





Characterization of the Trehalose Utilization Operon in *Streptococcus mutans* Reveals that the TreR Transcriptional Regulator Is Involved in Stress Response Pathways and Toxin Production

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ABSTRACT *Streptococcus mutans*, the organism most frequently associated with the development of dental caries, is able to utilize a diverse array of carbohydrates for energy metabolism. One such molecule is trehalose, a disaccharide common in human foods, which has been recently implicated in enhancing the virulence of epidemic strains of the pathogen *Clostridium difficile*. In this study, mutants with deletions of all three genes in the putative *S. mutans* trehalose utilization operon were characterized, and the genes were shown to be required for wild-type levels of growth when trehalose was the only carbohydrate source provided. Interestingly, the TreR transcriptional regulator appeared to be critical for responding to oxidative stress and for mounting a protective stress tolerance response following growth at moderately acidic pH. mRNA sequencing (RNA-seq) of a *treR* deletion mutant suggested that in *S. mutans*, TreR acts as a trehalose-sensing activator of transcription of the *tre* operon, rather than as a repressor, as described in other species. In addition, deletion of *treR* caused the downregulation of a number of genes involved in genetic competence and bacteriocin production, supporting the results of a recent study linking trehalose and the *S. mutans* competence pathways. Finally, deletion of *treR* compromised the ability of *S. mutans* to inhibit the growth of the competing species *Streptococcus gordonii* and *Lactococcus lactis*. Taking the results together, this study solidifies the role of the *S. mutans tre* operon in trehalose utilization and suggests novel functions for the TreR regulator, including roles in the stress response and competitive fitness.

IMPORTANCE *S. mutans* is the primary etiologic agent of dental caries, which globally is the most common chronic disease. *S. mutans* must be able to outcompete commensal organisms in its dental plaque niche in order to establish persistence and pathogenesis. To that end, *S. mutans* metabolizes a diverse array of carbohydrates to generate acid and impede its acid-sensitive neighbors. Additionally, *S. mutans* utilizes quorum signaling through genetic competence-associated pathways to induce production of toxins to kill its rivals. This study definitively shows that the *S. mutans* trehalose utilization operon is required for growth in trehalose. Furthermore, this study suggests that the *S. mutans* TreR transcriptional regulator has a novel role

Received 30 January 2018 Accepted 30 March 2018

Accepted manuscript posted online 9 April 2018

Citation Baker JL, Lindsay EL, Faustoferri RC, To TT, Hendrickson EL, He X, Shi W, McLean JS, Quivey RG, Jr. 2018. Characterization of the trehalose utilization operon in *Streptococcus mutans* reveals that the TreR transcriptional regulator is involved in stress response pathways and toxin production. *J Bacteriol* 200:e00057-18. <https://doi.org/10.1128/JB.00057-18>.

Editor Tina M. Henkin, Ohio State University

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in virulence through regulation of genes involved in genetic competence and toxin production.

KEYWORDS *Streptococcus mutans*, gene regulation, mutacin, oxidative stress, trehalose

Streptococcus mutans, the oral pathogen most frequently associated with the development of dental caries, relies chiefly on human host foodstuffs as an energy source (1). As the omnivorous human diet is quite diverse, it is to the benefit of *S. mutans* to be able to utilize as many host dietary macromolecules as possible. *S. mutans* is truly a master of carbohydrate metabolism, capable of exploiting a greater assortment of carbohydrate types than other sequenced Gram-positive organisms (2, 3). *S. mutans* encodes at least 14 phosphoenolpyruvate:phosphotransferase systems (PTS) and 2 ABC transporters for sugar transport (2, 3). After import, sugars are broken down by appropriate hydrolases and enter the Emden-Meyerhof-Parnas (EMP) glycolytic pathway, which is the primary method of ATP generation in *S. mutans*. The resulting pyruvate is then fermented to a number of mainly acidic end products, a process that regenerates the NAD^+ needed for a continuation of flux through the EMP pathway (*S. mutans* lacks a canonical electron transport chain). It is these acidic end products of fermentation that lower the local pH in dental plaque such that demineralization of the adjacent tooth surface occurs and the process of caries formation is initiated (4).

Trehalose is a disaccharide consisting of two α -glucose units joined by an α,α -1,1- α -glucosidic bond. Foods common in the human diet that naturally contain trehalose include shellfish, bread, beer, mushrooms, and seaweed. Following the discovery of a novel trehalose synthesis method in 2000, trehalose has seen increased use as a food additive and is used in a diverse array of foods, including pasta, ground beef, and ice cream. Trehalose is both an established solute in regulation of osmotic pressure/heat shock and a cryoprotectant in a variety of prokaryotic and eukaryotic species, including the Gram-positive organisms *Bacillus subtilis* and *Lactobacillus acidophilus* (5, 6). A *tre* operon, encoding the machinery to import and utilize trehalose as a carbon source, is widely conserved throughout Gram-positive bacteria (6). In the majority of cases, the operon consists of genes encoding a trehalose-specific PTS, a hydrolase capable of hydrolyzing trehalose to glucose and glucose-6-phosphate, and a transcriptional regulator that senses trehalose levels (6). In *Lactococcus lactis*, the *tre* operon also encodes a trehalose phosphate phosphorylase and a β -phosphoglucomutase (6). Regulation of this operon is most well understood in *B. subtilis*, where the multimeric TreR regulator binds to a *tre* operator in the promoter region of the operon and represses transcription of the operon when trehalose is absent. Trehalose-6-phosphate binds to TreR and inhibits multimerization of the protein and thus its ability to repress transcription. The *B. subtilis* *tre* operon is also regulated by carbon catabolite repression (CCR), through the global regulator carbon catabolite control protein (CcpA). Canonically, CCR represses use of nonpreferred sugars, such as trehalose, in the presence of glucose. In this manner, *B. subtilis* senses the presence of trehalose and accordingly upregulates the production of proteins needed to utilize it as an energy source (7–9). More recently, trehalose was shown to enhance virulence of the significant intestinal pathogen *Clostridium difficile*, likely through increased toxin production (10).

S. mutans UA159 is able to grow on trehalose as a sole carbon source, and its genome contains a putative *tre* operon encoding a trehalose-6-phosphate hydrolase (*treA* [alternatively annotated *treC*], SMU.2037), a trehalose-specific EIIABC PTS component (*treB* [alternatively annotated *treP* or *pttB*], SMU.2038), and a GntR family regulator of the trehalose operon (*treR*, SMU.2040) (2, 3). A previous study used cDNA microarray analysis to examine global gene expression in batch cultures of *S. mutans* UA159 grown in trehalose versus glucose, and it was observed that *treA* and *treB* were highly upregulated during growth in trehalose (3). The results suggested that the *S. mutans* *tre* operon is important for trehalose metabolism and is likely regulated via a trehalose-sensing mechanism, similar to the TreR-mediated repression observed in *B. subtilis* (3).

Another study indicated that although *S. mutans* could grow on trehalose, acid production was significantly reduced compared to that with growth on glucose, leading the authors to suggest the use of trehalose as a less-cariogenic sugar substitute (11). More recent analyses have shown that the expression of *S. mutans treA* and *treB* was elevated during growth in galactose compared to glucose and upon deletion of *ccpA*, indicating that CcpA may regulate *tre* expression through CCR in *S. mutans* (12). Further, it has been shown that growth in trehalose significantly increased the transformation efficiency of *S. mutans* by inducing expression of *comX*, *comS*, and *comYA* (13), indicating that trehalose may serve as an important signaling molecule in *S. mutans*.

To persist in plaque biofilm and cause disease, *S. mutans* must outcompete commensal streptococci that occupy the same niche. These commensals include peroxigenic streptococcal species such as *S. sanguinis* and *S. gordonii*, which produce bactericidal levels of H₂O₂ to kill rivals, such as *S. mutans* (14, 15). Therefore, the ability of *S. mutans* to contend with oxidative stress generated by H₂O₂ is paramount to its ability to prevail as a significant player in the dental plaque community (16–18). In addition to reducing the local environmental pH to impair less-acid-tolerant competitors, *S. mutans* utilizes toxins, including the nonantibiotic mutacins IV, V, and VI, to kill adversaries (19). In *S. mutans*, production of these toxins is intertwined with competence activation pathways through the competence-stimulating peptide (CSP) quorum-sensing peptide (20–22).

In this study, the regulation and function of the *tre* operon in *S. mutans* were investigated and characterized. All three genes in the operon were required for wild-type levels of growth when trehalose was the sole carbon source provided. In *S. mutans*, TreR appeared to function as an activator of transcription of the *tre* operon, rather than a repressor, under the conditions tested. Surprisingly, deletion of *treR* severely impacted the ability of *S. mutans* to tolerate oxidative stress and mount an effective stress tolerance response. Further investigation via mRNA sequencing (RNA-seq) of a *treR* deletion mutant confirmed that loss of *treR* affected transcription of a number of genes outside the *tre* operon, particularly several involved in competence and toxin production. Additionally, loss of *treR* compromised the ability of *S. mutans* to inhibit growth of the competing organisms *Streptococcus gordonii* and *Lactococcus lactis*. Taking the results together, this study confirms the role of the *S. mutans tre* operon in trehalose utilization and suggests that the *S. mutans* TreR transcriptional regulator acts in a disparate manner, and has an expanded role, compared to its previously characterized counterpart in *B. subtilis*.

RESULTS

The *S. mutans tre* operon is required for trehalose utilization. As a resident of the oral cavity, *S. mutans* derives a significant portion of its energy from carbohydrates harvested during transient contact with food being consumed by the human host. *S. mutans* is well adapted to the diverse human diet and is able to utilize numerous carbon sources present in human food, including trehalose. In *S. mutans*, the predicted trehalose utilization (*tre*) operon consists of three genes: a 1,629-bp putative trehalose-6-phosphate hydrolase gene (*treA*, SMU.2037), a 1,968-bp trehalose-specific EIIABC PTS component gene (*treB*, SMU.2038), and a 714-bp gene for a putative GntR family repressor of the trehalose operon (*treR*, SMU.2040) (2).

Although previous work determined that growth in trehalose resulted in upregulation of *treA* and *treB* (3), it remained unclear whether the *tre* operon was required for the growth of *S. mutans* in trehalose. To elucidate the role of the *tre* operon genes in sugar metabolism, a *treR* deletion mutant ($\Delta treR$ strain) was generated as described in Materials and Methods. *treA* and *treB* deletion mutants ($\Delta treA$ and $\Delta treB$ strains) have been previously described (23). Cultures of *S. mutans* UA159 and the $\Delta treA$, $\Delta treB$, and $\Delta treR$ mutants were grown in a Bioscreen C in either tryptone-yeast extract (TY) plus 1% glucose or TY plus 1% trehalose. During growth in glucose-containing medium, all four strains had nearly identical growth rates (Fig. 1A). However, when cultures were grown

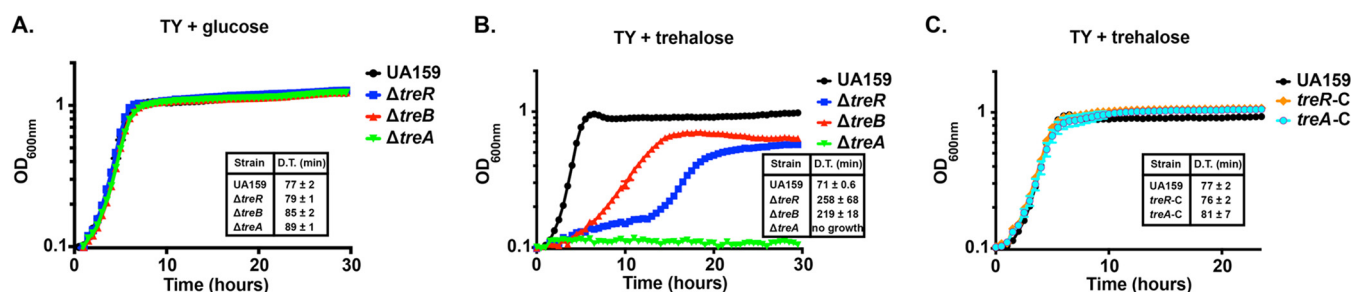


FIG 1 The *tre* operon is required for wild-type growth on trehalose. (A and B) Growth of the parent strain *S. mutans* UA159 and its $\Delta treR$, $\Delta treB$, and $\Delta treA$ derivatives in TY plus 1% (vol/vol) glucose (A) or TY plus 1% (vol/vol) trehalose (B). (C) Growth of the parent strain *S. mutans* UA159 and the *treR* and *treA* complement strains (*treR*-C and *treA*-C strains) in TY plus 1% (vol/vol) trehalose. $n = 10$ for each condition. Insets in each panel provide the mean doubling time (D.T.) \pm standard deviation for each strain, if applicable.

in TY medium containing trehalose, compared to UA159, the $\Delta treB$ strain was significantly impaired in its ability to grow, the $\Delta treR$ strain was even more severely impaired in its ability to grow, and the $\Delta treA$ strain was completely unable to grow (Fig. 1B). Meanwhile, UA159 exhibited nearly identical growth kinetics during growth in trehalose versus glucose. These assays were also performed under anaerobic conditions, which generated similar results (data not shown). Taken together, these data show that *treA* is absolutely required for growth in medium containing trehalose and that disruption of *treB* or *treR* significantly impairs the ability of *S. mutans* to grow in trehalose. To confirm specifically that the loss of these genes was indeed preventing growth of the strains in trehalose, complemented strains of the $\Delta treA$ and $\Delta treR$ strains were generated as described in Materials and Methods and designated *treA*-C and *treR*-C strains, respectively. Complementation of both *treR* and *treA* faithfully restored the phenotype of the parent strain during growth in trehalose (Fig. 1C). Construction of a *treB* complement strain was recalcitrant to our methods, likely due to toxicity of the transmembrane *treB* gene in *Escherichia coli*.

In *S. mutans*, *treR* is critical for tolerance of oxidative stress. Previous screening of an *S. mutans* mutant library indicated that deletion of *treA* or *treB* modestly impaired the growth of *S. mutans* under several conditions of acid and oxidative stress (23). In this study, growth of the $\Delta treR$ strain was characterized under acid and oxidative stress. Surprisingly, compared to that of UA159, growth of $\Delta treR$ strain was severely impaired in the presence of 0.5 mM H_2O_2 (Fig. 2A), while growth of the *treR*-C strain was nearly identical to that of the parent strain. Growth of the $\Delta treR$ strain was similar to that of UA159 in medium buffered to pH 5.4 or pH 5.6 (data not shown). Typically, growth of *S. mutans* at the moderately acidic pH of 5 induces the acid tolerance response (ATR), which protects the cells from further insult, including extreme acid and oxidative stress. Compared to UA159, after steady-state growth at pH 5 to induce the ATR, the $\Delta treR$

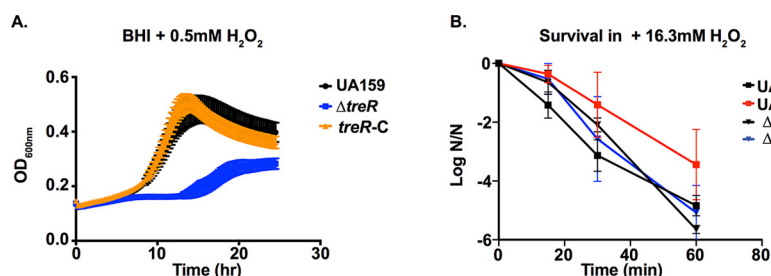


FIG 2 The $\Delta treR$ strain is impaired in its ability to grow in the presence of oxidative stress. (A) The parent strain, *S. mutans* UA159, the $\Delta treR$ strain, and the *treR*-C (*treR* complement) strain were grown in triplicate in BHI plus 0.5 mM H_2O_2 ($n = 10$, biological replicates). A representative graph is shown. (B) Sensitivity of *S. mutans* to H_2O_2 challenge following growth at steady-state pH 7 or steady-state pH 5 as indicated. Results are expressed as the $\log_{10}(n/n_0)$ where n is the viable cell count at each time point and n_0 is the viable cell count at time zero. Error bars represent the standard deviation.

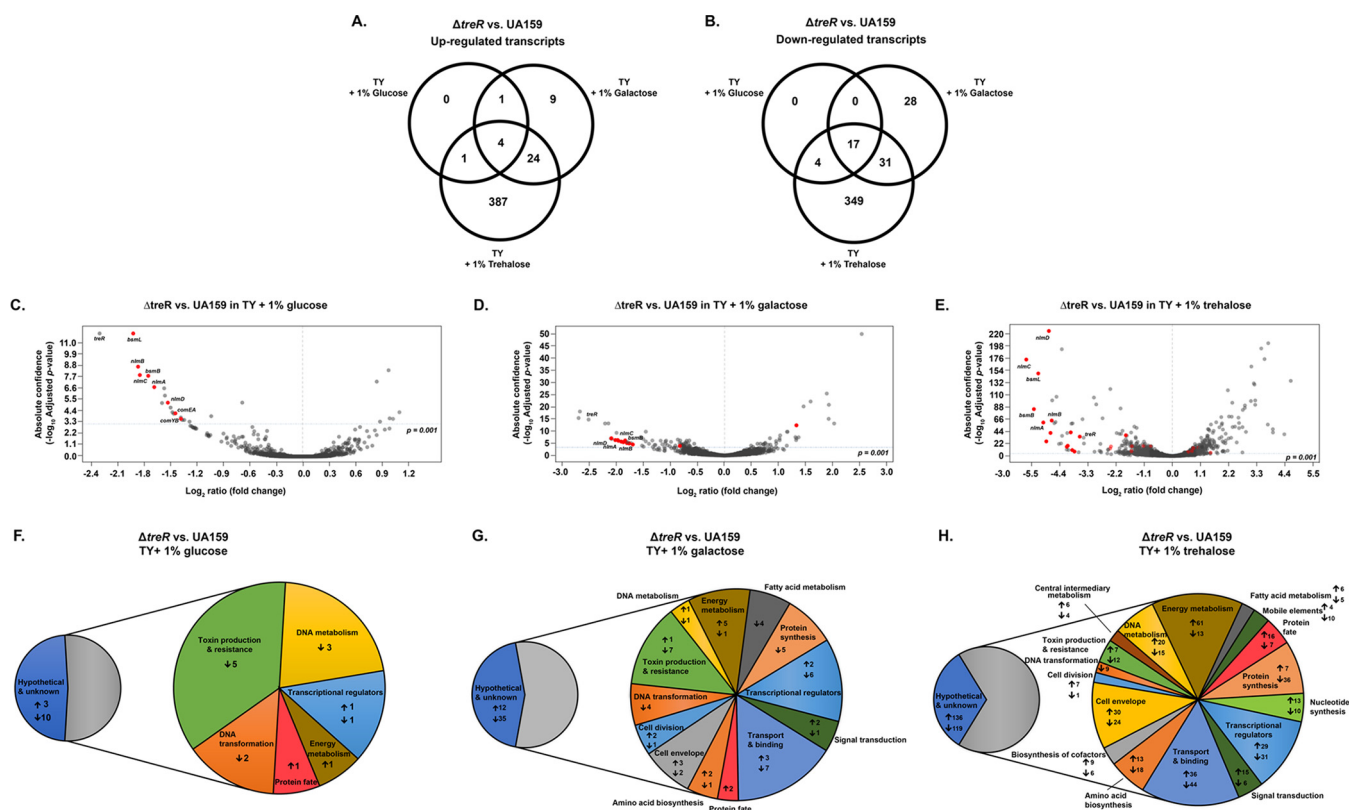


FIG 3 Differentially regulated genes in the $\Delta treR$ strain compared to *S. mutans* UA159. (A and B) Venn diagrams illustrating the number of genes significantly ($P < 0.001$; Benjamini-Hochberg procedure) upregulated (A) or downregulated (B) in the $\Delta treR$ strain, compared to UA159, during growth in TY plus 1% glucose, galactose, or trehalose, based on results from RNA-seq. (C to E) Volcano plots illustrating the transcriptome of the $\Delta treR$ strain versus UA159 during growth in TY plus 1% glucose (C), 1% galactose (D), or 1% trehalose (E). The vertical dashed line indicates 0-fold change in expression, and the horizontal dashed line indicates a Benjamini-Hochberg adjusted P value of 0.001, which was used as the cutoff in our analyses. Data points representing transcripts assigned to the functional classes “toxin production and resistance” or “DNA transformation” are colored red for visualization. Specific data points representing genes of interest are individually labeled. (F to H) Pie charts of functional classes with altered expression during growth of the $\Delta treR$ strain, compared to UA159, in TY plus 1% glucose (F), TY plus 1% galactose (G), or TY plus 1% trehalose (H). The numbers of genes upregulated are indicated next to the up arrows, and the numbers of downregulated genes are indicated next to the down arrows. Genes encoding hypothetical or unknown proteins are separated out in the smaller pie charts to allow for better visualization of genes with known functions.

strain was able to survive an acid challenge (pH 2.5) at a similar rate (data not shown); however, the $\Delta treR$ strain was significantly compromised in its ability to survive a peroxide challenge (16.3 mM H_2O_2), indicating a defect in the ATR in the absence of *treR* (Fig. 2B). These results were remarkable, as TreR is predicted to be simply a local regulator with a role strictly in trehalose utilization.

Loss of *treR* impacts the *S. mutans* transcriptome during growth on several carbon sources, and TreR is likely an activator of transcription of *treA* and *treB*.

Since the $\Delta treR$ strain was, unexpectedly, defective in its ability to tolerate oxidative stress, we hypothesized that in *S. mutans*, TreR may be involved in the regulation of genes outside its operon. Although *S. mutans* does not appear to accumulate trehalose in response to stress, the disaccharide may serve as an important signaling molecule (13). To examine the effects of the loss of TreR-mediated regulation on the *S. mutans* transcriptome, RNA-seq was performed on *S. mutans* UA159 or $\Delta treR$ strains grown in TY plus 1% glucose, 1% galactose, or 1% trehalose. In the $\Delta treR$ strain, compared to UA159, at a Benjamini-Hochberg adjusted P value cutoff of <0.001 , there were 6 genes upregulated and 21 genes downregulated during growth in glucose (see Table S1 in the supplemental material), 136 genes upregulated and 80 genes downregulated during growth in galactose (see Table S2 in the supplemental material), and 426 genes upregulated and 401 genes downregulated during growth in trehalose (see Table S3 in the supplemental material). A summary of the number of genes and functional classes differentially regulated is provided in Fig. 3A, B, and F to H, and a list of genes of interest

TABLE 1 Selected genes of interest differentially regulated in the $\Delta treR$ strain compared to UA159 during growth in the indicated media

Locus tag	Product	Functional class	Gene(s)	Differential expression, $\Delta treR$ /UA159 (\log_2 ; $P < 0.001$) in:		
				TY + 1% glucose	TY + 1% galactose	TY + 1% trehalose
SMU_1906c	Hypothetical protein	Cellular processes: toxin production and resistance	<i>bsmB</i>	−1.74	−1.96	−5.41
SMU_1905c	Putative bacteriocin secretion protein	Cellular processes: toxin production and resistance	<i>bsmL</i>	−1.91	−1.70	−5.24
SMU_1915	Competence-stimulating peptide, precursor	Hypothetical proteins: conserved	<i>comC</i>	NS ^a	NS	−2.43
SMU_1917	ComE response regulator	Signal transduction: two-component systems	<i>comE</i>	NS	NS	−1.09
SMU_625	Putative competence protein	Cellular processes: DNA transformation	<i>comEA</i>	−1.44	−1.73	−3.83
SMU_498	Putative late competence protein	Unknown function: general	<i>comF</i>	NS	NS	−3.62
SMU_1987	Putative ABC transporter, ATP-binding protein ComYA; late competence protein	Cellular processes: DNA transformation	<i>comYA</i>	NS	NS	−4.76
SMU_1985	Putative ABC transporter ComYB; probably part of the DNA transport machinery	Cellular processes: DNA transformation	<i>comYB</i>	−1.37	−1.53	−3.91
SMU_1984	Putative competence protein ComYC	Cellular processes: DNA transformation	<i>comYC</i>	NS	NS	−4.12
SMU_1983	Putative competence protein ComYD	Cellular processes: DNA transformation	<i>comYD</i>	NS	NS	−4.06
SMU_1913c	Putative immunity protein, BLpL-like	Hypothetical proteins	<i>immA</i>	NS	NS	−3.97
SMU_150	Hypothetical protein	Cellular processes: toxin production and resistance	<i>nImA</i>	−1.68	−2.02	−5.04
SMU_151	Hypothetical protein	Hypothetical proteins: conserved	<i>nImB</i>	−1.86	−1.87	−4.72
SMU_1914c	Hypothetical protein	Cellular processes: toxin production and resistance	<i>nImC</i>	−1.84	−1.91	−5.71
SMU_423	Hypothetical protein	Cellular processes: toxin production and resistance	<i>nImD</i>	−1.52	−2.09	−4.83
SMU_2037	Putative trehalose-6-phosphate hydrolase TreA	Energy metabolism: biosynthesis and degradation of polysaccharides	<i>treA</i>	NS	NS	−2.98
SMU_2038	Putative PTS, trehalose-specific IIABC component	Signal transduction: PTS	<i>treB</i>	NS	NS	−3.07
SMU_2040	Putative transcriptional regulator; repressor of the trehalose operon	Regulatory functions: DNA interactions	<i>treR</i>	−2.30	−2.52	−3.47

^aNS, not significant.

that were differentially regulated in the $\Delta treR$ strain compared to UA159 is provided in Table 1. As an internal control, *treR* was significantly downregulated under all 3 conditions, congruent with the fact that the $\Delta treR$ strain is a deletion mutant and no *treR* transcript should be present (Table 1). Interestingly, *treA* and *treB* were significantly downregulated in the $\Delta treR$ strain compared to UA159 during growth in trehalose and did not have significantly altered expression in the $\Delta treR$ strain compared to UA159 during growth in glucose or galactose (Table 1). These observations suggest that under the conditions tested, TreR acts as an activator of transcription of the *tre* operon in the presence of trehalose, rather than as a repressor that is derepressed in the presence of trehalose (as in *B. subtilis* [9]). If *S. mutans* TreR is indeed a trehalose-sensitive activator of *tre* transcription, a likely explanation for the large number of genes differentially regulated in the $\Delta treR$ strain compared to UA159 during growth in trehalose is general stress of the organism due to an inability to efficiently import and utilize the only available carbon source. This is supported by the impaired growth phenotype (Fig. 1B), as well as the vast number of genes involved in energy metabolism and protein synthesis that are differentially regulated in the $\Delta treR$ strain during growth in trehalose (Fig. 3H).

In the $\Delta treR$ strain, compared to UA159, all of the genes differentially regulated during growth in medium containing glucose, and the vast majority of genes differentially regulated during growth in medium containing galactose, were also differentially regulated during growth in at least one of the other two sugars (Fig. 3A and B). During growth in glucose, several genes involved in competence as well as toxin production resistance and DNA metabolism were downregulated in the $\Delta treR$ strain compared to UA159 (Fig. 3C and F; Tables 1 and S1). These included *comEA* and *comYB*, as well as the genes encoding the nonantibiotic toxins mutacin IV (*nImA* and *nImB*), mutacin V (*nImC*), and mutacin VI (*nImD*), which are known to kill other oral *Lactobacillales* (19). These trends continued, and were even more striking, in the $\Delta treR$ strain

during growth in medium containing galactose (Fig. 3D and G; Tables 1 and S2). In addition, a number of transcriptional regulators and genes involved in signal transduction, including *spxA2* (formerly *spxB*), *ciaH*, and *cpsY*, were differentially regulated during growth in galactose (Fig. 3G; Table S2). Although differential expression of nearly half of the organism's transcriptome was observed in the $\Delta treR$ strain grown in medium containing trehalose, compared to UA159, major trends included a downregulation of genes involved in protein synthesis and competence and an upregulation of genes involved in protein fate and degradation, as well as energy metabolism (Fig. 3H; Table S3). Taken together, the results from RNA-seq indicate that the *S. mutans* TreR regulator appears to function differently than described in other species: as an activator of *tre* transcription, rather than a repressor. In addition, the genes differentially regulated in the $\Delta treR$ strain, compared to UA159, during growth in glucose and galactose suggest that TreR acts, either directly or indirectly, as a more global regulator in *S. mutans* than previously thought and is involved in competence and toxin production.

Deletion of *treR* significantly reduces the ability of *S. mutans* to kill competing species. To determine if the reduced expression of the genes encoding mutacins IV, V, and VI in the $\Delta treR$ strain actually impaired the ability of *S. mutans* to kill and compete with neighboring species, a deferred-antagonism assay (24, 25) was performed using rich medium (brain heart infusion [BHI]) or defined medium (TY plus 1% glucose or TY plus 1% trehalose). *S. mutans* UA159, the $\Delta treR$ strain, or the *treR* complement strain (*treR*-C strain) was plated as an initial colonizer, and an overlay of *S. gordonii* (which is susceptible to mutacins IV and V) or *Lactococcus lactis* (which is susceptible to mutacins V and VI) was plated as a secondary colonizer (Fig. 4). While UA159 produced a marked zone of inhibition in the *S. gordonii* and *L. lactis* overlays, the $\Delta treR$ strain produced a greatly reduced zone of inhibition in both overlays, indicating a defect in the ability of the $\Delta treR$ strain to inhibit the growth of its competitors, most likely through a reduction in mutacin expression. The *treR*-C strain produced zones of inhibition that were larger than those of the $\Delta treR$ strain but smaller than those of the parent strain, UA159. The difference in apparent mutacin production between UA159 and the *treR*-C strain may be due to the fact that the complemented *treR* gene, while present, is expressed from a different genetic locus in the *treR*-C strain. All strains produced a larger zone of inhibition during growth on BHI compared to TY medium. The $\Delta treR$ strain produced no zone of inhibition during growth on TY plus 1% trehalose and, in fact, allowed the overlaid species to grow across the top of the colony. In all other cases, the primary colony, at least, prevented growth of *S. gordonii* or *L. lactis* across its top, even if there was no discernible clearing of the surrounding lawn. It is unclear whether this is due to poor growth of the $\Delta treR$ strain when trehalose is the only sugar provided (i.e., as seen in Fig. 1), a lack of mutacin production, or a combination of the two. In the case of the *L. lactis* overlay, growth on TY plus 1% trehalose produced larger zones of inhibition than growth on TY plus 1% glucose in the UA159 and *treR*-C strains, indicating that exposure to trehalose may increase mutacin production in *S. mutans*, though more detailed studies exploring a titration of trehalose concentrations and growth in multiple sugars are needed to confirm this hypothesis.

DISCUSSION

The disaccharide trehalose consists of two α -glucose molecules linked by an α,α -1,1-glucosidic bond. A number of bacterial and eukaryotic species utilize trehalose as a carbon source, as well as as a protectant from heat shock, osmotic stress, and cold (5, 6). Among Gram-positive bacteria, the machinery needed to import and consume trehalose as an energy source is most well understood in *Bacillus subtilis*. *B. subtilis* is thought to sense the presence of trehalose and upregulate the machinery needed to import and prepare the disaccharide for use as an energy source. The *B. subtilis* *tre* operon is also subject to CCR, mediated by the CcpA protein, and the presence of glucose (7).

In the oral pathogen *S. mutans*, the ability to metabolize a wide variety of carbohydrates is of significant interest, such that full advantage can be taken of the diverse

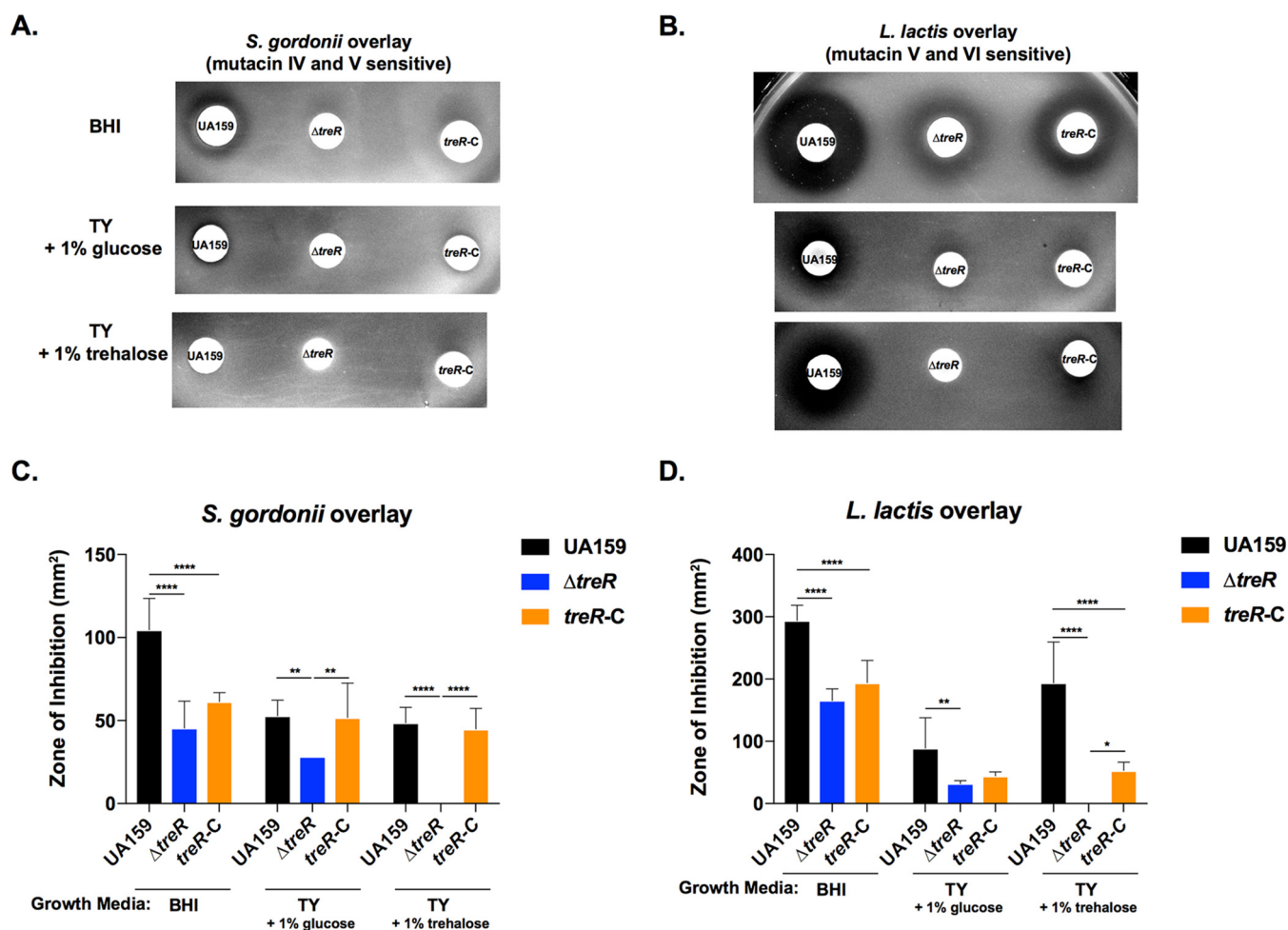


FIG 4 Loss of *treR* drastically reduces the ability of *S. mutans* UA159 to inhibit its competitors, *S. gordonii* and *L. lactis*. (A and B) Deferred-antagonism assay, performed as described in Materials and Methods, to observe mutacin production. Cultures of *S. mutans* UA159, $\Delta treR$, and *treR-C* strains were spotted on BHI agar, TY plus 1% glucose agar, and TY plus 1% trehalose agar and incubated overnight. Cultures of *S. gordonii* (A) or *L. lactis* (B) were added to 5 ml BHI soft agar and used to overlay the plates containing the *S. mutans* strains. Zones of inhibition were measured 24 h after addition of the overlay. Six replicate assays were performed, and representative images are shown. (C and D) Areas of the zones of inhibition for the indicated strains in the indicated media for *S. gordonii* (C) and *L. lactis* (D). Error bars represent standard deviation, and asterisks denote statistical significance between indicated pairs as determined by Tukey's multiple-comparison test following a two-way analysis of variance (ANOVA) (*, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$) ($n = 6$).

human host diet (1, 2). Trehalose is among the numerous sugars which *S. mutans* can exploit, and the *S. mutans* genome is predicted to encode TreA, TreB, and TreR (2). Similar to the case in *B. subtilis*, the *S. mutans tre* operon was upregulated during growth in trehalose or galactose and upon deletion of *ccpA* (3, 12), suggesting that the operon was regulated in a manner similar to that in *B. subtilis*. A more recent study showed that in *S. mutans*, exposure to trehalose significantly increased genetic competence, and suggested that in *S. mutans*, trehalose may function as a signaling molecule, as well as an energy source (13). In this study, the role of the *S. mutans tre* operon was characterized, and the role of the TreR regulator was further explored.

To confirm that the *tre* operon was indeed crucial for utilization of trehalose by *S. mutans*, the $\Delta treA$, $\Delta treB$, and $\Delta treR$ strains were all tested for their ability to grow in medium where trehalose was the sole carbon source provided. Although all three genes were required for wild-type levels of growth in trehalose (Fig. 1B), *treA* appears to be the most essential gene for trehalose utilization, as the $\Delta treA$ strain was unable to grow under these conditions. This finding is logical, as without TreA, it is likely that *S. mutans* was unable to break trehalose down to glucose for use in energy metabolism. The $\Delta treR$ and $\Delta treB$ mutants were able to grow, but at a greatly reduced rate and with lower final yields (Fig. 1B). The loss of TreB most likely affected the ability of *S. mutans*

to import trehalose in a high-affinity, specific manner. Instead, other nonspecific, lower-affinity (and less efficient) transport systems may be used. It was initially less clear why the $\Delta treR$ strain was significantly impaired in its ability to grow on trehalose, because if it functioned merely as a repressor of the *tre* operon, the deletion strain should exhibit increased expression of the machinery needed to utilize trehalose and thus would be unlikely to have impaired growth in trehalose.

Further characterization of the $\Delta treR$ strain revealed a surprising phenotype: increased sensitivity to H_2O_2 (Fig. 2A). This was unexpected, as TreR has thus far only been described as a local regulator governing trehalose utilization. Typically, induction of the *S. mutans* ATR protects against both acid and reactive oxygen species (ROS) toxicity; however, deletion of *treR* appeared to hinder the capacity of *S. mutans* to mount an ATR that protected against oxidative stress, following growth at the moderately acidic pH of 5 (Fig. 2B). The full arsenal of the ATR is crucial in the capacity of *S. mutans* to cause disease, as this robust stress response gives *S. mutans* a significant competitive advantage against less aciduric commensal rivals, several of which produce H_2O_2 to kill *S. mutans* (26).

To further examine the phenotype observed in the $\Delta treR$ strain, RNA-seq was performed on the UA159 and $\Delta treR$ strains grown in glucose, trehalose, or galactose (a nonpreferred sugar other than trehalose, i.e., alleviating CCR). RNA-seq revealed that *treA* and *treB* were not upregulated upon deletion of *treR*, as was expected. In fact, upon deletion of *treR*, *treA* and *treB* were significantly downregulated during growth in trehalose and were not differentially regulated during growth in glucose or galactose (Table 1). This suggests that in *S. mutans*, TreR actually serves as a transcriptional activator of the *tre* operon when trehalose is present. This hypothesis also explains the growth defect of the $\Delta treR$ strain during growth in trehalose: without TreR-mediated activation of *treA* and *treB* expression, *S. mutans* struggles to import and generate energy from the only carbon source provided. The stress generated from this condition would also serve to explain the vast number of genes (>800) that are differentially regulated in the $\Delta treR$ strain compared to UA159 during growth in trehalose, particularly the upregulation of genes involved in energy metabolism and protein degradation and the concurrent downregulation of genes involved in protein synthesis (see Table S3 in the supplemental material). While this theory appears likely, further work is needed to confirm that TreR acts directly as a transcriptional activator. Elucidation of the *S. mutans* *tre* promoter, including downstream effects of the binding and stoichiometry of multiple regulators, will also assist in obtaining a more complete understanding of *tre* regulation. Indeed, putative binding motifs for the CcpA and FabT regulators have been identified in the *S. mutans* *tre* promoter, in addition to the predicted TreR motif. In-depth biochemical studies exploring these regulators and *tre* expression are in progress.

The majority of the most significantly downregulated genes in the RNA-seq data for the $\Delta treR$ strain were involved in toxin production and the competence signaling pathways (Fig. 3C to E; Table 1). This was particularly interesting given that (i) trehalose increases genetic competence in *S. mutans* (13), (ii) competence coordinates toxin production in *S. mutans* (27), and (iii) trehalose was also recently shown to increase toxin production in *C. difficile* (10). This collective evidence suggests that in *S. mutans*, TreR may indirectly control expression of toxins through involvement in the competence signaling pathway. Several previous studies have reported that *S. mutans* strains with deficiencies in competence signaling are also defective in the ATR, stringent response, and tolerance of oxidative stress (including H_2O_2), which likely explains the oxidative-stress-sensitive phenotype of the $\Delta treR$ strain (28–30). Indeed, analysis using virtual footprint regulon prediction (31) revealed putative TreR binding motifs in the promoters of *comC* and *comEA*, supporting the possibility that these genes are regulated by trehalose via TreR (data not shown). The *comC* gene encodes the precursor for the competence-stimulating peptide (CSP), and thus TreR could have far-reaching effects on *S. mutans* signaling and transcription. Additional experiments illustrating the

binding of TreR to promoters outside the *tre* operon are needed to confirm the direct regulation of noncanonical targets.

Regardless of the mechanism, it appears that the reduction in expression of the genes encoding mutacins IV, V, and VI in the $\Delta treR$ strain is sufficient to drastically reduce inhibition of *S. gordonii* or *L. lactis*, as shown in the antagonism assay (Fig. 4). This finding indicates that regulation by TreR is likely to play a critical role in competition with rival species and therefore the persistence and virulence of *S. mutans*. The apparent involvement of *S. mutans* TreR in the competence signaling pathways and mutacin expression is reminiscent of a recent study illustrating that the *S. mutans* Spx global regulators (SpxA1 and SpxA2) are also crucial for proper competence development and, therefore, mutacin expression (32). Indeed, *spxA2* had reduced expression in the $\Delta treR$ strain during growth in galactose, indicating possible overlap of the Spx and TreR regulons (see Table S2 in the supplemental material). SpxA2 is involved in regulation of the oxidative stress response, and genes involved in management of ROS were differentially regulated in the $\Delta treR$ strain, including *dpr*, *ahpF*, and *nox* (Table S3). Both this study and the study by Galvao et al. (32) highlight that much work remains to be done to fully understand the highly complex and convoluted regulatory pathways that have evolved in *S. mutans* as it continues to contribute to disease in the majority of humans.

In summary, the results of the present study confirm that the *S. mutans tre* operon is required for growth in trehalose. TreR appears to function as an activator of expression for the *tre* operon, rather than as a repressor (as is the case in *B. subtilis*). TreR also appears to be intertwined with competence signaling pathways, which has farther-reaching effects on toxin production and survival during oxidative stress and, accordingly, the overall competitive fitness and virulence of *S. mutans*. Additional biochemical studies, confirming the direct action of TreR as an activator of expression and its novel role in global signaling, stress response, and toxin production, are currently in progress. The link between trehalose and toxin production (and therefore competitive fitness), demonstrated here with *S. mutans* and in the recent study with *C. difficile* (10), provides an avenue for discovering similar associations in other *Firmicutes*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains used in this study are listed in Table 2. The *Streptococcus mutans* genomic type strain UA159 has been described previously (2, 33). The *S. mutans* $\Delta treA$ and $\Delta treB$ mutant strains are derivatives of UA159 and have been described previously (23). *S. mutans* was maintained on brain heart infusion (BHI) agar plates (BD/Difco, Franklin Lakes, NJ) at 37°C in a 5% (vol/vol) CO₂–95% air environment. Where applicable, antibiotics were added to a final concentration of 5 μ g ml^{−1} for erythromycin and 1 mg ml^{−1} for kanamycin. Organisms were cultured in TY medium (3% tryptone, 0.1% yeast extract, 0.5% KOH, 1 mM H₃PO₄) plus 1% (wt/vol) glucose and were grown in liquid culture or in continuous culture in a BioFlo 2000 fermentor (New Brunswick Scientific, Edison, NJ) as described previously (34, 35). Continuous cultures were grown at a dilution rate of 0.24 h^{−1} under glucose-limiting conditions (2.3 mM) with a continuous impeller speed of 200 rpm. Steady-state growth at pH 7.0 or 5.0 was maintained by the addition of 2 N KOH. The culture pH was continuously monitored throughout the experiment by an indwelling pH probe (Mettler Toledo, Columbus, OH). After continuous cultures had been maintained for a minimum of 10 generations under a given condition, aliquots of the culture were removed and cells were collected by centrifugation. Cell pellets were washed and stored frozen at −80°C.

Growth curves were recorded using an automated growth-monitoring device, a Bioscreen C (Growth Curves USA, Haverhill, MA), as described previously (36). The optical density at 600 nm (OD₆₀₀) was measured every hour with a 10-s shaking period before each reading to evenly suspend cultures. Microaerobic cultures were created using a 50- μ l mineral oil overlay of the Bioscreen cultures. Growth in glucose versus trehalose or galactose was measured in TY medium plus 1% glucose, 1% trehalose, or 1% galactose. Where applicable, H₂O₂ was added to 0.5 mM.

Oxidative stress challenge. Cultures of *S. mutans* UA159 and the $\Delta treR$ mutant were grown in continuous culture to steady-state conditions of pH 7 and pH 5 as described above. Aliquots of cells were harvested by centrifugation and subjected to an oxidative stress (16.3 mM H₂O₂), as previously described (36). Briefly, steady-state samples harvested from the chemostat, at culture pH values of 7 and 5, were resuspended in BHI medium, and hydrogen peroxide was added to a final concentration of 16.3 mM. Aliquots were removed at 0, 15, 30, and 60 min, serially diluted, and plated on BHI agar medium. Viable cells from each condition were counted and used to calculate log (n/n_0), where n is the number of colonies obtained at a specific time point and n_0 is the number of colonies at time zero.

TABLE 2 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description (reference[s] or source, if not this study)
Strains	
<i>Streptococcus mutans</i>	
UA159	Genomic type strain (2, 33)
$\Delta treA$ strain	<i>treA</i> deletion strain (23)
$\Delta treB$ strain	<i>treB</i> deletion strain (23)
$\Delta treR$ strain	<i>treR</i> deletion strain
<i>treR</i> -C strain	<i>treR</i> complement strain
<i>treA</i> -C strain	<i>treA</i> complement strain
<i>Escherichia coli</i>	
XL-1 Blue	Host for p <i>treR</i> -C-Topo (Stratagene)
XL10-Gold	Host for p <i>treR</i> -C (Stratagene)
Stellar	Host for p <i>treA</i> -C (Clontech)
<i>Streptococcus gordonii</i> DL1	Laboratory stock
<i>Lactococcus lactis</i> ATCC 11454	Laboratory stock
Plasmids	
p <i>treR</i> -KO	Construct to make the $\Delta treR$ strain (GenScript)
p <i>treR</i> -C-TOPO	Construct for subcloning of <i>treR</i> -C strain
pBGE	Vector backbone containing erythromycin resistance cassette to make <i>treR</i> -C strain (gift from José Lemos)
p <i>treR</i> -C	Construct to make <i>treR</i> complement strain [<i>treR</i> -C strain]
pSUGKBgIII	Vector backbone containing kanamycin resistance cassette to make <i>treA</i> -C strain (36)
p <i>treA</i> -C	Construct to make <i>treA</i> complement strain [<i>treA</i> -C]
Primers	
5' <i>treR</i> -C-XbaI-F	Forward primer to make p <i>treR</i> -Topo, <u>TCTAGAAAATTTTCTAGGTTAACCTAGTC</u> ^a
3' <i>treR</i> -C-BsrGI-R	Reverse primer to make p <i>treR</i> -Topo, <u>TGTACATTCTTTGTGCTTTCCTTGTGTC</u> ^b
3' <i>treA</i> -C	Primer to make p <i>treA</i> -C, ATTTAAAAATAGATCTGATCAATTATTGTAAGGAGAATAA
5' <i>treA</i> -C	Primer to make p <i>treA</i> -C, GAGCTCGAATAGATCTTCAGACTTCTGCTAAATCGC

^aThe XbaI site is underlined.^bThe BsrGI site underlined.

DNA manipulations. Chromosomal DNA was isolated from *S. mutans* as previously described (37). Plasmid DNA was isolated from *E. coli* as previously described using a E.Z.N.A plasmid minikit (Omega Bio-Tek, Norcross, GA). PCR was carried out with Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Amplicons were isolated and purified via gel electrophoresis as previously described (38). *S. mutans* UA159 was transformed by previously described methods (33, 39).

Generation of recombinant $\Delta treR$, *treR*-C, and *treA*-C strains. (i) $\Delta treR$ strain. A UA159 derivative was constructed with the *treR* open reading frame (ORF) replaced by a kanamycin resistance gene (*ΔtreR*). A construct designated p*treR*-KO was synthesized using the pUC57 vector backbone with the EcoRV site in the multiple-cloning site containing the kanamycin resistance gene flanked on the left side by a NotI restriction site followed by 500 bp upstream of *treR* and flanked on the right side by 500 bp downstream of *treR*, followed by an XhoI restriction site (GenScript, Piscataway, NJ). The construct was digested with NotI and XhoI, and the resulting insert was gel purified and transformed into *S. mutans* UA159. Transformants were selected for Kan^r, and one isolate was designated the $\Delta treR$ strain.

(ii) *treR*-C strain. *ΔtreR* was complemented using a single-copy genomic insertion of the SMU.2040 (*treR*) locus, including the intergenic region between *treR* and *treB*, into the *gtfA* (SMU.881) locus. Under the growth conditions tested, no physiological impact from the disruption of the *gtfA* locus was detected, as reported previously (40). Primers 5'*treR*-C-XbaI-F and 3'*treR*-C-BsrGI-R (Table 2) were used to amplify the *treR* and cognate promoter, as well as to add XbaI (5') and BsrGI (3') restriction sites to the amplicon. The resulting amplicon was gel purified and subcloned into pCR2.1 using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) to produce p*treR*-C-Topo. The TOPO ligation reaction product was transformed into *E. coli* XL-1 Blue (Stratagene, La Jolla, CA). The streptococcal integration vector pBGE was a gift from José Lemos and has been previously described (41). p*treR*-C-Topo and pBGE were linearized using XbaI and BsrGI, and the fragment of p*treR*-C-Topo containing the *treR* ORF was ligated into the vector backbone of pBGE to produce p*treR*-C. The ligation reaction product was transformed into *E. coli* XL-10 Gold (Stratagene, La Jolla, CA), and positive clones were selected on LB agar medium containing erythromycin (500 μg ml⁻¹). The integrity of the construct was confirmed by sequencing. p*treR*-C was transformed into the $\Delta treR$ strain and selected on BHI agar medium containing erythromycin. The complemented strain was designated the *treR*-C strain, and the integrities of both the deletion and complementation loci were confirmed by sequencing.

(iii) *treA*-C strain. *ΔtreA* was complemented using a single-copy genomic insertion of the SMU.2037 (*treA*) locus, including the intergenic region between *treA* and *treB*, into the *gtfA* (SMU.881) locus. Under the growth conditions tested, no physiological impact from the disruption of the *gtfA* locus was detected, as reported previously (40). The streptococcal integration vector pSUGKBgIII, described previously (36), was linearized with the restriction enzyme BgIII. Primers 3'*treA*-C and 5'*treA*-C (Table 2) were used to

amplify *treA* and the intergenic region between *treA* and *treB*. The resulting amplicon was ligated into the linearized pSUGKBgIII using an In-Fusion HD cloning kit (Clontech, Mountain View, CA) to produce p*treA*-C. The cloning reaction product was transformed into *E. coli* Stellar (Clontech, Mountain View, CA), and positive clones were selected on LB agar medium containing kanamycin (50 $\mu\text{g ml}^{-1}$). The integrity of the construct was confirmed by sequencing. p*treA*-C was transformed into the Δ *treA* strain and selected on BHI agar medium containing kanamycin. The complemented strain was designated *treA*-C, and the integrities of both the deletion and complementation loci were confirmed by sequencing.

RNA-seq. For mRNA sequencing (RNA-seq), triplicate samples of *S. mutans* UA159 or the Δ *treR* strain were grown at 37°C in a 5% (vol/vol) CO₂-95% air atmosphere to mid-log phase in TY medium supplemented with 1% glucose (OD₆₀₀ of ~0.6), 1% galactose (OD₆₀₀ of ~0.5), or 1% trehalose (for UA159, OD₆₀₀ of ~0.7; for the Δ *treR* strain, OD₆₀₀ of ~0.3). Cells were harvested by centrifugation, and cell pellets were stored at -80°C. The protocols for transcriptomic sequencing were modified from those described by Edlund et al. (42). Briefly, total RNA extraction was performed using the Qiagen RNeasy Power Microbiome kit according to the manufacturer's instructions (Qiagen, Chatsworth, CA). rRNA was removed from the total RNA using the Ribo-Zero (Bacteria) kit (Epicentre, Madison, WI). mRNA was purified using the RNA Clean and Concentrator kit (Zymo Research, Irvine, CA). RNA concentration and integrity were monitored before and after rRNA removal using a Qubit 3.0 fluorometer (Life Technologies, Waltham, MA) and the Agilent RNA ScreenTape assay for the 4200 TapeStation system (Agilent Technologies, Santa Clara, CA). cDNA libraries from rRNA-depleted RNA were generated using random-primed cDNA synthesis methods according to the ScriptSeq v2 RNA-seq library preparation protocol (Epicentre, Madison, WI). Prior to second-strand cDNA synthesis, the ditagged cDNA was purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, CA). ScriptSeq index primers were added to the libraries, which were then PCR amplified for 15 cycles. The final libraries were run on the Illumina NextSeq 550 platform using the High Output v2 150-cycle kit (2 × 75-bp paired-end reads) (Illumina, San Diego, CA).

Barcodes were trimmed, and low-quality and short sequences (<100 bp) were filtered using BBDuk (<https://sourceforge.net/projects/bbmap>) implemented in Geneious version 11 (Biomatters, Auckland, New Zealand) (43). Expression values for each mRNA sample were generated by mapping of paired reads onto the annotated reference genome of *S. mutans* UA159, resulting in 175 to 275× mean coverage per sample (range of 4.2 to 6.4M reads mapped per sample). Expression level counts and differential expression calculations in triplicate between conditions were performed using Geneious version 11 (Biomatters) (43) with DESeq2 normalization (44). DESeq2 uses a model based on the negative binomial distribution with variance and mean linked by local regression (45).

Deferred-antagonism assay. The deferred-antagonism assay was performed as previously described (24, 25). Briefly, cultures of *Streptococcus mutans* UA159, the Δ *treR* derivative strain, and the *treR*-C complement strain were grown in BHI broth, in triplicate, at 37°C in a 5% (vol/vol) CO₂-95% air atmosphere. Following overnight incubation, the cultures were diluted in fresh BHI medium to an OD₆₀₀ of ~0.3. An aliquot (8 μl) of each culture was spotted on BHI agar, TY agar plus 1% glucose, or TY agar plus 1% trehalose and incubated for 18 h, following which colonies were exposed to UV light in a Stratalinker (Stratagene, San Diego, CA) for 3 min. Stationary-phase cultures (0.5 ml) of *Streptococcus gordonii* DL1 (mutacin IV and V sensitive) and *Lactococcus lactis* ATCC 11454 (mutacin V and VI sensitive) were added to 5 ml BHI agar (0.75%) and used to overlay the BHI agar plate containing the primary colonizer (*S. mutans* UA159, Δ *treR*, or *treR*-C strain). Plates were incubated overnight before photographing to document zones of inhibition. Areas of the zones of inhibition were measured and recorded. Statistical analysis was performed using a two-way analysis of variance (ANOVA) test followed by Tukey's test of multiple comparisons.

Accession number(s). RNA-seq data have been deposited in the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) with accession number GSE110275.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00057-18>.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank José Lemos for the gift of the pBGE plasmid.

This study was supported by NIH/NIDCR grants T90-DE021985 (J.L.B. and E.L.L.), F32-DE026947 (J.L.B.), R01-DE020102 and R01-DE026186 (J.S.M., X.H., and W.S.), and R01-DE013683 and R01-DE017425 (R.G.Q.).

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