minimal ratio of 1.5 fold and at a statistical significance level of *P* < 0.001 were then identified. For RealTime-PCR analysis, cDNA was synthesized with 1 µg of total RNA using the iScript cDNA synthesis kit (Bio-Rad) by following the procedures recommended by the...
RESULTS AND DISCUSSION

Rex is part of the BrpA-regulon

Previously, we reported that deficiency of BrpA (for biofilm regulatory protein A) in S. mutans caused major defects in the ability of the deficient mutants to tolerate acid and oxidative stresses and the ability to accumulate biofilms (Wen & Burne, 2002, Wen, et al., 2006). The rex gene was found to be significantly decreased in the BrpA-deficient mutant, TW14D, during the early-exponential phase of growth (data not shown), suggesting that rex expression is influenced by BrpA and that rex may be involved in regulation of stress tolerance response and/or biofilm formation by S. mutans. To verify that rex is indeed a part of the BrpA-regulon, the expression of rex was analyzed using RealTime-PCR with total RNAs extracted from cultures grown in BHI and harvested during early (OD<sub>600nm</sub>≈0.2)-, mid (OD<sub>600nm</sub>≈0.4)-, and late (OD<sub>600nm</sub>≈0.6)-exponential phase, respectively. The expression of rex in the wild-type strain was at its highest level during early-exponential phase, averaging 7.85E+07 copies per µg of total RNA, although the underlying mechanism governing the regulation remains unclear. Consistent with microarray data, rex expression in TW14D was decreased by more than 6-fold during this period of growth, with an average of only 1.00E+07 copies per µg of total RNA (P<0.001). However, no significant differences were observed in cells from mid- or late-exponential phase cultures (data not shown).

The Rex-deficient mutant had a decreased ability to form biofilms

To investigate whether Rex could be associated with phenotypes observed in BrpA-deficient mutants, an internal fragment (nucleotides 136 to 584 relative to the translational initiation site) of the rex gene was deleted and replaced with a non-polar kanamycin resistance element (Zeng, et al., 2006). Rex-deficiency did not have a major impact on the morphology and growth rate in planktonic cultures in BHI (Figure 1A). However, when biofilm formation in 96-well culture plates was analyzed (Loo, et al., 2000, Wen & Burne, 2002), the Rex-deficient mutant, TW239 was shown to accumulate only a small fraction of the biofilms of the wild-type, UA159. Following staining with 0.1% crystal violet after 24 hours, the OD<sub>575nm</sub> of mutant biofilms was 3.5-fold (P<0.001) less than that of the wild-type strain when grown on glucose (Figure 1B) and decreased by more than 3-fold (P<0.001) when sucrose was the carbohydrate source (Abstr. 87<sup>th</sup> IADR Annu. Conf. #2652). When grown on glass slides in BMGS (Nguyen, et al., 2002, Wen, et al., 2010), the biofilms formed by TW239 after 3-days were about 6.2-fold less abundant than those formed by UA159, with an average of 1.82E7±1.02E7 colony-forming-units (CFU) for TW239 vs 1.13E8±2.88E7 (P<0.001) for UA159. Similar results were also observed with biofilms grown on HA discs, a commonly used in vitro tooth model. As compared to the wild-type, biofilms of the Rex-deficient mutant also had an altered structure. While UA159 formed compact, more evenly distributed biofilms, the Rex-deficient mutant biofilms appeared to be loose (more easily washed off the substratum) and porous (filled with bigger gaps between cell clusters), especially when sucrose was present in the culture medium (Figure 2). Interestingly, patches of wool-like extracellular polysaccharides were apparently in larger quantities in TW239 biofilms than UA159 biofilms.

To further evaluate the production of glucose polymers, 3-day biofilms grown on HA discs were treated with Alexa Fluor 488 conjugated concanavalin A lectin (Invitrogen, CA) by following the supplier’s instructions. Concurrently, SYTO 59 (Invitrogen, CA) was used to stain nucleic acids, conferring the bacteria with red fluorescence. Consistent with SEM analysis, TW239 biofilms were porous and contained significantly more glucans than the
wild-type (Figure 3). Complementation with a wild-type copy of rex, including its promoter region, on shuttle vector pDL278 (LeBanc & Lee, 1991) partially restored the phenotype of the wild-type (Figure 3). A phenol-sulfuric acid assay was also used to measure total glucans in the biofilms (Mukasa, et al., 1985, Kumada, et al., 1987, Ausubel, et al., 1992, Werning, et al., 2008). As expected, TW239 biofilms contained more than 2-fold glucose polymers than the parent strain, with an average of 30.62 (±5.7) µg/ml for UA159 and 72.45 (±15.85) µg/ml for TW239 (P<0.001), respectively. The complement strain, TW239C contained 41.91(±10.07) µg/ml.

Rex-deficiency increases susceptibility to oxidative stress

When compared to the wild-type strain, the Rex-deficient mutant, TW239 displayed an extended lag phase when 25 mM, methyl viologen (MV, also paraquat, Sigma) was included in the growth medium (Figure 1A). TW239C, a mutant carrying a wild-type copy of rex, showed resistance levels to MV similar to the wild-type, UA159. Incubation of the bacterial cells in buffer containing hydrogen peroxide (Fisher) at 0.2% (58 mM) resulted in a survival rate for TW239 that was more than 1-log lower than that of the wild-type after 90 minutes (Data not shown). The effect was particularly evident especially in 3-day biofilms. The complemented strain, TW239C, had an enhanced survival rate after hydrogen peroxide killing, compared to TW239 (data not shown). Effort was also made to assess whether Rex-deficiency had any impact on acid tolerance by acid killing, but no major differences were detected between the wild-type and the mutant. Collectively, the results suggest that Rex plays a major role in oxidative stress tolerance in S. mutans.

Rex-deficiency caused alterations in expression of genes central to energy metabolism, NAD+ regeneration and oxidative homeostasis

When analyzed using DNA microarray analysis with total RNA extracted from mid-exponential phase cultures grown in BHI (Abranches, et al., 2006, Wen, et al., 2006, Wen, et al., 2010), 53 genes were found to be differentially expressed in TW239, with 25 up- and 28 down-regulated by at least 1.5-fold (P<0.001) (Tables 2&S1). Among the down-regulated genes were mleS (SMU.137) for a malolactic enzyme, mleP (SMU.138) for malate permease, gshR (SMU.140) for a putative glutathione reductase, and pflC (SMU.490) for pyruvate formate-lyase activating enzyme. The up-regulated genes included pgk (SMU.361) for phosphoglycerate kinase, adhAB (SMU.127/8) for acetoin dehydrogenase, pdhAB (SMU.1422/3) for pyruvate dehydrogenase, adhE (SMU.148) for alcohol-acetaldehyde dehydrogenase, and frdC (SMU.1410) for fumarate reductase.

Malolactic enzyme MleS catalyzes decarboxylation of malic acid, yielding lactate. It was recently shown that malolactic fermentation is a major system for alkali production and that deficiency of MleS as well as MleP in S. mutans resulted in loss of protection against acid killing (Sheng, et al., 2010). In addition, the malolactic fermentation system was also found to be protective against oxidative stress and starvation. Glutathione reductase, GshR, is known to play a significant role in defense against oxidative stress in both eukaryotes and Gram-negative bacteria, and similar results were also reported in S. mutans (Yamamoto, et al., 1999). Down regulation of mleSP and gshR will certainly have an impact on the ability of the deficient mutants to survive oxidative stress, which could at least in part attribute to the observed defects in tolerance against MV and H2O2, and consequently to the decreased ability to form biofilms by TW239.

Pyruvate formate-lyase activating enzyme (PflC or Act) is shown to be the sole enzyme able to activate pyruvate formate lyase (Yamamoto, et al., 2000), which is known to be highly sensitive to oxygen and play a critical role in sugar fermentation, ATP synthesis and NAD+ and/or NADH recycling under anaerobic conditions (Yamada, et al., 1985). Acetoin
dehydrogenase (AdhAB), pyruvate dehydrogenase (PdhAB), alcohol-acetaldehyde dehydrogenase (AdhE) and fumarate reductase (FrdC) all are key enzymes in heterofermentation, ATP synthesis, and NAD⁺ and/or NADH regeneration. Unlike S. aureus, but similar to B. subtilis (Larsson, et al., 2005, Pagels, et al., 2010), the lactate dehydrogenase gene ldh was not among the genes aberrantly expressed in TW239. Coupled with the increased expression of adhAB, pdhAB, adhE and frdC and the down-regulation of pflC in response to Rex-deficiency, the data presented here also support an important role for Rex in the regulation of glycolysis and acid production by S. mutans in the plaque.

Recently, it has been shown that exposure of S. mutans to aeration causes a substantial alteration in expression of genes involved in oxidative stress (e.g., nox for NADH oxidase), energy metabolism and fermentation (e.g., pdhAB and adhE) and biofilm formation (e.g., gtfB) (Ahn, et al., 2007). Cross-referencing of these two transcriptional profiles (aeration vs rex mutation) revealed that of the genes identified in TW239, eleven (10 up- and 1 down-regulated, respectively) were also found to be consistently altered in S. mutans stressed by aeration (Tables 2&S1), indicating that Rex-mediated regulation could be part of the pathway that S. mutans employs to cope with the oxidative stress induced by exposure to oxygen. Computer-aided analysis of the affected genes also revealed the presence of inverted repeats highly similar to the conserved Rex-binding site, -TTGTGAAW₄TTCACAA-, in the promoter regions of most, but not all genes identified by microarray (Schau, et al., 2004, Gyan, et al., 2006, Pagels, et al., 2010). Efforts to investigate whether Rex can bind to the promoter of the targeted genes and how NAD⁺/NADH balances affect Rex-regulated gene expression are ongoing.

**Better understanding the role of Rex in biofilm formation**

It is apparent that Rex-deficiency did not have any significant effect on the morphology and growth rate of the deficient mutant when grown planktonically under the conditions studied (Figure 1A). However, the deficient mutant did show a decreased ability to develop biofilms on a surface, and it formed biofilms with an altered structure (Figures 2&3). These defects could be in part attributed to the altered expression of genes central to carbohydrate fermentation and energy metabolism (e.g., pflC and pdhAB), NAD⁺/NADH recycling (e.g., adhE, adhAB and frdC) and oxidative homeostasis (mleSP and gshR) (Tables 2&S1).

One particularly interesting observation of the Rex-deficient mutant is that while it had a decreased ability to form biofilms, it also appeared to generate more glucans (Figures 2&3). S. mutans possesses at least three glucosyltransferases (GtfB, -C, and -D) and one fructosyltransferase (Ftf). The enzymes use sucrose as the primary substrate, assembling glucans and fructans from the glucose- and fructose-moiety of sucrose, respectively (Burne, 1998). At a significant level of P<0.01, gtfC was also identified by DNA microarray analysis to be up-regulated by 1.56-fold in TW239, but not gtfB, gtfD and ftf (data not shown). When analyzed by RealTime-PCR, the expression of gtfC was found to be increased by more than 13-fold in TW239 (Table 2), but again no significant differences were detected in expression of either gtfB, gtfD or ftf. Similar observations were also made recently in S. mutans grown with aeration (Ahn, et al., 2007). Consistent with the severely impaired ability to form biofilms, S. mutans grown in the presence of oxygen showed major changes in the amount and localization of the Gtf enzymes. In particular, the cell surface-associated GtfC was found by Western blotting to be dramatically increased in cells grown aerobically, as compared to those prepared under anaerobic conditions. However, it remains to be investigated whether the localization of any of the Gtf enzymes were altered in S. mutans as a result of Rex-deficiency.

Glucosyltransferase GtfB is known to produce α1,3-linked, water-insoluble glucans that play a central role in S. mutans adherence and accumulation on surfaces, whereas the glucan
products of GtfC contain α1,3-linked, water-insoluble and a substantial amount of α1,6-linked water-soluble glucans (Bowen & Koo, 2011). However, limited information is available concerning the regulation of glucans biosynthesis and the impact of alterations in glucans structure (α1,3-linked v.s. α1,6-linked) on the ability of S. mutans to form biofilms (Banas & Vickerman, 2003, Banas, et al., 2007, Lynch, et al., 2007, Klein, et al., 2009, Koo, et al., 2010). Recent studies by us and some other labs have generated evidence that alteration of the glucans’ structure and/or the ratio of glucans to glucan-binding proteins could have a significant effect on S. mutans adherence and accumulation on a surface (Hazlett, et al., 1998, Hazlett, et al., 1999, Wen, et al., 2005), although the basis for this phenomenon remains unclear. Similarly, aberrant expression of GtfC in TW239 would likely cause alterations in glucan structures (more α1,6-linked, water-soluble glucans than the wild-type) and probably the ratio of glucans to glucan-binding proteins, possibly contributing to the observed decreases in biofilm formation by the deficient mutant.

In summary, this study clearly showed that transcriptional repressor Rex plays a significant role in regulation of central metabolism and energy generation, NAD+ regeneration, oxidative homeostasis, and biofilm formation by S. mutans. Current effort is directed to further investigation of the underlying mechanism of Rex-mediated regulation in oxidative stress response and biofilm formation.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

This work is supported in part by NIDCR grants DE13239 and DE19106 to RA Burne and DE19452 to ZT Wen and by South Louisiana Institute of Infectious Disease Research.

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Figure 1.
Growth study (A) and crystal violet staining of 24-hour biofilms (B). A: The impact of Rex-deficiency on growth rate was assessed using a Bioscreen C system with *S. mutans* strains growing on regular BHI broth with or without inclusion of methyl viologen (MV, 25 mM). Results show an extended lag phase for the Rex-deficient mutant and complementation of the mutant with a wild-type copy of *rex* plus its cognate promoter in multi-copy plasmid restored the phenotype. Data presented here are representatives of more than three independent experiments. B: Crystal violet staining of 24-hour biofilms was done on 96-well plates, and *S. mutans* UA159 and TW239 were grown on BM medium with glucose (G) and sucrose (S), respectively. Data represent average (±standard deviation) of more than three separate experiments.
Figure 2.
SEM analysis of biofilms. S. mutans strains were grown on BM medium with glucose, sucrose or glucose plus sucrose as the supplemental carbohydrate sources and biofilms were allowed to accumulate on HA discs. Images show representatives of 24 hour biofilms of UA159 (UA) and TW239 (TW) grown on BM glucose (G) and BM sucrose (S), respectively, with the respective magnifications as indicated.
Figure 3. 
Analysis of glucan production. *S. mutans* biofilms were grown on HA discs in BM medium with glucose and sucrose as the supplemental carbohydrate sources. After 3-days, biofilms were treated with concanavalin A lectin conjugated with Alexa Fluor 488 and SYTO 59, and green fluorescent glucans and red fluorescent cell clusters were visualized using a confocal microscope at magnification of 600×. Data presented here are representative images of BM sucrose-grown UA159 (UA), TW239 (TW) and TW239C (TWC), respectively.
### Table 1

**Bacterial strains, primers, and plasmids used in this study**

| Strains /Plasmid | Relevant characteristics | References |
|------------------|--------------------------|------------|
| *S. mutans* UA159 | wild-type                | (Ajdic, et al., 2002) |
| *S. mutans* TW14D | ΔbrpA (smu.410), Erm<sup>r</sup> | (Wen & Burne, 2002, Wen, et al., 2006) |
| *S. mutans* TW239 | Δsmu.1053, Kan<sup>r</sup> | this study |
| pDL278           | shuttle vector, Sp<sup>r</sup> | (LeBanc & Lee, 1991) |
| pDL278:rex       | shuttle carrying rex, Sp<sup>r</sup> | this study |
| *S. mutans* TW239C | TW239/pDL278:rex, Kan<sup>r</sup> and Sp<sup>r</sup> | this study |

**Primers for mutagenesis**

| Primer        | Sequence (5' to 3') | Sequence (5' to 3') | Flanking region amplified |
|---------------|---------------------|---------------------|---------------------------|
| SMU.1053 5F   | 55- tctaatgaagatgcgctc 53-tctaactgtgcatgcgatc 5' of *rex* for mutagenesis |
| SMU.1053 3F   | 35-tgttgctcaagctgatgcaaactgtaactag 33-atgcgaacaactcattattgtg 3' of *rex* for mutagenesis |
| Primers for qPCR | Forward (5' to 3') | Reverse (5' to 3') | Application |
| 16S-1 rRNA    | cacacgcagcctagctcacc  | cagccgacacgtaagac  | 16S rRNA fragment, 160 bp |
| SMU.1054      | accattggcatggctcactc | cgtcagccgacacgtaagac  | SMU.1054 fragment, 191 bp |
| SMU.1055      | agaagctgttgaagac  | cgcaggtcaggtaagac  | SMU.1055 fragment, 122 bp |
| SMU.148       | tgaattggaagcggtagtgc | atcagccgctgatgtcataactgtaaagcggctaagg | *adhE* fragment, 118 bp |
| *gtfB*        | agcaatgcagccctcatcacaat | agcagggcagggcttgtaaggc  | *gtfB* fragment, 98 bp |
| *gtfC*        | atgcaagccatgatgtaataa | cggatgaagggtaaaagaag | *gtfC* fragment, 172 bp |
| *gtfD*        | tagcagctggctgctaagctgtaa | ggtattgtgtgtaatgag | *gtfD* fragment, 98 bp |
| *ftf*         | gcgcagctgctgctgtgctgtgct | tacctcgcagcttcctac | *ftf* fragment, 94 bp |

Note: Kan<sup>r</sup>, Sp<sup>r</sup> and Erm<sup>r</sup>, for kanamycin, spectinomycin and erythromycin resistance, respectively. Sequences underlined are restriction sites engineered for cloning.
Table 2
RealTime-PCR analysis of selected genes

| Name or ID | Description and putative function# | UA159* | TW239 | Ratio@ (TW239/UA159) |
|------------|------------------------------------|--------|-------|----------------------|
| 16S rRNA   | 16S ribosomal RNA                  | 8.97E+08 | 1.12E+09 | 1.25                 |
| SMU.148    | putative alcohol-acetaldehyde dehydrogenase, AdhE | 1.03E+07 | 2.25E+08 | 21.8                |
| SMU.1054   | putative glutamine amidotransferase | 7.21E+07 | 3.53E+08 | 4.90                |
| SMU.1055   | DNA repair protein RadC             | 1.02E+07 | 2.31E+07 | 2.28                |
| ftf        | fructosyltransferase, Ftf           | 9.52E+06 | 8.00E+06 | 0.84                |
| gtfB       | glucosyltransferase B               | 5.92E+05 | 5.13E+05 | 0.867               |
| gtfC       | glucosyltransferase C               | 1.13E+07 | 1.56E+08 | 13.9                |
| gtfD       | glucosyltransferase D               | 2.49E+07 | 2.79E+07 | 1.12                |

Note:
# Description and putative function of the selected genes are based upon the published S. mutans database.
* expressed as copy number of respective transcripts per µg of total RNA.
@ defined as levels of expression in the Rex-deficient mutant TW239 relative to those of the wild-type UA159. Genes in italics are those identified in both the Rex-deficient mutant and cells stressed with aeration.