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Repression of the TreR transcriptional regulator in *Streptococcus mutans* by the global regulator, CcpA

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One sentence summary: The global regulator CcpA represses TreR in *Streptococcus mutans*, and in the oral pathogen *S. mutans*, the global regulator CcpA exhibits the ability to control expression of the trehalose utilization operon activator treR, suggesting an additional level of control over stress tolerance mechanisms in the organism.

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ABSTRACT

Streptococcus mutans, the etiologic agent of dental caries in humans, is considered a dominating force in the oral microbiome due to its highly-evolved propensity for survival. The oral pathogen encodes an elaborate array of regulatory elements, including the carbon catabolite-responsive regulator, CcpA, a global regulator key in the control of sugar metabolism and in stress tolerance response mechanisms. The recently characterized trehalose utilization operon, integral for the catabolism of the disaccharide trehalose, is controlled by a local regulator, TreR, which has been implicated in a number of cellular functions outside of trehalose catabolism. Electrophoretic mobility shift assays demonstrated that CcpA bound a putative *cre* site in the *treR* promoter. Loss of *ccpA* resulted in elevated expression of *treR* in cultures of the organism grown in glucose or trehalose, indicating that CcpA not only acts as a repressor of trehalose catabolism genes, but also the local regulator. The loss of both CcpA and TreR in *S. mutans* resulted in an impaired growth rate and fitness response, supporting the hypothesis that these regulators are involved in carbon catabolism control and in induction of components of the organism's stress response.

Keywords: *Streptococcus mutans*; regulation; trehalose; CcpA; TreR; repressor

INTRODUCTION

Streptococcus mutans, the primary causative agent of dental caries, is at the forefront of global research to improve oral health. As public healthcare costs for treatments continue to rise, and the incidence of carious lesions in adults and children increase, there is a need to further the understanding of how *S. mutans* causes disease (Marcenes *et al.* 2013). The Gram-positive cocci is a highly-evolved pathogen with fine-tuned metabolic processes and stress responses that allow it to dominate its niche, resulting in tooth decay (Bowden and Hamilton 1998).

Carbohydrate utilization is the epicenter of pathogenesis by *S. mutans*. The importance of sugar metabolism is illustrated by the diverse array of import and catabolism machinery found in the genome (Ajdic *et al.* 2002). The organism encodes 14 phosphoenolpyruvate-phosphotransferase (PEP-PTS) systems and three ABC transporters dedicated to the uptake of sugar (Ajdic and Pham 2007). Further demonstrating the devotion by the cell to energy generation, numerous operons for the catabolism of preferred and non-preferred sugars have been identified in the genome. Apart from energy generation, sugar

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catabolism arms *S. mutans* with its most powerful weapon: acid secretion (Quivey et al. 1995; Baker, Faustoferri and Quivey 2017). The consequence of the lack of a traditional electron transport chain results in ATP generation via fermentative processes (Takahashi and Nyvad 2011). This process generates lactic (and other) acid by-products which are subsequently secreted into the extracellular matrix, lowering the pH of the environment to values as low as 3.7 (Bowden and Hamilton 1998). The non-aciduric bacteria coexisting in the biofilm succumb to the acidic stress, allowing *S. mutans* to predominate.

Of the many catabolism operons found in the genome of *S. mutans*, one recently annotated is the trehalose (*tre*) utilization operon (Baker et al. 2018). Trehalose is a lesser-known disaccharide common to yeast-based foods and is currently becoming more popular in preservation of frozen meats and ice cream (Neta, Takada and Hirasawa 2000; Duong et al. 2005). The *tre* operon consists of the trehalose-specific hydrolase, *treA*, a trehalose-specific PTS importer, *treB* and an activator of *tre* gene expression, *treR*. Upon characterization of the operon, it was determined that the regulator, TreR, was integral not only in catabolic function, but was shown to be important in the stress tolerance abilities of the organism, as suggested by research in other Gram-positive organisms (Schock and Dahl 1996; Baker et al. 2018). When *S. mutans* was grown in medium without trehalose, loss of *treR* negatively impacted the oxidative stress tolerance and mutacin production of the organism (Baker et al. 2018). In the absence of *treR*, when cultures were grown in trehalose-containing medium, the catabolic functions of *treA* and *treB* were not completely mitigated; however, the authors observed significant effects on the expression of key competence-related genes (Baker et al. 2018). These findings solidified the suggestion of a regulator(s) with the potential to control expression of the operon, in addition to TreR itself.

The major global regulatory protein, carbon catabolite protein A (CcpA), is a carbon-responsive regulator found in many Gram-positive species (Zeng et al. 2013). A member of the LacI family of proteins, CcpA identifies and binds to the catabolite repressive element (*cre*) site found in promoter regions across the genome (Ramseier et al. 1995). Previous work has extensively described the role of CcpA regulation in major virulence factors, within the stringent response, and in the acid tolerance response (ATR; Abranches et al. 2008; Faustoferri et al. 2015; Moye et al. 2016). Control of key regulatory systems by CcpA is indicative of the organism's ability to adapt dynamically to its constantly fluctuating carbohydrate availability. Earlier studies have also demonstrated a role for CcpA in controlling expression of the trehalose operon genes, *treA* and *treB*, even in the absence of trehalose as the major growth carbohydrate (Ajdic and Pham 2007; Abranches et al. 2008).

We hypothesized that CcpA, by virtue of its ability to influence stress tolerance and catabolic systems within *S. mutans*, regulated expression of *treR*, thereby affecting the TreR regulon. The present study demonstrates that CcpA represses *treR* and that loss of both CcpA and TreR reduce the competitive fitness of *S. mutans*, particularly when trehalose is the sole carbon source available for growth.

MATERIALS AND METHODS

Bacterial strains and growth conditions

All strains used in this study are listed in Table 1. The *S. mutans* genomic-type strain UA159 has been described previously (Ajdic et al. 2002). The *S. mutans* mutant strains Δ ccpA and Δ treR are derivatives of UA159 and have been described previously (Quivey

et al. 2015; Baker et al. 2018). For generation of the Δ treR Δ ccpA double mutant, the Δ ccpA strain was transformed with pTreRKO (Baker et al. 2018) to replace *treR* with a kanamycin resistance cassette, and Kan^R colonies were selected and screened via sequencing. A positive clone was denoted Δ treR Δ ccpA. *S. mutans* was maintained on brain heart infusion agar plates (BHI; BD/Difco, Franklin Lakes, NJ) at 37°C in a 5% (v/v) CO₂/95% air environment. Organisms were cultured in TY medium (3% tryptone, 0.1% yeast extract, 0.5% KOH and 1 mM H₃PO₄) + 1% (w/v) glucose or 1% (w/v) trehalose, where specified.

Chloramphenicol acetyltransferase gene reporter fusions

The intergenic region preceding SMU.2040 (*treR*) was amplified from UA159 genomic DNA via PCR using primer pair treRCATBgl (5'-AGTTGCAGATCTAAGCAATTCAATCTAATTGTGC-3') and treRCATSacI (5'-TTTCATGAGCTCAAATTTTCCTAGGTTAAC C-3'). The amplicon was digested with BglII and SacI, gel-purified and cloned into the promoterless chloramphenicol acetyltransferase reporter plasmid pJL84, digested with BamHI and SacI. Transformants were selected on LB agar medium containing kanamycin (LB Kan; 50 µg/mL). Clones were screened by colony PCR and a correct construct was named pJLtreR.

To create the mutated *cre* binding site in the *treR* promoter, pJLtreR was the template for InFusion site-directed mutagenesis (Takara Bio USA, Mountain View, CA) using the primer pair treR Δ creFwd (5'-ACTTTTTC AATGAAGAATACCAG TTTTGTGTGGCAAGTTGTCC-3') and treR Δ creRev (5'-GCCAA CAAA AACTGGTATCTTCATTGAAAAAGTAAACGTACTG-3'), where the boldface type indicates mutated bases. The resulting ligation mixture was transformed into *E. coli* Stellar cells (Takara), and transformants were selected on LB agar medium containing kanamycin (LB Kan; 50 µg/mL). The mutated promoter sequence was verified via nucleotide sequencing and the mutant CAT construct was designated as pJLtreR Δ cre.

The *S. mutans* parent strain UA159 was transformed with pJLtreR or pJLtreR Δ cre to generate a single-copy, integrated reporter fusion in the intergenic region between *mtlA* (SMU.1185c)/*glmS* (SMU.1187c) and *mtlD* (SMU.1182c)/*phnA* (SMU.1180c) (Faustoferri et al. 2015). Kan^R colonies were screened for proper integration into the chromosome by PCR and the resulting strains were named UR382 and UR394, respectively (Table 1). The *S. mutans* Δ ccpA strain was also transformed with pJLtreR and pJLtreR Δ cre and the strains containing the properly integrated promoter-cat construct were named UR395 and UR396, respectively (Table 1). Three independent cultures of strains carrying pJLtreR or pJLtreR Δ cre were grown, in triplicate, to mid-logarithmic phase (OD₆₀₀ ~ 0.5) in TY medium supplemented with 1% (w/v) glucose.

Measurement of *treR* promoter activity in each condition was performed in triplicate using a previously described CAT assay method (Shaw 1975; Kuhnert et al. 2004). Reactions were initiated by addition of 0.1 mM chloramphenicol. Optical density measurements at 412 nm were monitored over 3 min. Reaction rate and quantitation of total protein were used to determine CAT activity, represented as nmol chloramphenicol acetylated/min/mg total protein.

Real time PCR

Quantitative Real Time PCR was performed as previously described (Faustoferri et al. 2015). Briefly, four independent cultures of *S. mutans* UA159 or Δ ccpA were grown in TY medium

Table 1. Strains used in this study.

Strain name	Description (reference, if not this study)
<i>Streptococcus mutans</i>	
UA159	Genomic type strain (Ajdic et al. 2002; Murchison et al. 1986)
$\Delta treR$	<i>treR</i> deletion strain (Baker et al. 2018)
$\Delta ccpA$	<i>ccpA</i> deletion strain (Quivey, Jr. et al. 2015)
$\Delta treR\Delta ccpA$	<i>ccpA</i> deletion strain with Kanamycin cassette replacing <i>treR</i>
UR382	<i>S. mutans</i> UA159 carrying integrated <i>treR</i> -cat reporter fusion
UR394	$\Delta ccpA$ strain carrying integrated <i>treR</i> -cat reporter fusion
UR395	<i>S. mutans</i> UA159 carrying integrated <i>treR</i> -cat reporter fusion with mutated <i>cre</i> site
UR396	$\Delta ccpA$ strain carrying integrated <i>treR</i> -cat reporter fusion with mutated <i>cre</i> site
<i>Streptococcus sanguinis</i> 10904	Laboratory Stock

supplemented with 1% (w/v) glucose to mid-logarithmic phase ($OD_{600} \sim 0.5$) and then harvested. RNA was extracted from harvested cells by previously described methods (Abranches et al. 2006; Baker et al. 2014). Extracted RNA was used along with random primers to synthesize cDNA with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Primer pair *treR*qPCRfwd (5'-TGGATAAAGCATTGATTCCTCA-3') and *treR*qPCRrev (5'-AATCAACTTAAGCTGGACATTG-3') was used to amplify *treR*-specific mRNA using Power SYBR Green Master Mix (Life Technologies) from three replicates of each sample. Reactions were carried out in a Step One Plus Real Time PCR System (Applied Biosystems, Foster City, CA). The *treR* template concentration was used to determine copy number and serial dilutions were performed to create a standard curve. The mRNA copy number was quantified based on a standard curve of *treR* as previously described (Faustoferri et al. 2015).

Regulon binding motif prediction

Putative DNA-binding regulatory motifs in the intergenic region preceding *treR* were identified using the consensus IUPAC sequence described in RegPrecise (<http://regprecise.sbpdiscovers.org:8080/WebRegPrecise/>) for CcpA (TGWAARCGYTWN CW) to search the *S. mutans* genome sequence via Virtual Footprint (http://www.prodoric.de/vfp/vfp_regulon.php) (Munch et al. 2003).

CcpA protein expression and isolation

CcpA was isolated as described by Santiago et al. (2013). Briefly, an *E. coli* M15 strain carrying the expression construct pQEc-cpA (Abranches et al. 2008) was grown, and native protein expression was induced with the addition of isopropyl- β -D-thiogalactopyranoside (IPTG), added to a concentration of 1 mM. The culture was incubated at room temperature, shaking at 200 rpm, for 4 h. Protein concentrations were estimated according to the method of Bradford (Bio-Rad, Hercules, CA).

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays to assess CcpA binding were performed as described previously (Santiago et al. 2013). A DNA fragment containing the promoter region of *treR* (SMU.2040) was amplified by PCR using primer pair *treR*CATBgl and *treR*CAT-SacI. The DNA product was purified and end-labeled with T4 polynucleotide kinase (PNK) (New England Biolabs, Ipswich, MA) and [γ - 32 P] ATP (PerkinElmer, Waltham, MA) for 10 min at 37°C. Quantitation of labeled DNA was performed by scintillation counting. Binding reaction mixtures were performed in 25 μ L and consisted of the following: binding buffer (20 mM Tris-HCl,

pH 8; 8.7% [v/v] glycerol; 1 mM EDTA; 5 mM $MgCl_2$; 250 mM KCl; 0.5 mM dithiothreitol [DTT]; 2 μ g bovine serum albumin [BSA]), radiolabeled DNA probe (3000 cpm) and 2 mM fructose 1,6 bisphosphate (F-1,6-P). Purified CcpA was used at final concentrations of 250, 500, 750 nM. Binding reaction mixtures were incubated at 37°C for 45 min.

CcpA-DNA binding was assessed using an 8% non-denaturing acrylamide gel in 1X Tris-glycine buffer. Poly (dG-dC) was used as a non-specific competitor for protein binding (Smith and Delbary-Gossart 2001; Hellman and Fried 2007). For competition reactions, unlabeled *treR* DNA was added in excess to binding reactions at 200X, 500X or 1000X, as compared to quantities of labeled probe. Radiolabeled *ilvE* promoter was included as a positive control and was prepared as described by Santiago et al. (2013). The gels were exposed to a phosphorimager screen, and binding was detected with a Molecular FX phosphorimager and Bio-Rad Quantity One software (Bio-Rad, Hercules, CA).

Spot competition assay

To assess the competitive fitness of the $\Delta treR$, $\Delta ccpA$ or $\Delta treR\Delta ccpA$ mutant strains, a spot-based competition assay was employed as described previously (Kreth et al. 2005; Kovacs et al. 2017). Briefly, the peroxigenic commensal bacteria *Streptococcus sanguinis* 10904 and the acidogenic *S. mutans* UA159 were used as primary cultures in competition assays to determine the susceptibility of *S. mutans* to niche-specific stresses. For the primary spots, bacteria were grown overnight in TY + 1% (w/v) sugar [glucose (TYG) or trehalose (TYT)] at 37°C in a 5% (v/v) CO_2 /95% air atmosphere, and then subcultured into fresh TYG or TYT medium until cultures reached an OD_{600} of 0.4. An 8.0 μ L aliquot was then spotted onto a prewarmed TY + 1% (w/v) sugar agar plate and incubated overnight at 37°C in a 5% (v/v) CO_2 /95% air atmosphere. Test strains were similarly grown overnight and subcultured, and an 8.0 μ L aliquot was spotted immediately adjacent to the primary spot. Plates were returned to 37°C in a 5% (v/v) CO_2 /95% air atmosphere and incubated overnight. The data are shown as representative images of outgrowth inhibition from three independent experiments performed in triplicate.

RESULTS

Identification of a CcpA binding motif within the *treR* promoter

Previous studies revealed elevated expression of *treA* and *treB* in cultures of $\Delta ccpA$ grown in media containing glucose (Abranches et al. 2008). The idea that CcpA would be capable of binding

Figure 1. CcpA regulates expression of *trcR*. (Panel A) Representation of the *trc* operon of *Streptococcus mutans* UA159 with predicted binding motifs for the TrcR regulator (gray circle) and the global regulator CcpA (red circle) indicated. (Panel B) DNA sequence of the intergenic region preceding *trcR* (SMU.2040). The start site of translation for the upstream coding region, *trcB* (SMU.2038), is indicated in green with a left-pointing arrow. The translational start site for *trcR* is likewise indicated with a right-pointing arrow. The predicted binding motifs for CcpA (red) and TrcR (blue) graphically depicted in Panel A are indicated here as underlined text. (Panel C) Electrophoretic mobility shift assay (EMSA) displaying binding of CcpA to the *trcR* promoter region (depicted in Panel B). Left to right: Lane 1: Radiolabeled *trcR_P* fragment alone; *trcR_P* fragment incubated with 250 nM (Lane 2), 500 nM (Lane 3) or 750 nM (Lane 4) purified recombinant CcpA, as described in Materials and Methods; unlabeled *trcR_P* fragment at 200X, 500X or 1000X molar quantity (Lane 5, 6 and 7, respectively), compared to Lane 3, used as cold competitor in binding reactions with 500 nM CcpA protein; *trcR_P* fragment bound to BSA (negative control) (Lane 8); poly-dG-dC used as a competitor to the *trcR_P* fragment for binding to 500 nM CcpA (negative control; Lane 9); *ilvE* (SMU.1203) promoter fragment bound to 500 nM CcpA (positive control; Lane 10) (Santiago et al. 2013). The image is representative of *n* = 3 experiments.

to the *trcR* promoter, and regulating expression, follows, intuitively, from these data. Using the motif predictive software, Virtual Footprint, a putative *cre* motif was identified within the intergenic region between *trcB* and *trcR*. The identified *cre* motif is located approximately 100 bp upstream of the *trcR* start codon (Fig. 1A and B). To demonstrate interaction of CcpA with the putative binding motif, purified recombinant CcpA was co-incubated with a DNA fragment containing the *trcR* promoter (*trcR_P*) region in an electrophoretic mobility shift assay (EMSA). The radiolabeled *trcR_P* DNA fragment was incubated in the presence of increasing concentrations of purified CcpA, and the required CcpA binding cofactors (Fig. 1C; Santiago et al. 2013). The results show that free *trcR_P* DNA displayed an evident shift in the presence of CcpA, confirming binding of the protein to the *trcR* promoter region. To further test binding, radiolabeled probe and CcpA were incubated with increasing concentrations of unlabeled *trcR* promoter (cold competitor). As the concentration of the cold competitor increased, the shift begins to reverse, with a greater quantity of free probe evident. Incubation of labeled *trcR_P* with BSA did not result in a shift, suggesting specificity of CcpA for the *trcR_P* DNA. Likewise, use of poly dG-dC to compete with *trcR_P* for CcpA binding did not result in a shift. As a positive control for the binding conditions used, the promoter region of *ilvE*, labeled with ³²P, was incubated with CcpA, resulting in a band with reduced mobility, as has been previously shown (Santiago et al. 2013).

Loss of *ccpA* alleviates repression of *trcR*

CcpA binding to the intergenic region between *trcB* and *trcR* suggested a role for the global regulator in expression of *trcR*. To determine the extent of regulation of *trcR* by CcpA, a chloramphenicol acetyltransferase (*cat*) fusion was used to measure *trcR* (*pJLtrcR*) promoter-driven activity in the presence or absence of *ccpA* (Fig. 2A). When CAT activity from the *trcR* promoter was measured in the *ccpA* deletion strain, there was a significant increase in expression, compared to baseline expression in the parent strain, UA159 (Fig. 2A). A *trcR*-*cat* fusion (*pJLtrcRΔcre*) with specific point mutations in the CcpA binding site (*cre*) was

constructed to demonstrate specificity of CcpA for the *cre* site in the *trcR* promoter region (Fig. 2A). Compared to the native *trcR*-*cat* fusion in UA159, the mutated *trcR*-*cat* construct exhibits significantly increased activity in both the UA159 and Δ *ccpA* background strains. This increase was a result of the inability of CcpA to bind the mutated *cre* site and thus, repress *trcR* activity. The increased activity resulting from the *cre* site mutation was similar to that observed for the native *trcR*-*cat* fusion in the *ccpA* deletion. The reporter fusion results were confirmed via qRT-PCR using RNA isolated from cultures of *S. mutans* UA159 or the Δ *ccpA* derivative grown with glucose as the sole carbon source (Fig. 2B). Loss of CcpA resulted in significantly elevated expression of *trcR*, compared to wild type expression levels. Notably, these expression levels were a direct result of *ccpA* deletion, as there was no trehalose present in the growth medium. The qRT-PCR results further substantiate the suggestion that CcpA represses *trcR*.

The Δ *trcRΔccpA* double mutant exhibits impaired growth in glucose and trehalose

Since previous work identified *trcA* and *trcB* as targets of CcpA (Abranches et al. 2008), and CAT assay data revealed CcpA regulatory control over *trcR*, the fitness of a *trcRccpA* double mutant strain was characterized to further understand the effects of concurrent regulator deletion. The wild type (UA159), Δ *trcR*, Δ *ccpA* and the double mutant strains were grown to mid-log phase in TY + 1% glucose, then sub-cultured into fresh TY medium containing either 1% glucose or 1% trehalose and grown for 15h at 37°C with OD₆₀₀ readings taken every 15 min. Compared to UA159 in TY + 1% glucose, the only strain that exhibited impaired growth was Δ *ccpA* (Fig. 3A). This growth deficit, compared to the wild type strain, was also observed in TY + 1% trehalose medium (Fig. 3B). The deletion of *trcR* had no effect on growth in glucose-containing media, though there was an apparent decrease in growth when Δ *trcR* was grown in media with trehalose as the sole carbon source (Fig. 3A and B), consistent with previous reports (Baker et al. 2018). This impaired growth phenotype in TY + 1% trehalose was also exhibited by

Figure 2. CcpA is a negative regulator of *trcR*. (Panel A) Expression of *trcR* or *trcRΔccpA*, as a function of CAT (chloramphenicol acetyltransferase) activity, in the parent strain, *Streptococcus mutans* UA159, or in the *ΔccpA* (SMU.1591c) derivative. Assays were performed as described in Materials and Methods using three independent cultures, in triplicate, and activity is expressed as nmol chloramphenicol acetylated per minute per mg total protein. Statistical significance determined by Student's t-test is indicated by **, *P*-value < 0.05. (Panel B) Expression of *trcR* determined by quantitative real-time PCR as described in Materials and Methods using RNA isolated from three independent cultures of *S. mutans* UA159 and *ΔccpA*, sampled in triplicate. Data is represented as number of copies of *trcR* in each strain as determined by a standard curve. Statistical significance determined by Student's t-test is indicated by **, *P*-value < 0.05.

the *ΔccpA* strain, suggesting that loss of either regulator handicaps the catabolism of trehalose in the bacterium. When deleted concurrently, the *ΔtrcRΔccpA* double mutant exhibits the largest growth defect in either medium, compared to the wild type or either single mutant (Fig. 3A and B). The maximum OD₆₀₀ of the double mutant strain was only capable of achieving approximately half of the maximum OD₆₀₀ of the wildtype strain, displaying the difficulty of *ΔtrcRΔccpA* to metabolize either carbohydrate in the absence of the regulators.

Double deletion of *ΔtrcR* and *ΔccpA* resulted in decreased competitive fitness

An essential element for robust colonization of the oral cavity by *S. mutans* is the organism's ability to survive the competitive niche in which it subsists. In this study, each test strain

Figure 3. Growth of test strains in different carbohydrate sources. Cultures of *S. mutans* UA159 (black line), its derivatives *ΔtrcR* (blue line), *ΔccpA* (red line), or the double mutant strain, *ΔtrcRΔccpA* (magenta line), were grown in either tryptone-yeast extract medium containing 1% (w/v) glucose (Panel A) or 1% (w/v) trehalose (Panel B) in a Bioscreen C plate reader, as described in Materials and Methods. Each culture was grown in triplicate and sampled 10 times. OD_{600nm} readings were taken every hour for 15 h.

Figure 4. Spot competition of test strains. The test strains *S. mutans* UA159 and its derivatives *ΔtrcR*, *ΔccpA*, or the double mutant strain, *ΔtrcRΔccpA*, were spotted on tryptone-yeast extract agar medium containing either 1% (w/v) glucose (upper panels) or 1% (w/v) trehalose (lower panels) adjacent to either an acidic challenge (left panels) (*S. mutans* UA159) or a peroxigenic challenge (right panels; *Streptococcus sanguinis* 10904), as described in Materials and Methods, and incubated in a 37°C 95% air/5% CO₂ atmosphere for 24 h.

was grown on an agar plate in the presence of a primary, established, culture of *S. mutans* (acidic challenge) or *S. sanguinis* (peroxigenic challenge) (Fig. 4). The challenges were performed with either glucose or trehalose as the sole carbon source to determine the necessity of the carbohydrate to induce acid or oxidative stress tolerance mechanisms. The nature of this assay not

only tested the acid/oxidative stress sensitivity of the strains, but by design, also indicated susceptibility of the test strains to other products secreted by the primary strain, such as bacteriocins. When challenged with an acidic stress (via *S. mutans*), the double mutant strain exhibited the least tolerance to the induced stress, compared to the other test strains, as evidenced by a slightly larger zone of inhibition and a decreased density of the cells. Though minor, this defect implicates both regulators in stress tolerance or stress tolerance activation. When challenged with the peroxigenic *S. sanguinis*, again the double mutant strain showed a reduced tolerance to oxidative stress. Overall, there was little difference in the zone of inhibition between the test strains and the wild type in response to oxidative stress. While other secreted products from the primary spots may account for some of the sensitivity results, it is most likely that the observed results are primarily from the acid or oxidative stress present. The greatest effects on stress tolerance were most prevalent when strains were grown on trehalose-containing medium. In each scenario, the $\Delta treR$ strain was less tolerant than either the wild type or the $\Delta ccpA$ strain. These findings support the conclusion that the regulators each play a role in the activation of stress tolerance cascades and, further, suggest that this activation may be growth condition-dependent.

DISCUSSION

The skilled bacterial competitor, *S. mutans*, establishes dominance over oral commensals in biofilms through an array of mechanisms (Takahashi and Nyvad 2011). This adept organism produces acid as a by-product of ATP breakdown, resulting in the decay of dentin and enamel. Due to the harsh conditions of the low pH generated by secreted acid, *S. mutans* has developed strategies to tightly control metabolic processes. Carbon catabolite repressor A (CcpA), the global regulator, is one such mechanism that allows *S. mutans* to respond metabolically to environmental cues such as carbon availability, as well as regulating key competitive fitness mechanisms (Abranches et al. 2008). In characterizing the trehalose utilization (*tre*) operon (Baker et al. 2018), a putative DNA binding sequence for CcpA was discovered in the promoter region of *treR* (Fig. 1A and B). Putative binding was confirmed by an EMSA with purified recombinant CcpA and isolated *treR* promoter DNA. The band shift that resulted after incubation of the *treR* promoter region with CcpA indicated an interaction between the regulatory protein and the predicted intergenic motif. Negative regulation of *treR* by CcpA was elucidated using a *treR*-promoter-cat fusion to examine *treR* expression in the wild type and $\Delta ccpA$ backgrounds, and further confirmed via qRT-PCR measurement of *treR* copy number in the same background strains.

Growth of the $\Delta treR$ strain in medium containing trehalose was impaired, due to the inability of the organism to regulate trehalose catabolism machinery via the local regulator (Baker et al. 2018). While growth of the $\Delta ccpA$ strain is compromised in medium containing either trehalose or glucose, growth trends did not differ between the two substrates (Fig. 3A and B). However, strikingly, loss of both *ccpA* and *treR* resulted in a more severe impact on growth of the double mutant strain in either carbohydrate source (Fig. 3), suggesting a compensatory mechanism for activation of the *tre* system or trehalose catabolism. When we examined the competitive fitness of the $\Delta treR$ and $\Delta ccpA$ strains in an acidic challenge (spot competition against *S. mutans* UA159) or in a peroxigenic challenge (spot competition against *S. sanguinis*), both single deletion strains

responded equally on medium containing either glucose or trehalose, with a greater sensitivity to the peroxigenic challenge. Yet, the $\Delta treR \Delta ccpA$ double mutant was less able to stave off attack by hydrogen peroxide released by the commensal *S. sanguinis* (Fig. 4), particularly when the strains were grown on trehalose-containing medium. The *treR* deletion strain exhibited increased stress sensitivity when trehalose was the sole carbon source, as observed by the decreased growth of the bacterial spot. This could be attributed to a decreased growth phenotype of the strain when trehalose is present, but the growth curves (Fig. 3) revealed similar growth for the *ccpA* mutant strain cultured in the same conditions. There was no major defect in the stress tolerance of $\Delta ccpA$ as observed for the *treR* deletion strain (Fig. 4); thereby suggesting that CcpA regulation of *treR* is dynamic. This hypothesis allows for a hierarchy of regulation by CcpA for the major stress tolerance mechanisms and for carbohydrate catabolism.

These results suggest that trehalose metabolism is controlled by the global regulator, CcpA, as well as the local regulator, TreR, though the precise mechanism is yet to be elucidated. The cooperativity of these two regulatory proteins in the stress response cascade is also yet to be determined, though the likelihood is that the hierarchy of the response by each protein is dependent on the carbohydrate present or the stress to which the bacterium is exposed.

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Conflicts of Interest. None declared.

REFERENCES

- Abranches J, Candella MM, Wen ZT et al. Different roles of EIIAB-Man and EIIGlc in regulation of energy metabolism, biofilm development, and competence in *Streptococcus mutans*. *J Bacteriol* 2006;188:3748–56.
- Abranches J, Nascimento MM, Zeng L et al. CcpA regulates central metabolism and virulence gene expression in *Streptococcus mutans*. *J Bacteriol* 2008;190:2340–9.
- Ajdic D, McShan WM, McLaughlin RE et al. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A* 2002;99:14434–9.
- Ajdic D, Pham VT. Global transcriptional analysis of *Streptococcus mutans* sugar transporters using microarrays. *J Bacteriol* 2007;189:5049–59.
- Baker JL, Derr AM, Karuppaiah K et al. *Streptococcus mutans* NADH oxidase lies at the intersection of overlapping regulons controlled by oxygen and NAD⁺ levels. *J Bacteriol* 2014;196:2166–77.
- Baker JL, Faustoferri RC, Quivey RG, Jr. Acid-adaptive mechanisms of *Streptococcus mutans*-the more we know, the more we don't. *Mol Oral Microbiol* 2017;32:107–17.
- Baker JL, Lindsay EL, Faustoferri RC et al. 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* 2018;200.

- Bowden GH, Hamilton IR. Survival of oral bacteria. *Crit Rev Oral Biol Med* 1998;9:54–85.
- Duong T, Barrangou R, Russell MW et al. Characterization of the *tre* locus and analysis of trehalose cryoprotection in *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol* 2005;72:1218–25.
- Faustoferri RC, Hubbard CJ, Santiago B et al. Regulation of fatty acid biosynthesis by the global regulator CcpA and the local regulator FabT in *Streptococcus mutans*. *Mol Oral Microbiol* 2015;30:128–46.
- Hellman LM, Fried MG. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat Protoc* 2007;2:1849–61.
- Kovacs CJ, Faustoferri RC, Quivey RG, Jr. RgpF is required for maintenance of stress tolerance and virulence in *Streptococcus mutans*. *J Bacteriol* 2017;199. DOI: 10.1128/JB.00497-17.
- Kreth J, Merritt J, Shi W et al. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. *J Bacteriol* 2005;187:7193–203.
- Kuhnert WL, Zheng G, Faustoferri RC et al. The F-ATPase operon promoter of *Streptococcus mutans* is transcriptionally regulated in response to external pH. *J Bacteriol* 2004;186:8524–8.
- Marcenes W, Kassebaum NJ, Bernabe E et al. Global burden of oral conditions in 1990–2010: a systematic analysis. *J Dental Res* 2013;92:592–7.
- Moye ZD, Son M, Rosa-Alberty AE et al. Effects of carbohydrate source on genetic competence in *Streptococcus mutans*. *Appl Environ Microbiol* 2016;82:4821–34.
- Munch R, Hiller K, Barg H et al. PRODORIC: prokaryotic database of gene regulation. *Nucleic Acids Res* 2003;31:266–9.
- Murchison HH, Barrett JF, Cardineau GA et al. Transformation of *Streptococcus mutans* with chromosomal and shuttle plasmid (pYA629) DNAs. *Infect Immun* 1986;54:273–82.
- Neta T, Takada K, Hirasawa M. Low-cariogenicity of trehalose as a substrate. *J Dent* 2000;28:571–6.
- Quivey RG, Jr., Faustoferri RC, Clancy KA et al. Acid adaptation in *Streptococcus mutans* UA159 alleviates sensitization to environmental stress due to RecA deficiency. *FEMS Microbiol Lett* 1995;126:257–61.
- Quivey RG, Jr., Grayhack EJ, Faustoferri RC et al. Functional profiling in *Streptococcus mutans*: construction and examination of a genomic collection of gene deletion mutants. *Mol Oral Microbiol* 2015;30:474–95.
- Ramseier TM, Reizer J, Kuster E et al. In vitro binding of the CcpA protein of *Bacillus megaterium* to cis-acting catabolite responsive elements (CREs) of gram-positive bacteria. *FEMS Microbiol Lett* 1995;129:207–13.
- Santiago B, Marek M, Faustoferri RC et al. The *Streptococcus mutans* aminotransferase encoded by *ilvE* is regulated by CodY and CcpA. *J Bacteriol* 2013;195:3552–62.
- Schock F, Dahl MK. Expression of the *tre* operon in *Bacillus subtilis* 168 is regulated by the repressor TreR. *J Bacteriol* 1996;178:4576–81.
- Shaw WV. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. *Methods Enzymol* 1975;43:737–55.
- Smith MF, Jr., Delbary-Gossart S. Electrophoretic Mobility Shift Assay (EMSA). *Methods Mol Med* 2001;50:249–57.
- Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. *J Dent Res* 2011;90:294–303.
- Zeng L, Choi SC, Danko CG et al. Gene regulation by CcpA and catabolite repression explored by RNA-Seq in *Streptococcus mutans*. *PLoS One* 2013;8:e60465.