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Effects of Carbohydrate Source on Genetic Competence in *Streptococcus mutans*

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ABSTRACT

The capacity to internalize and catabolize carbohydrates is essential for dental caries pathogens to persist and cause disease. The expression of many virulence-related attributes by *Streptococcus mutans*, an organism strongly associated with human dental caries, is influenced by the peptide signaling pathways that control genetic competence. Here, we demonstrate a relationship between the efficiency of competence signaling and carbohydrate source. A significant increase in the activity of the promoters for *comX*, *comS*, and *comYA* after exposure to competence-stimulating peptide (CSP) was observed in cells growing on fructose, maltose, sucrose, or trehalose as the primary carbohydrate source, compared to cells growing on glucose. However, only cells grown in the presence of trehalose or sucrose displayed a significant increase in transformation frequency. Notably, even low concentrations of these carbohydrates in the presence of excess glucose could enhance the expression of *comX*, encoding a sigma factor needed for competence, and the effects on competence were dependent on the cognate sugar:phosphotransferase permease for each carbohydrate. Using green fluorescent protein (GFP) reporter fusions, we observed that growth in fructose or trehalose resulted in a greater proportion of the population activating expression of *comX* and *comS*, encoding the precursor of *comX*-inducing peptide (XIP), after addition of CSP, than growth in glucose. Thus, the source of carbohydrate significantly impacts the stochastic behaviors that regulate subpopulation responses to CSP, which can induce competence in *S. mutans*.

IMPORTANCE

The signaling pathways that regulate development of genetic competence in *Streptococcus mutans* are intimately intertwined with the pathogenic potential of the organism, impacting biofilm formation, stress tolerance, and expression of known virulence determinants. Induction of the gene for the master regulator of competence, ComX, by competence-stimulating peptide (CSP) occurs in a subpopulation of cells. Here, we show that certain carbohydrates that are common in the human diet enhance the ability of CSP to activate transcription of *comX* and that a subset of these carbohydrates stimulates progression to the competent state. The cognate sugar:phosphotransferase permeases for each sugar are needed for these effects. Interestingly, single-cell analysis shows that the carbohydrates that increase *com* gene expression do so by enhancing the proportion of cells that respond to CSP. A mathematical model is developed to explain how carbohydrates modulate bistable behavior in the system via the ComRS pathway and ComX stability.

The initiation and progression of dental caries in humans are associated with changes in the composition of oral microbial biofilms: from a microbiota composed of greater proportions of health-associated phylotypes, including *Streptococcus gordonii* and *Streptococcus sanguinis*, to a pathogenic population comprised of *mutans* streptococci, lactobacilli, and other strongly aciduric organisms (1). The introduction of carbohydrate-containing foodstuffs into the host's diet leads to an accumulation of acidic end products in oral biofilms generated by the metabolic activity of microbes. These acidic conditions select for organisms that can grow at low pH (2, 3), many of which are particularly efficient at metabolizing a spectrum of different carbohydrates to organic acids. The organisms that have been associated by conventional and metagenomic analyses with the initiation and progression of dental caries are diverse, and many remain poorly described. However, the well-characterized caries pathogen *Streptococcus mutans* has shown one of the strongest associations with sites of active disease (4, 5). The primary virulence characteristics that allow cariogenic organisms, particularly *S. mutans*, to thrive in oral microbial biofilms under conditions conducive to caries development are their ability to establish and persist in oral biofilms, to metabolize a wide array of carbohydrates, to produce large quantities of acidic end products, and to outcompete acid-sensitive organisms as the local pH declines (6–8). In the years since *S. mutans* was confirmed to be an effective dental caries pathogen, intensive efforts have been directed at dissecting the physiology and genetics of this organism. *S. mutans* has proven to be a valuable model organism, especially in the fields of biofilm formation, stress responses, and regulation of the development of genetic competence (9).

Expression of many virulence properties of *S. mutans*, including biofilm formation and acid tolerance (10), is strongly influ-

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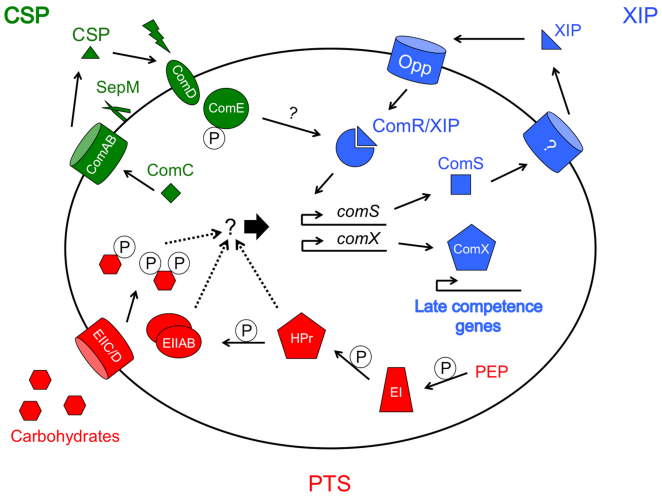

FIG 1 Schematic representation of competence initiation (green and blue), carbohydrate uptake via the PTS (red), and possible mechanisms by which the transport and metabolism of certain carbohydrates influence competence gene expression (black) by altering *comS* and *comX* expression in *S. mutans*. Genetic competence is initiated by the alternate sigma factor ComX, which directs RNA polymerase to the promoters of late competence genes. Expression of *comX* is regulated by the ComR/XIP complex, consisting of the Rgg-type regulator ComR and XIP, a 7-aa peptide derived from ComS. In addition to *comX*, the ComR/XIP complex activates the promoter of *comS*, creating a positive-feedback loop. The CSP-ComDE pathway is one of several extracellular signaling systems capable of influencing expression of the ComRS circuit, subsequently affecting genetic competence. After the product of *comC* has been exported and modified to produce CSP, this peptide is detected by ComDE, and through an unknown mechanism, the ComRS circuit is activated. Finally, carbohydrate uptake via the PTS is mediated by the transfer of a phosphate group from phosphoenolpyruvate (PEP) to the incoming carbohydrate through EI, HPr, and membrane-bound PTS permeases. During transport of different carbohydrates, particular glycolytic intermediates can accumulate, the cognate EIIAB permeases exist in a dephosphorylated state, and the phosphorylation status of HPr changes. We hypothesize that particular metabolic intermediates and/or differentially phosphorylated PTS enzymes arising from the transport and metabolism of carbohydrates analyzed in this study (Fig. 2) may interact with factors that influence *com* gene expression and, in some cases, the progression to the competent state. See the text for more details.

enced by the regulatory pathways that control the development of genetic competence, which allows the organism to internalize DNA from its environment. Competence is tightly regulated in a complex manner, presumably due to the fact that a substantial bioenergetic investment is needed to produce the components of the DNA uptake and metabolism machinery and that triggering of competence under certain circumstances can induce a form of programmed cell death (11–13). Genetic competence was first shown to develop naturally in *S. mutans* by Perry and Kuramitsu (14). Since then, numerous signal molecules, regulatory proteins, and checkpoints have been shown to govern whether organisms progress to the competent state, as measured by the capacity of cells to take up DNA. In *S. mutans*, inputs for the activation of genetic competence converge on the production of the alternative sigma factor ComX (SigX), which directs RNA polymerase to promoters of genes required for the internalization of extracellular DNA and incorporation of sufficiently homologous DNA into the bacterial chromosome (Fig. 1). ComR, an Rgg-type regulator, serves as the transcriptional activator for *comX* and for the *comS* gene located directly downstream of *comR*; *comS* encodes a 17-amino-acid (aa) hydrophobic peptide (15). ComS is exported and processed to remove 10 residues from the N terminus (15) to yield the 7-aa *sigX*-inducing peptide (XIP). XIP can be reinternalized by the Opp oligopeptide ABC transporter, where it is believed to form a ComR/XIP complex, which activates binding of ComR to its target genes (15). Activation of *comS* and *comX* by ComR/XIP generates a positive-feedback loop and stimulates late competence gene expression, respectively (15, 16). Current evidence suggests a model in which the ComRS circuit is the proximal regulator for *comX* activation, with other factors that influence genetic competence acting upstream of ComRS (15).

The early genes of the competence-signaling pathway in *S. mutans* are regulated by inputs from peptide signals, as well as by multiple sensors of environmental stress and cellular homeostasis (17–24). Among the signaling pathways, perhaps the best characterized is the ComDE two-component system (TCS), which detects a specific peptide signal and activates genetic competence and the production of bacteriocins and immunity proteins (25, 26). Competence-stimulating peptide (CSP) serves as the signal for the ComDE system and is encoded as an immature propeptide by *comC* (21). Two different proteins act on the 46-aa ComC protein to generate mature CSP. ComAB (NlmTE) cleaves CSP at a double glycine (GG) motif during export to yield a 21-aa peptide (27). Subsequently, SepM, located at the cell surface, removes three residues from the C terminus to create an 18-aa version of CSP that stimulates ComDE more effectively than the 21-aa peptide (27, 28). Once engaged by CSP, the histidine kinase ComD presumably autophosphorylates and subsequently transfers its phosphoryl group to the response regulator ComE. Activated ComE then directly stimulates the promoters of multiple genes encoding bacteriocins and bacteriocin immunity proteins (29, 30). Importantly, *comX* gene expression is also upregulated in response to treatment of cells with CSP (31), but the *comX* promoter region does not bear the canonical direct-repeat motif required for binding by ComE. Furthermore, purified ComE does not bind this region *in vitro*. Thus, activation of *comX* and competence by CSP is not directly regulated by ComE.

Due to the intimate association of genetic competence with multiple stress response pathways in several well-characterized Gram-positive bacterial species (25, 32), it is not surprising to find that environmental factors directly impact the competence cascade. For example, pH and medium composition (33, 34) and the presence of saliva (35) affect the ability of exogenously supplied CSP and XIP to modify gene expression and induce the competent state in *S. mutans*. In addition, the phase of growth of batch-cultured cells influences CSP- and XIP-mediated signaling, with cells experiencing a transient state of responsiveness to signal pheromones in early exponential phase but becoming refractile to signals as they progress further into exponential phase (33), with acidification of the environment during growth contributing to this behavior.

The introduction of a spectrum of nutrients into the oral cavity via host secretions and the diet mandates that members of oral microbial communities deploy multiple strategies to cope with a "feast or famine" lifestyle (36). For many of the most abundant oral bacteria, the ability to monitor the availability of carbohydrates in the oral cavity and to metabolize an array of sugars is essential for their survival and persistence and is a major contributor to the virulence of cariogenic organisms (37). Carbohydrates are internalized by *S. mutans* primarily by the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS), which

transports a wide variety of mono- and disaccharides. ABC transporters also participate in uptake of a few carbohydrates, such as melibiose and raffinose, but the PTS is the dominant route of intake of the carbohydrates that are most commonly found in host secretions and the human diet. A functional PTS is composed of the general proteins EI and HPr and a sugar-specific EII enzyme (38). EII enzymes consist minimally of cytoplasmically localized EIIA and EIIB domains with a carbohydrate-specific, membrane-spanning EIIC, although some EII enzymes require an EIID domain for functionality. As many as 15 different PTS EII enzymes are encoded in the genomes of *S. mutans* isolates, with several of these enzymes being present in all isolates and a few restricted to certain strains (39–41). Phosphoenolpyruvate (PEP) acts as the phosphoryl donor for autophosphorylation of the EI protein. Phosphorylated EI then donates its phosphoryl group to HPr, which can transfer the phosphate group to the EIIA domain, where it is transferred to the EIIB domain, then ultimately by the EIIC, and EIID if required, to the incoming carbohydrate.

The vast majority of studies of genetic competence in *S. mutans* have been conducted using media supplemented with glucose, while a few have utilized sucrose. In this study, we sought to determine the impact of other carbohydrate sources on competence signaling through the CSP pathway and on transformation efficiency. We report that cultures of *S. mutans* grown in media supplemented with certain common dietary carbohydrates displayed significantly altered competence signaling and, in a subset of cases, transformability. Single-cell studies revealed that enhancement of *com* signaling was the result of a greater subpopulation of cells activating competence genes. Overall, these results provide compelling evidence that the carbohydrates to which oral biofilms are commonly exposed modify the circuits connecting CSP pheromone sensing with the competence activation cascade.

MATERIALS AND METHODS

Bacterial growth and genetic manipulation. *Streptococcus mutans* UA159 and its derivatives (for a complete list of strains utilized in this study, see Table S1 in the supplemental material) were routinely grown in brain heart infusion (BHI) broth (Difco) or a 9:1 (vol/vol) mixture of tryptone-vitamin medium (42) and BHI broth, designated TVB, which was supplemented with the desired carbohydrates. When required, antibiotics were added to the growth media: 500 μg ml^{-1} spectinomycin, 500 μg ml^{-1} kanamycin, 5 μg ml^{-1} erythromycin, or 5 μg ml^{-1} tetracycline. Reporter gene fusion strains used in β-galactosidase assays were engineered by transformation of *S. mutans* with the promoter elements of *comX*, *comS*, *comR*, *comYA*, or *cipB* fused to a *lacZ* gene in plasmid pMC340B (35, 43). The *lacZ* gene of pMC340B is derived from *Streptococcus salivarius* 57.I, and the cloning sites for creating gene fusions are flanked by *S. mutans* gene fragments that allow for integration of the reporter gene construct into the chromosome at the *phn-mtlA* locus via double crossover recombination. Insertions of the gene fusions into the *S. mutans* chromosome and mutants created by allelic replacement of sequences with antibiotic resistance markers were confirmed by PCR and sequencing. For gene expression analysis of single cells, *S. mutans* strains were transformed with plasmid pDL278 containing the promoter elements of *comX*, *comS*, or *comR* fused to a gene encoding a green fluorescent protein (GFP), as detailed elsewhere (35).

β-Galactosidase assay. Cultures were grown overnight in BHI medium with antibiotics, if needed, and diluted 1:20 into fresh TVB supplemented with the carbohydrates of interest. Cultures were grown to an optical density at 600 nm (OD_{600}) of 0.1 at 37°C in a 5% CO_2 aerobic atmosphere, and then 0.1 or 1 μM synthetic peptide corresponding to the 21-aa CSP (synthesized and purified by Biomatik Corp., Cambridge, Ontario, Canada) was added to cultures. Cultures were grown for an additional 2 h, and β-galactosidase assays were performed as previously described, with minor modifications (44). Briefly, cells were collected by centrifugation and washed once in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, and 1 mM $MgSO_4$ with 50 mM β-mercaptoethanol freshly added). Pelleted cells were resuspended in 1.3 ml of Z buffer, and 500 μl of the suspension was mixed with 25 μl of 1:9 toluene-acetone, while the remaining volume was used to determine the OD_{600} of the suspension. The 500-μl suspensions were mixed with 100 μl of *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG [4 mg/ml]) in 0.1 M sodium phosphate buffer (pH 7.5) and incubated at 37°C. Once a change in color was observed, 500 μl of 1 M Na_2CO_3 was added to terminate the reaction, and the duration of the reaction was recorded. After brief centrifugation, the OD_{420} and OD_{550} of the supernates were measured and used to quantify the β-galactosidase activity, which was reported as Miller units (44).

Transformation assay. *S. mutans* UA159 and its derivatives were grown overnight in BHI broth and diluted 1:20 into TVB supplemented with different carbohydrates. Cultures were grown to an OD_{600} of 0.1, and 1 μM CSP and 100 ng of the plasmid pBGS, which was generated by replacing the kanamycin marker of pBGK2 (45) with a marker conferring resistance to spectinomycin, were added to cultures. After 3 h of additional growth, cultures were diluted and plated on BHI agar and on BHI agar containing spectinomycin (1 mg ml^{-1}). After 24 to 48 h of incubation at 37°C in a 5% CO_2 aerobic atmosphere, CFU were enumerated. Transformation efficiency is expressed as the number of spectinomycin-resistant colonies divided by the total number of viable colonies multiplied by 100 (i.e., percentage of transformants).

Monitoring of gene expression in single cells. Cultures of *S. mutans* UA159 and derivatives containing *gfp* reporter plasmids were grown overnight in BHI containing any necessary antibiotics and were diluted 1:20 into TVB medium containing the desired carbohydrates and antibiotics, if needed. Once cultures reached an OD_{600} of 0.1, CSP was added to a final concentration of 1 μM, and cells were incubated for 2 h. At this point, cultures were prepared for single-cell experiments, and the images were analyzed as described elsewhere (46). Briefly, cultures were mildly sonicated to disrupt cell chains and aggregates, the cell suspensions were pipetted onto glass slides, and then phase-contrast and green fluorescence images were obtained using a microscope equipped with a motorized stage, shutter, and cooled charge-coupled device (CCD) camera. Custom Matlab codes were developed to control the imaging sequence and to analyze the images (46). The analysis compared the intensity of the phase-contrast image with the brightness of green fluorescence image pixel by pixel for an individual cell. The data are expressed as the unitless parameter R, which is proportional to the concentration of GFP within the cell.

RESULTS

Development of a modified base medium for the study of effects of carbohydrates on competence. Induction of *comX* and enhancement of transformation efficiency by CSP have not been observed in chemically defined medium. Instead, optimal induction of competence by CSP occurs in complex media, and often BHI is the medium used. Unfortunately, BHI and a number of other complex media that support the growth of streptococci were found not to be suitable for analyzing the effects of carbohydrate source on competence, presumably because they contain relatively high concentrations of glucose (for example, see Fig. S1 in the supplemental material). Conversely, TV medium, composed only of tryptone and a vitamin mixture (42), has proven to be very useful for studying carbohydrate-specific effects in *S. mutans* as TV base medium will not support growth of *S. mutans* unless metabolizable carbohydrates are added. However, when the P_{comX}-*lacZ* strain was grown in TV base medium supplemented with glucose, cells displayed poorer induction of the *comX* promoter in response to addition of CSP (see Fig. S2A, dark bars, in

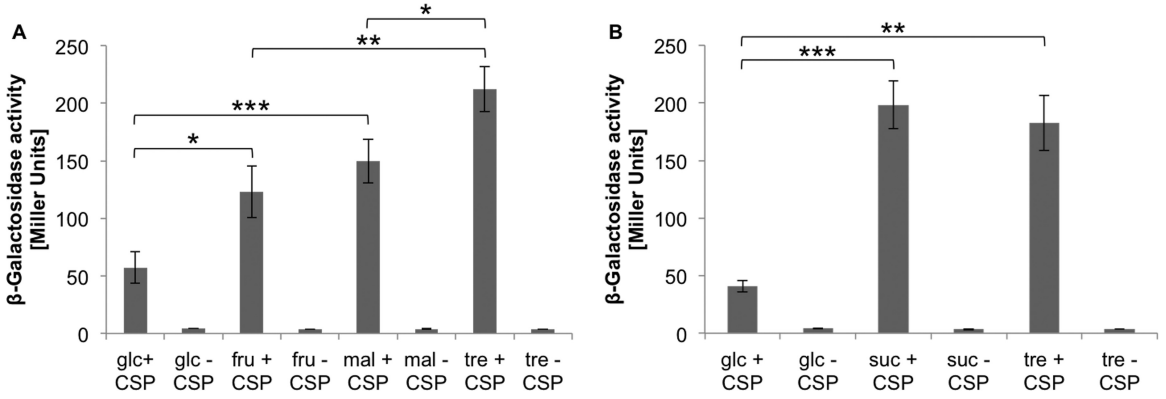

FIG 2 Effect of carbohydrate type on the expression of *comX* after CSP stimulation. A derivative of *S. mutans* UA159 (A) containing P_{comX}-*lacZ* was grown in TVB base medium supplemented with 20 mM glucose (glc), 20 mM fructose (fru), 10 mM maltose (mal), or 10 mM trehalose (tre). (B) A derivative of *S. mutans* UA159 carrying a deletion of the tandemly arranged *gtfBC* genes and containing the P_{comX}-*lacZ* fusion was grown in TVB supplemented with 20 mM glucose (glc), 10 mM sucrose (suc), or 10 mM trehalose (tre). In all cases, cultures were grown to an OD_{600} of 0.1 and treated with 1 μM CSP or left untreated. After an additional 2 h of incubation, *comX* expression was determined by performing LacZ assays. The data represent the mean of three independent replicates, with error bars indicating the standard deviation. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.005$, by the Student *t* test.

the supplemental material) than was previously observed for cells cultured in BHI broth. A variety of media and admixtures of media were tested to identify one that did not support robust growth of *S. mutans* unless supplemental carbohydrate was added but which would better support induction of *comX* by CSP. We found that the use of TV base medium that had been augmented with 1/10 volume of normal-strength BHI (i.e., 9 volumes of TV base medium plus 1 volume of BHI broth), heretofore referred to as TVB medium, allowed for significantly more efficient induction of *comX* by CSP signaling (see Fig. S2A). However, similar to TV (see Fig. S2B and -C), TVB did not support growth of *S. mutans* beyond an OD_{600} of 0.1 unless supplemental carbohydrates were added. Thus, at the time of assaying gene expression or competence, cells would have exhausted any carbohydrate present in the base medium (predominantly glucose from BHI medium) and would have been growing on the carbohydrate of interest.

Carbohydrate source impacts *comX* expression in response to CSP. To investigate the impact of carbohydrate source on induction of genetic competence by CSP, an *S. mutans comX* reporter gene fusion (P_{comX}-*lacZ*) strain was cultivated on TVB supplemented with various carbohydrates to screen for sugars that could modify *comX* expression. When cells entered early exponential phase, 1 μM CSP was added to the cultures, and after 2 h of additional incubation, LacZ assays were performed. Of the carbohydrates tested, growth of the reporter strain in TVB supplemented with 20 mM fructose, 10 mM maltose, or 10 mM trehalose demonstrated significantly higher induction of the *comX* promoter than in cells grown with 20 mM glucose (Fig. 2A). (Note that different molar concentrations of mono- or disaccharides were used to provide the same amount [wt/vol] of carbohydrate in the cultures.) Trehalose consistently allowed for the highest level of expression of the *comX* promoter, and cells grown with fructose or maltose expressed similar levels of *comX* promoter activity to one another. During our initial screening of the carbohydrates, we noted that sucrose was capable of inducing a high level of expression from the P_{comX}-*lacZ* reporter strain (data not shown). However, the production of water-insoluble glucans by the GtfB and GtfC glucosyltransferase enzymes led to the aggregation of cells, which negatively impacted proper normalization of LacZ activity. Therefore, to study the effects of sucrose on *com* gene expression, the P_{comX}-*lacZ* reporter plasmid was introduced into a *gtfBC* deletion strain of *S. mutans* UA159, MMZ945 (47), which does not produce water-insoluble glucans. This strain was grown in TVB supplemented with 20 mM glucose, 10 mM sucrose, or 10 mM trehalose, and when cells reached an OD_{600} of 0.1, 1 μM CSP was added. The expression of *comX* in the *gtfBC* deletion strain grown with sucrose was significantly greater than that in cells grown with glucose (Fig. 2B), with LacZ activities similar to that expressed in cells growing on trehalose.

Previous studies demonstrated that the optimal activation of *comX* expression by CSP occurred in early exponential phase at an approximate OD_{600} of 0.1 to 0.2 (33). We therefore investigated whether growth-phase-dependent induction of *comX* expression was observed as a function of carbohydrate source. Interestingly, cells grown in TVB supplemented with 20 mM glucose, 20 mM fructose (see Fig. S3A in the supplemental material), or 10 mM sucrose (see Fig. S3B) displayed optimal *comX* induction in early exponential phase, with the highest level of expression being achieved when CSP was added at an optical density of 0.1. In fact, expression of *comX* was significantly reduced when addition of CSP was delayed until the culture reached an OD_{600} of 0.2. As noted for cells grown in BHI broth (33), expression from the *comX* promoter in response to CSP dropped off sharply for all sugars tested if CSP was added later in the exponential phase. Based on these results, all subsequent experiments were conducted by adding CSP at an OD_{600} of 0.1.

Low concentrations of certain carbohydrates are sufficient to enhance *comX* expression. We next sought to determine if the expression of *comX*, as monitored via the P_{comX}-*lacZ* reporter strain, was sensitive to lower concentrations of fructose, trehalose, maltose, or sucrose in the growth media than those used above. To perform these experiments, we titrated these carbohydrates in TVB medium, using glucose to keep the final concentration (wt/vol) of carbohydrate in each sample constant. As little as 0.5 mM fructose (Fig. 3A), trehalose (Fig. 3B), or maltose (Fig. 3C) was capable of inducing significantly higher expression from the P_{comX}-*lacZ* reporter strain than from cells grown on glucose alone. In addition, the *gtfBC* deletion strain bearing the P_{comX}-*lacZ* re-

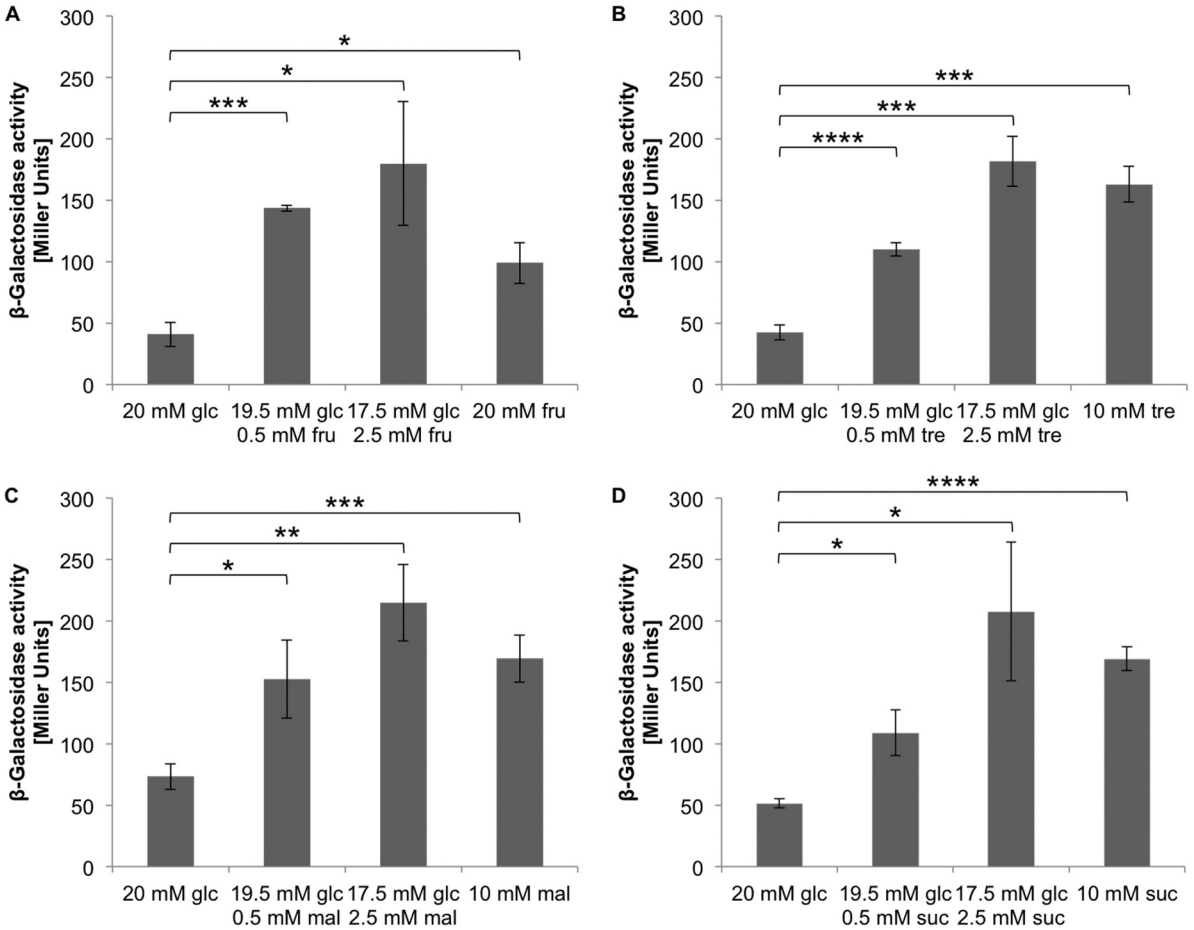

FIG 3 Relatively low concentrations of carbohydrates are capable of enhancing CSP-mediated activation of *comX*. *S. mutans* UA159 (or a *gtfBC* mutant for sucrose-grown cells) containing P_{comX}-*lacZ* was cultured in TVB medium supplemented with 20 mM glucose (glc), 19.5 mM glucose and 0.5 mM inducing carbohydrate, 17.5 mM glucose and 2.5 mM inducing carbohydrate, or 20 mM inducing carbohydrate (10 mM for inducing disaccharides). The inducing carbohydrates included fructose (fru) (A), trehalose (tre) (B), maltose (mal) (C), and sucrose (suc) (D). When cultures reached an OD_{600} of 0.1, they were treated with 1 μM CSP. Cultures were incubated an additional 2 h, and LacZ assays were performed to monitor *comX* promoter activity. Each bar represents the mean of three independent replicates, and error bars represent the standard deviation. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.005$, and ****, $P < 0.001$, by the Student *t* test.

porter gene fusion was grown in TVB supplemented with various concentrations of sucrose, again using glucose to maintain a constant concentration (wt/vol) of carbohydrate between samples. In this case, as little as 0.5 mM sucrose (Fig. 3D) allowed for induction of high levels of *comX* expression, to a magnitude similar to that noted for the other tested carbohydrates. Concentrations lower than 0.5 mM inducing carbohydrate were not tested. Interestingly, cells grown with a combination of 2.5 mM fructose, trehalose, maltose, or sucrose plus glucose to maintain an equivalent amount (wt/vol/) of carbohydrate between samples yielded consistently higher, though not statistically significant, *comX* expression than that of cells growing with fructose (20 mM), trehalose (10 mM), maltose (10 mM), or sucrose (10 mM) alone (Fig. 3).

Carbohydrate source impacts the induction of genetic competence by CSP. In order to further investigate how different carbohydrate sources influence competence signaling, we tested whether other genes in the competence-signaling pathway could be influenced by carbohydrate source, including *comR* and *comS* and the late competence gene *comYA*. Using the reporter strain UA159 P_{comS}-*lacZ*, it was observed that growth in TVB supplemented with fructose, maltose, or trehalose induced a higher level of *comS* expression than that of cells grown with glucose (Fig. 4A). Importantly, while cells growing on fructose or maltose had higher *comS* promoter activity than glucose-grown cells, cells grown on trehalose showed even higher *comS* promoter activity than cells growing on fructose or maltose. Using a P_{comR}-*lacZ* reporter strain, we found no consistent effect of carbohydrate source on *comR* expression (data not shown). When a strain carrying *lacZ* fused to the *comYA* promoter (P_{comYA}-*lacZ*) was tested, cells grown with 20 mM fructose, 10 mM maltose, or 10 mM trehalose exhibited significantly higher *comYA* expression after CSP addition than those grown with 20 mM glucose (Fig. 4B), with cells grown on trehalose again displaying the highest level of induction.

We next tested whether enhanced expression of *com* genes in cells grown with selected carbohydrates resulted in corresponding increases in the transformability of *S. mutans*. When the transformation efficiency of the organism grown in TVB supplemented with a variety of carbohydrate sources was assessed, growth with 20 mM fructose or 10 mM maltose did not result in a significant increase in the percentage of transformants recovered after stim-

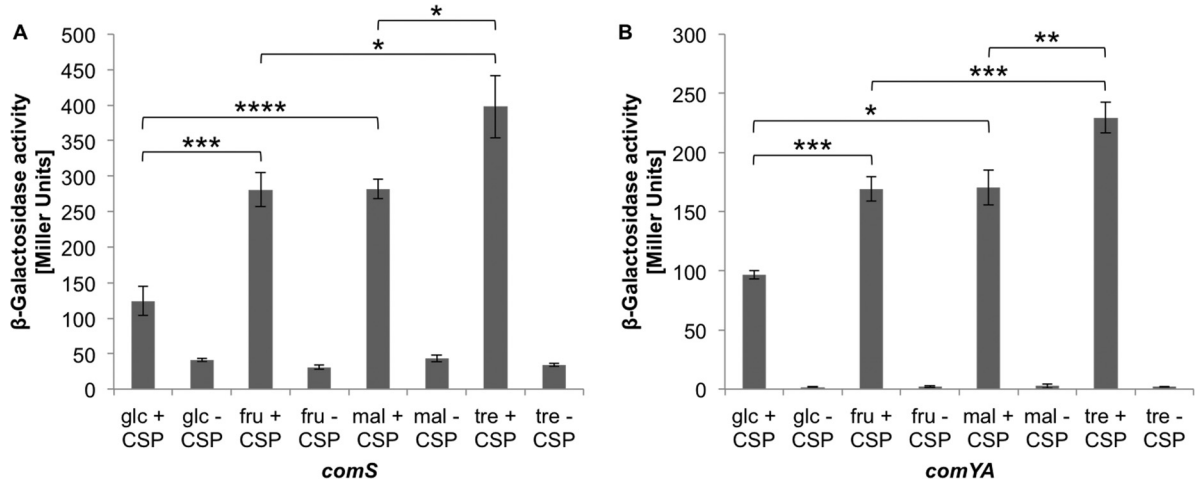

FIG 4 Effect of carbohydrate type on CSP-mediated expression of *comS* and *comYA*. Derivatives of *S. mutans* UA159 containing P_{comS}-*lacZ* (A) or P_{comYA}-*lacZ* (B) were grown in TVB supplemented with 20 mM glucose (glc), 20 mM fructose (fru), 10 mM maltose (mal), or 10 mM trehalose (tre). When cultures reached an OD_{600} of 0.1, half of the cultures were treated with 1 μM CSP, while the other half were left untreated. All cultures were grown for an additional 2 h, and activity of the *comS* or *comYA* promoter was monitored by performing LacZ assays. Data represent the mean of three independent replicates, with error bars indicating the standard deviation. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.005$, and ****, $P < 0.001$, by the Student *t* test.

ulation with 1 μM CSP, compared to cells grown with 20 mM glucose (Fig. 5A and B). In contrast, growth of *S. mutans* UA159 in TVB containing 10 mM trehalose yielded significantly more transformants than cultures grown with 20 mM glucose (Fig. 5B). As sucrose was able to stimulate *comX* promoter activity to levels similar to those observed for cells grown in trehalose, we tested whether sucrose could also enhance the transformability of *S. mutans*. When the glucosyltransferase mutant strain *gtfBC* was grown in TVB supplemented with either 20 mM glucose or 10 mM sucrose, a slight but significant increase in the number of transformants recovered from cells grown in 10 mM sucrose was noted compared to that in cells grown in 20 mM glucose (Fig. 5C). Importantly, although the absolute percentage of transformants recovered varied in some cases from day to day (e.g., compare

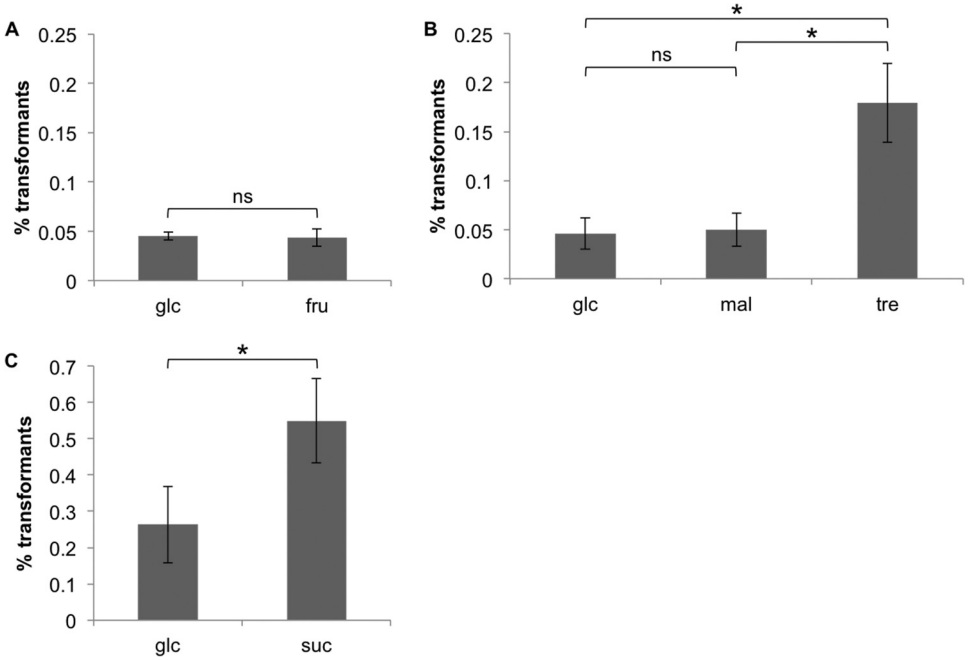

FIG 5 Impact of carbohydrate type on CSP-mediated transformability. *S. mutans* UA159 was grown in TVB base medium supplemented with 20 mM glucose (glc) or 20 mM fructose (fru) (A) or 20 mM glucose (glc), 10 mM maltose (mal), or 10 mM trehalose (tre) (B). A *gtfBC* deletion derivative of *S. mutans* UA159 was grown in TVB supplemented with 20 mM glucose (glc) or 10 mM sucrose (suc) (C). All cultures were grown to an OD_{600} of 0.1, and then 1 μM CSP and 100 ng of the plasmid pBGS were added. Cultures were incubated for an additional 3 h and then diluted and plated on BHI agar with or without 1 mg ml^{-1} spectinomycin. Plates were incubated for 24 to 48 h, and CFU were enumerated. Data represent the mean of three independent replicates, with error bars expressing the standard deviation. ns, not significant. *, $P < 0.05$, by the Student *t* test.

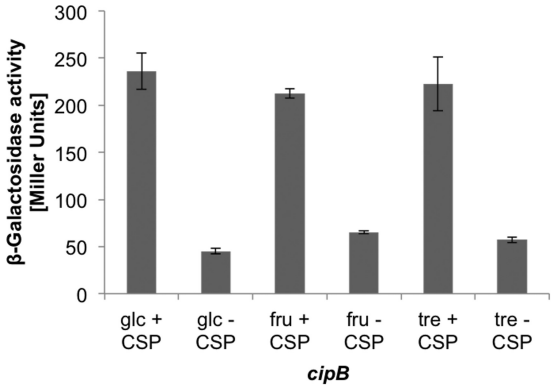

FIG 6 Effect of carbohydrate type on activation of the *cipB* promoter after CSP stimulation. A derivative of *S. mutans* UA159 containing P_{cipB}-*lacZ* was grown in TVB medium supplemented with 20 mM glucose (glc), 20 mM fructose (fru), or 10 mM trehalose (tre). Once an OD_{600} of 0.1 was achieved, cultures were treated with 0.1 μM CSP and incubated for an additional 2 h. Expression of the *cipB* promoter was monitored by performing LacZ assays. The data presented are the mean of three independent replicates, with error bars expressing the standard deviation.

the cultures grown with glucose in Fig. 5A and C), growth with sucrose or trehalose always resulted in increased transformation efficiency (~2- or 3.5-fold, respectively) compared to the level in cells grown with glucose in experiments performed on the same day.

Sugar-specific PTS transporters are required for the effects of carbohydrates on competence signaling. Since the ComDE TCS is responsible for sensing and responding to CSP, we first determined if the changes in *com* gene expression and transformability elicited by growth on certain carbohydrate sources were due to alterations in the ComDE-CSP signaling pathway. We therefore monitored the expression of the *cipB* gene, which encodes a bacteriocin and is directly activated by the ComE response regulator. When the P_{cipB}-*lacZ* reporter strain was grown in TVB supplemented with 20 mM glucose, 20 mM fructose, or 10 mM trehalose and stimulated with 0.1 μM CSP, we observed no significant difference in the expression from the *cipB* promoter (Fig. 6). Growth in the presence of CSP has also been shown to result in apparent growth inhibition, and an intact ComDE-CSP signaling pathway is required for this effect. Similar to the response of *cipB* expression to CSP, growth inhibition by CSP (see Fig. S4 and S5 in the supplemental material) resulted in lower final optical densities of ~0.1 U in all carbohydrate sources tested (see Table S2 in the supplemental material). Therefore, it does not appear as though carbohydrate source alters the ability of ComE to activate its targets in response to addition of CSP.

We next investigated whether the enhancements in *comX* expression required the cognate PTS transporters of the carbohydrates under investigation using mutant strains with deletions in the various PTS transporters for the sugars under investigation. We observed that concomitant mutation of the three PTS permeases that are primarily responsible for fructose uptake, encoded by *fruI*, *fruCD*, and *levD*, resulted in substantially reduced growth on TVB supplemented only with fructose (data not shown). The P_{comX}-*lacZ* reporter was introduced into a *fruI fruCD levD* deletion strain, and the strain was grown in TVB with 2.5 mM fructose and 17.5 mM glucose, conditions that resulted in robust growth and high levels of *comX* expression in strain UA159 (Fig. 3A). When contrasted with the parental strain, the *fruI fruCD levD* triple mutant showed no enhancement of expression from the *comX* promoter (Fig. 7A) after stimulation with CSP when fructose was present. Interestingly, the *fruI fruCD* P_{comX}-*lacZ* double mutant reporter strain and *levD* P_{comX}-*lacZ* single mutant reporter strain retained enhanced expression of *comX* in response to fructose, albeit the magnitude of the enhancement was lower than in the wild-type strain (see Fig. S6 in the supplemental material). Thus, all three high-affinity fructose transporters must be deleted to block the enhancement of *comX* expression by fructose.

When *comX* expression was monitored in a strain bearing a deletion of the gene for the trehalose PTS permease, *treB*, and growing in TVB with 2.5 mM trehalose and 17.5 mM glucose, *comX* promoter activity was dramatically lower in the *treB* deletion mutant than in the strain with an intact trehalose PTS (Fig. 7B). Similar results were obtained when the P_{comX}-*lacZ* reporter was moved into a *malT* mutant background, with deletion of the EII maltose permease resulting in loss of enhanced induction of *comX* expression in cells grown in the presence of maltose (Fig. 7C). Finally, we investigated the impact of deleting the gene for the sucrose PTS permease, *scrA*, on *comX* expression by using a *gtfBC scrA* deletion mutant containing the P_{comX}-*lacZ* reporter. Similar to what was observed with the other PTS transporter mutants tested, there was a significant difference in *comX* expression between the *gtfBC scrA* deletion mutant and the *gtfBC* strain when grown in TVB with 2.5 mM sucrose and 17.5 mM glucose (Fig. 7D). However, the degree to which inactivation of *scrA* blunted the enhancement of *comX* expression was less than those observed for other PTS mutants. We attribute this finding to the facts that sucrose can also be transported by the TreB PTS permease (47, 48) and that cleavage of sucrose by extracellular enzymes other than GtfBC (e.g., GtfD and FruA) can release fructose into the medium. Notably, in our experiments with PTS mutants, we observed day-to-day variations in the magnitude of *comX* promoter activity (e.g., compare the *comX* expression for UA159 grown with glucose in Fig. 7A and C), but for each carbohydrate tested, deletion of the cognate transporter consistently resulted in diminished expression of the *comX* promoter that was significantly lower than that in the parental strain in assays performed on the same day.

Catabolite repression and genetic competence. Multiple studies have demonstrated the importance of the glucose/mannose-type PTS transporter $EIIAB^{Man}$ and the general PTS protein HPr in the regulation of CcpA-independent catabolite repression in *S. mutans* (49, 50). Interestingly, it has been observed that deletion of the gene for $EIIAB^{Man}$, *manL*, resulted in decreased transformation efficiency compared to that of the parental strain when cells were grown in BHI broth supplemented with heat-inactivated horse serum in the presence or absence of exogenously added CSP (51). We therefore investigated if deletion of the EII^{Man} PTS permease would impact competence signaling and if changes in the carbohydrate content of the media could alter competence-related behaviors in the ManL-deficient strain. To monitor the expression of the *comX* promoter, we moved the P_{comX}-*lacZ* reporter into a strain with deletion of the *manLMN* genes (52), which encode $EIIAB^{Man}$, $EIIC^{Man}$, and $EIID^{Man}$, respectively, and grew cells in TVB containing 20 mM glucose, 20 mM fructose, or 10 mM trehalose. Expression from the *comX* promoter trended lower in the sugars tested, albeit not significantly so for fructose and trehalose, in the *manLMN* deletion strain for all carbohydrates tested, with almost no expression in cells growing on glu-

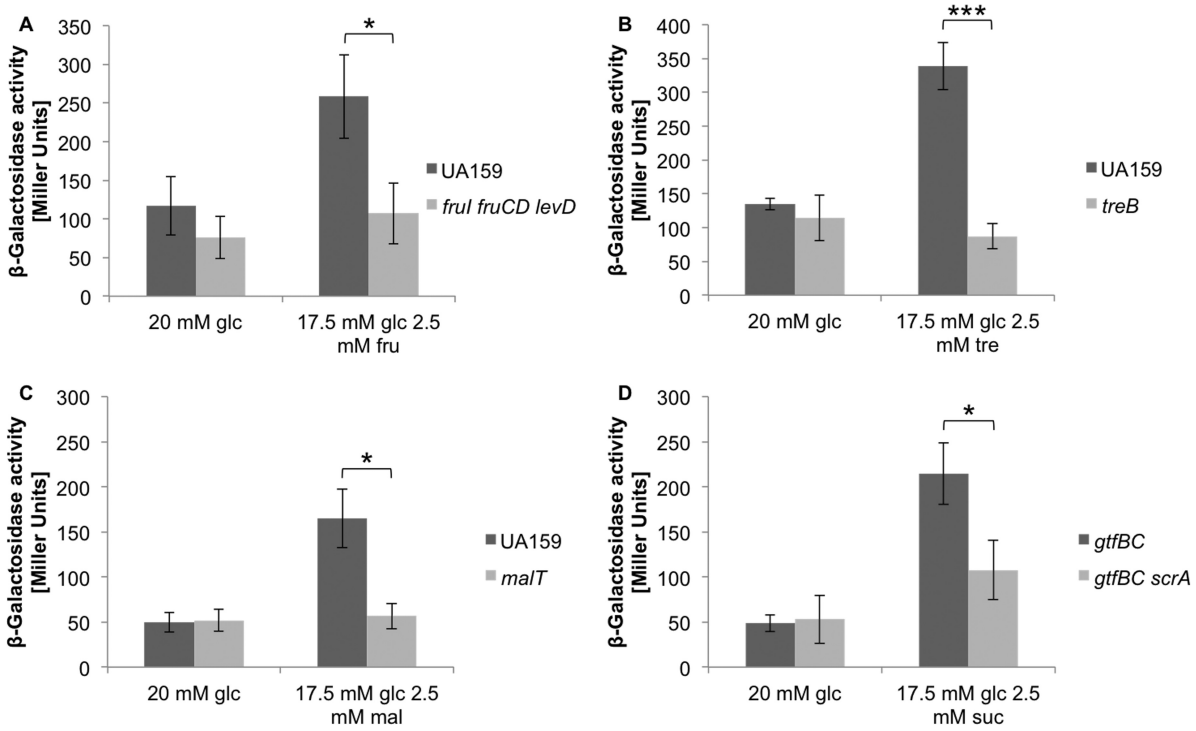

FIG 7 Enhancement of CSP-mediated *comX* expression by carbohydrates requires their cognate PTS transporter(s). Cells of *S. mutans* UA159 (or the *gtfBC* mutant in the case of sucrose) as well as a *fruI fruCD levD* (A), *treB* (B), *malT* (C), or *gtfBC scrA* (D) derivative of UA159 containing P_{comX}-*lacZ* were grown in TVB supplemented with 20 mM glucose (glc) or 17.5 mM glucose and 2.5 mM inducing carbohydrate. The inducing carbohydrates were fructose (fru) (A), trehalose (tre) (B), maltose (mal) (C), and sucrose (suc) (D). Cultures were grown to an OD_{600} of 0.1, treated with 1 μM CSP, and incubated for an additional 2 h. LacZ assays were performed to determine the expression of *comX*. Data represent the mean of three independent replicates, with error bars indicating the standard deviation. *, $P < 0.05$, and ***, $P < 0.005$, by the Student *t* test.

cose in the absence of ManLMN (Fig. 8A). However, the overall pattern of expression in response to carbohydrate source was the same as in the parental strain, with cells grown in trehalose expressing the highest level of *comX* promoter activity. Similar results were observed for transformation efficiencies (see Fig. S7A and C in the supplemental material).

Unlike in most low-G+C Gram-positive bacteria, deletion of the gene for the global regulator CcpA in *S. mutans* does not alleviate catabolite repression. However, CcpA has been shown to negatively regulate the expression of certain EII enzymes (49), and deletion of *ccpA* results in a slight decrease in transformation efficiency of *S. mutans* (L. Zeng and R. A. Burne, unpublished data). To evaluate the impact of deletion of *ccpA* on the expression of *comX* in the context of different carbohydrates, we transformed a *ccpA* deletion strain with P_{comX}-*lacZ*. The resulting reporter strain was grown in TVB supplemented with 20 mM glucose, 20 mM fructose, or 10 mM trehalose and stimulated with 1 μM CSP. We found that, compared to the parental strain, the *ccpA* deletion

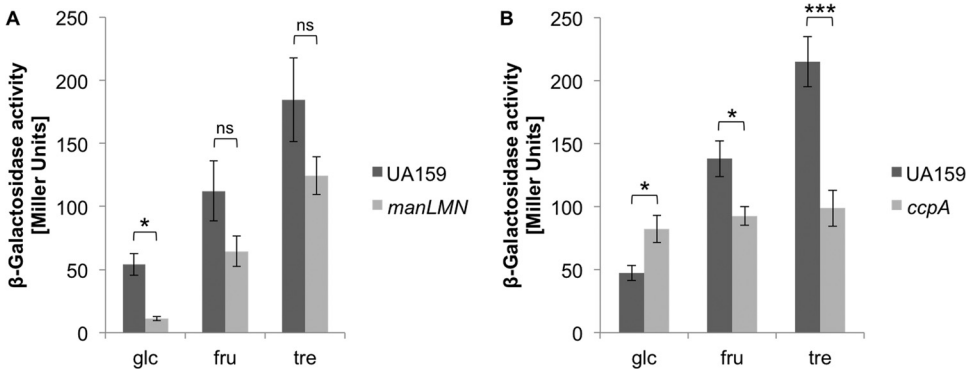

FIG 8 Effect of deletion of regulators of carbohydrate utilization on CSP-mediated activation of *comX*. *S. mutans* UA159 derivatives carrying *manLMN* (A) or *ccpA* (B) deletions and the P_{comX}-*lacZ* fusion were grown in TVB supplemented with 20 mM glucose (glc), 20 mM fructose (fru), or 10 mM trehalose (tre). When cultures reached an OD_{600} of 0.1, they were treated with 1 μM CSP and then incubated for an additional 2 h. LacZ assays were performed to determine the expression of *comX*. Data represent the mean of three independent replicates, with error bars indicating the standard deviation. ns, not significant. *, $P < 0.05$, and ***, $P < 0.005$, by the Student *t* test.

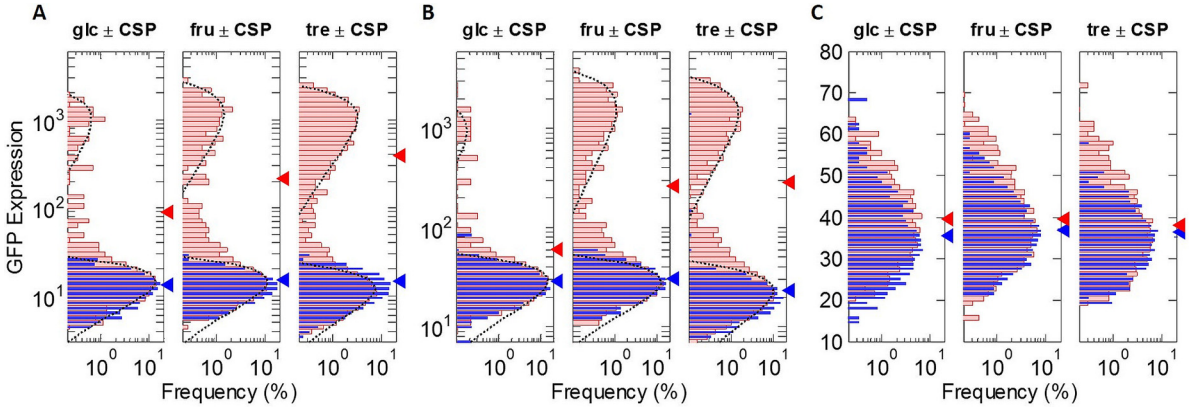

FIG 9 Effect of carbohydrate type on the proportion of *S. mutans* cells activating *com* genes after CSP stimulation. Derivatives of *S. mutans* UA159 containing P_{comX}-*gfp* (A), P_{comS}-*gfp* (B), or P_{comR}-*gfp* (C) were grown in TVB supplemented with 20 mM glucose (glc), 20 mM fructose (fru), or 10 mM trehalose (tre). When cultures reached an OD_{600} of 0.1, half of the cultures were treated with 1 μM CSP (red), while the other half were left untreated (blue). After an additional 2 h of incubation, the expression of *comX*, *comS*, and *comR* was determined by measuring the intensity of GFP using a fluorescence microscope. Image analysis is described in Materials and Methods. An arrow represents the mean GFP florescence for cells incubated with CSP (red) or left untreated (blue). The black dotted line indicates the best-fit normal curve for the turned-on/off group in CSP samples. The mean and standard deviation of each curve were estimated using the method of least squares, and the area under each curve was calculated to estimate the fraction of turned-on/off cells.

mutant displayed a slight elevation of *comX* promoter activity when grown in glucose and a significant decrease in *comX* promoter when grown on fructose or trehalose, compared to the wild-type strain growing on the same carbohydrates (Fig. 8B). Transformation efficiency assays revealed that, compared to the parental strain, the *ccpA* mutant displayed a decrease in the percentage of transformants when grown in glucose or trehalose, with the transformation efficiencies being similar for these two carbohydrates (see Fig. S7B in the supplemental material). Thus, while CcpA does not play a dominant role in catabolite repression in *S. mutans* (37), loss of CcpA did have a significant impact on the response of this organism to CSP in the sugars tested.

Carbohydrate source impacts the proportion of cells that activate competence signaling. CSP-mediated activation of *comX* in *S. mutans* grown in complex media occurs in a bimodal fashion, with only a subpopulation of cells activating *comX* expression. Thus, the presence of certain carbohydrates could cause increases in *comX* promoter activity by inducing higher levels of expression in the subset of responding cells or by allowing for a greater proportion of the population to respond. To discriminate between these two possibilities, we utilized strains carrying P_{comX}-*gfp* or P_{comS}-*gfp* promoter reporter fusions (35), each carried on the shuttle plasmid pDL278 in the wild-type genetic background. Cells were grown in glucose (baseline), fructose, which enhanced *com* expression but did not alter transformability, or trehalose, which increased *com* expression and transformation efficiency. The reporter strains were grown in TVB with 20 mM glucose, 20 mM fructose, or 10 mM trehalose, and 1 μM CSP was added at an OD_{600} of 0.1. After cells had grown for an additional 2 h, the proportion and intensity of the response of the population were examined by fluorescence microscopy. Carbohydrates that enhanced *comX* expression, fructose and trehalose, resulted in a greater proportion of cells activating *comX* (Fig. 9A) or *comS* (Fig. 9B), compared with glucose-grown cells. Consistent with LacZ assays, growth in trehalose resulted in the greatest proportion of cells activating *comX* expression, 47%, and cells grown in fructose exhibited a much lower proportion of activated cells, 25.9%, which was still higher than the proportion in glucose-grown cells (12.6%).

The proportions of cells expressing *comS* were comparable between cells grown in fructose and trehalose (21.9 and 24.5%, respectively), which deviates from what was observed for LacZ assays; however, these proportions were much higher than the 3% of cells showing activation when cells were grown in glucose. Importantly, there was very little difference in the overall intensity of expression compared to glucose. We also tested a P_{comR}-*gfp* reporter strain and noted no difference in expression of this gene when cells were stimulated with CSP or when the carbohydrate source was altered (Fig. 9C), similar to the results seen with the P_{comR}-*lacZ* reporter (data not shown). Finally, as the activation of the *comS* gene results in the production of a positive-feedback loop that leads to stimulation of *comX* expression, we wanted to investigate if the carbohydrates tested altered the initial stimulation of the *comS* promoter. To accomplish this, we moved the P_{comS}-*gfp* reporter plasmid into a *comS*-deficient strain. This strain presumably receives signals from CSP stimulation, but it is not capable of producing ComS, and subsequently XIP, to activate the positive-feedback loop. When we grew the *comS* P_{comS}-*gfp* strain in TVB supplemented with 20 mM glucose, 20 mM fructose, or 10 mM trehalose and added CSP, we did not observe activation of the *comS* promoter, and there were no differences in expression among the carbohydrates tested (see Fig. S8 in the supplemental material). However, robust expression of *comS* was observed when the strain was grown in defined medium, FMC (53) supplemented with 20 mM glucose, after XIP stimulation, indicating that the reporter was functional and the ComR-XIP pathway was operational in these cells.

DISCUSSION

Growth on the monosaccharide fructose and disaccharides maltose, trehalose, or sucrose altered CSP-dependent activation of competence gene expression in the dental caries pathogen *S. mutans* compared to that in cells grown on glucose. One key finding in this study was that deletion of the cognate PTS EII enzymes for

each enhancing carbohydrate essentially eliminated the increased expression of *comX* for cells grown on fructose, maltose, or trehalose (Fig. 7). There was also a dramatic reduction in the degree to which sucrose enhanced *comX* responsiveness to CSP in a strain lacking the primary PTS permease ScrA, but the decrease was not as dramatic as for other permeases; presumably because sucrose and fructose liberated from sucrose extracellularly can be internalized by other PTS permeases. Notably, no enhancement in *com* gene expression was seen with sugars that are internalized exclusively by ABC transporters, and deletion of an essential gene for the function of the multiple sugar metabolism (*msm*) ABC, *msmE*, had no effect on *comX* induction in TVB media supplemented with glucose, fructose, or trehalose (data not shown). Furthermore, while fructooligosaccharides and fructose were capable of enhancing *comX* expression, the trisaccharide raffinose, which contains fructose and is internalized by the *msm* transporter, did not alter the induction of *comX* promoter activity (data not shown). Collectively, then, PTS-dependent internalization of the carbohydrates by their cognate PTS permeases appears to be necessary for the modification of *com* gene expression stimulated by exogenous CSP.

In the absence of its cognate sugar, PTS EII enzymes generally remain phosphorylated, and during carbohydrate transport, the pool of unphosphorylated EII enzymes increases. We have previously demonstrated that certain EII enzymes, when participating in carbohydrate transport and predominately unphosphorylated, can profoundly influence the expression of a wide variety of genes in *S. mutans* (49). It is therefore possible that the specific PTS permeases studied here are able to exert an effect, directly or through other regulatory pathways, on competence gene expression and transformability. In addition to possible influences by unphosphorylated EII enzymes, the impact on *com* gene expression and transformation may be associated with the presence of particular phosphorylated metabolic intermediates, thus explaining the need for both PTS sugars and permeases to elicit the enhancement in CSP-dependent upregulation of *comX*. However, the various sugars used in this study, when internalized by the PTS, yield multiple different phosphorylated intermediates. In the case of glucose, this monosaccharide is transported into the cell and phosphorylated to produce glucose-6-phosphate, which, as part of the Embden-Meyerhoff-Parnas pathway, is isomerized to fructose-6-phosphate and then phosphorylated to form fructose-1,6-bisphosphate and further metabolized. Unlike for glucose, uptake of fructose via the PTS yields fructose-1-phosphate or fructose-6-phosphate (54). These sugar phosphates are then acted on by 1-phosphofructokinase or 6-phosphofructokinase, respectively, to produce fructose-1,6-bisphosphate. In either case, glucose-6-phosphate levels in cells growing on fructose would be much lower than those in cells growing on glucose. Maltose is a disaccharide composed of two glucose moieties in an α-1,4 linkage and is phosphorylated during transport to form maltose-6-phosphate. Traditionally, it has been thought that maltose-6-phosphate is acted on by a maltose-6-phosphate hydrolase, probably encoded by SMU.2046c (55), to produce glucose and glucose-6-phosphate, and the molecule of glucose can be subsequently phosphorylated by glucokinase. Recently, Sato and colleagues proposed a mechanism for maltose metabolism involving glucose expulsion and uptake of free glucose by the cell (56). Thus, glucose-6-phosphate levels should be high in cells growing on maltose, compared to fructose. The disaccharide trehalose is also composed of two glucoses moieties, but in an α-1,1 linkage. Internalization via a PTS porter results in the production of trehalose-6-phosphate, and this intermediate is cleaved to form glucose and glucose-6-phosphate by the action of trehalose-6-phosphate hydrolase, apparently encoded by SMU.2037 (*treA*). Finally, *S. mutans* encodes a multitude of enzymes capable of metabolizing sucrose; several extracellular enzymes generate glucose and fructose polymers from sucrose, while sugar transporters, primarily the PTS permeases ScrA and TreB, internalize sucrose to produce sucrose-6-phosphate. Sucrose-6-phosphate is split into fructose and glucose-6-phosphate via the activity of the sucrose-6-phosphate hydrolase, ScrB, encoded by SMU.1843. The free fructose could be phosphorylated by the action of a fructokinase, although recently it was shown that the fructose moiety from sucrose-6-phosphate may be expelled from cells, where it could be acted on by several PTS enzymes for reuptake as fructose-1-phosphate or fructose-6-phosphate (57). It becomes evident, therefore, that the enhancements in the competence pathway observed for the sugars of interest cannot easily be explained by the generation of a particular glycolytic intermediate or sugar phosphate.

Of note, we have conducted some preliminary experiments to explore whether a balance in sugar phosphate levels is required for optimal *comX* expression. Specifically, we attempted to disrupt the pools of intermediates generated via trehalose metabolism by overexpressing *treA* using the expression vector pIB184, which would presumably accelerate the conversion of trehalose-6-phosphate to glucose and glucose-6-phosphate. Notably, we did not observe an impact on *comX* expression or growth in trehalose compared to the vector-only control (data not shown). It is, however, an important observation that a *ccpA* mutant displayed altered competence phenotypes when grown on the carbohydrates tested. CcpA serves as an important regulator of multiple virulence attributes of *S. mutans* and of pathways that control carbon flow and the utilization of a variety of carbohydrates (58). Interestingly, the entanglement of genetic competence pathways and CCR has also been observed in the gammaproteobacteria *Vibrio cholerae* and *Haemophilus influenzae*, where it was demonstrated that the major regulator of catabolite repression, cyclic AMP (cAMP) receptor protein (CRP), and its cofactor, cAMP, are absolutely required to achieve transformation (59–61). Current evidence suggests that the CRP/cAMP complex directly activates the promoters of *com* genes in these organisms (62, 63), through binding of catabolite response elements (CREs), which importantly, does not appear to be the case for CcpA in *S. mutans*. However, it was notable that the inclusion of PTS sugars in the growth media reduced transformation efficiency in *V. cholerae*, and this was proposed to result from an accumulation of unphosphorylated EII enzyme (due to the transfer of phosphate groups to incoming sugars) and a reduction of phosphorylated EII species (59). Importantly, it is the phosphorylated EII enzymes that stimulate adenylate cyclase (CyaA), producing cAMP, and this decrease of cAMP, resulting from transport by the PTS, diminished the affinity of CRP for *com* gene promoters. In short, signals of the environmental nutrient status (via pools of EII enzymes) can modulate the abundance of a cofactor for a major CCR regulator and subsequently regulate competence initiation. In *S. mutans*, engagement of CcpA with its target operons is strongly dependent on the levels of fructose-1,6-bisphosphate and possibly glucose-6-phosphate, which enhance HPr kinase activity to increase the levels of serine-phosphorylated HPr, the cofactor for CcpA binding.

Consequently, the rate of metabolism of the sugars that enhance competence, which would impact the pools of key glycolytic intermediates, may affect competence phenotypes via CcpA. The fact that competence is sensitive to growth phase, where carbohydrate availability, growth rate, and pH can vary widely, may add further support to the idea that sugar phosphate pools, and possibly associated factors, like ATP levels or NAD/NADH balances, act as important signals to integrate the competence decision network with cellular physiology.

It is also of interest that enhanced *com* gene expression in response to CSP was observed for *S. mutans* cultures grown with fructose or maltose but that the transformation efficiency of the organism was only altered when the disaccharide sucrose or trehalose was used as the primary carbohydrate source. We hypothesize that these particular carbohydrates are capable of enhancing transformability due to evolutionary adaptations associated with the unique roles and influences that these carbohydrates can have on oral biofilms (see Fig. S9 in the supplemental material). In the case of sucrose, *S. mutans* produces no fewer than seven extracellular enzymes capable of metabolizing this common dietary carbohydrate. Three glucosyltransferase enzymes utilize sucrose as a substrate for the construction of a largely water-insoluble exopolymeric matrix that facilitates bacterial adherence and accumulation, and these enzymes contribute in major ways to development of dental caries on smooth surfaces (64). As sucrose is metabolized, *S. mutans* and other dental plaque-forming microorganisms produce thick, heterogeneous biofilms, and it is likely that nutrient diffusion becomes restricted. Such conditions may allow for accumulation of CSP, which in turn would activate the mutacins of *S. mutans*, lysing sensitive species and releasing substantial quantities of extracellular DNA (eDNA) into the environment. Under those conditions, progression to competence would be beneficial to *S. mutans* because there would be considerable quantities of eDNA available as a nutrient or for genome diversification. While admittedly somewhat more speculative than the rationale we propose for why sucrose enhances transformability, we note that trehalose is accumulated by many bacteria in response to stress (65), especially osmotic stress, although this does not appear to be the case for streptococci. Thus, the enhanced transformation of *S. mutans* in the presence of trehalose may reflect an evolutionary adaptation in which the presence of trehalose confers a signal that nearby bacteria have lysed and released cellular contents, including DNA.

One important observation arising from this study is that *comS* expression can be enhanced by the same carbohydrates that influence *comX* activation. This is a critical finding because signaling through CSP-ComDE does not directly activate *comX* expression, and CSP-dependent activation of *comS* is bimodal (occurs only in a subpopulation). The model that is most consistent with the available data for cross talk between CSP and competence, and for the bimodality, posits that CSP exerts its influence on competence through the ComRS circuit. It is also important to note that XIP reinternalization triggers a positive-feedback loop to activate competence, as evidenced by enhanced expression of *comS* in cells treated with either CSP or XIP. Thus, we incorporate here a hypothesis for how carbohydrate source may influence the competence behaviors of *S. mutans*. As described by Son et al., two prerequisite assumptions must be made by the model in order for the bimodality of this auto-feedback circuit to be achieved: (i) stochastic gene expression in *comR* and/or *comS* and (ii) either higher-order multimer formation by ComR/XIP (or ComR/S) complex or a higher cooperativity term for the complex activation of the *comS* promoter (35). Many studies have shown the stochastic nature of gene expression (66, 67), and recent work by Parashar et al. has demonstrated that Rgg proteins, which include ComR, form multimers (68). After considering these assumptions, we evaluated parameters of this model (see Table S3 in the supplemental material) that, when altered, could elicit similar changes to the ComRS circuit as observed after growth in fructose or trehalose (i.e., a decrease in the threshold for ComRS activation). Our results exclude certain possibilities. For example, the data do not support that the type of carbohydrate in the growth medium influences the basal level of expression of *comS* (see Fig. S8 in the supplemental material). Additionally, we have excluded simulations where the intensity of *com* gene expression (number of GFP^+ pixels per cell) is significantly affected, as these possibilities are contrary to our observations. That is, in our single-cell experiments only the subpopulation of responding cells was altered and not the magnitude of *com* gene expression within individual, activated cells. After eliminating these alternatives, we adjusted parameters of the model (see Table S3) representing certain parts of the ComRS circuit by 40%, chosen to represent a large change in *com* gene expression (growth in trehalose) or 20%, which represents an intermediate change in gene expression (growth in fructose), with baseline values for the parameters representing growth on glucose. We found that decreasing the value of the terms representing the degradation of ComR or ComS or reducing the dissociation constant for the formation of the ComR/XIP (or ComR/S) complex or for the binding of this complex to the *comS* promoter by the percentages indicated lowered the threshold for positive-feedback activation, which would result in a larger fraction of cells activating *comS*. Among the parameters tested, a decrease in the degradation rate of ComS or the dissociation constant for formation of the ComR/XIP complex resulted in the greatest impact on the threshold (Fig. 10). Such modeling of the possible impact of carbohydrates on competence after CSP stimulation is helping to guide ongoing experiments to probe the underlying mechanisms in more detail.

In conclusion, recent studies have demonstrated the importance of environmental influences on the signaling pathways that control genetic competence in *S. mutans*. We present evidence here that specific carbohydrates affect the connection of the CSP-ComDE circuit with *com* gene expression and transformability. Furthermore, the PTS EII permeases responsible for internalizing these carbohydrates were required to observe increases in *com* gene expression and competence. Analysis of populations at the single-cell level revealed that growth on certain carbohydrates resulted in an increase in the proportion of cells capable of activating *comX* and *comS* in response to CSP. Experimental evidence and mathematical modeling support that the change in subpopulation response arises from a decrease in the threshold for activation of the ComRS circuit. Collectively, the results illustrate the intimate link between metabolism and competence activation in *S. mutans*. Further investigation is warranted to explore in more detail the molecular mechanisms by which carbohydrate uptake modifies the signaling pathways of genetic competence.

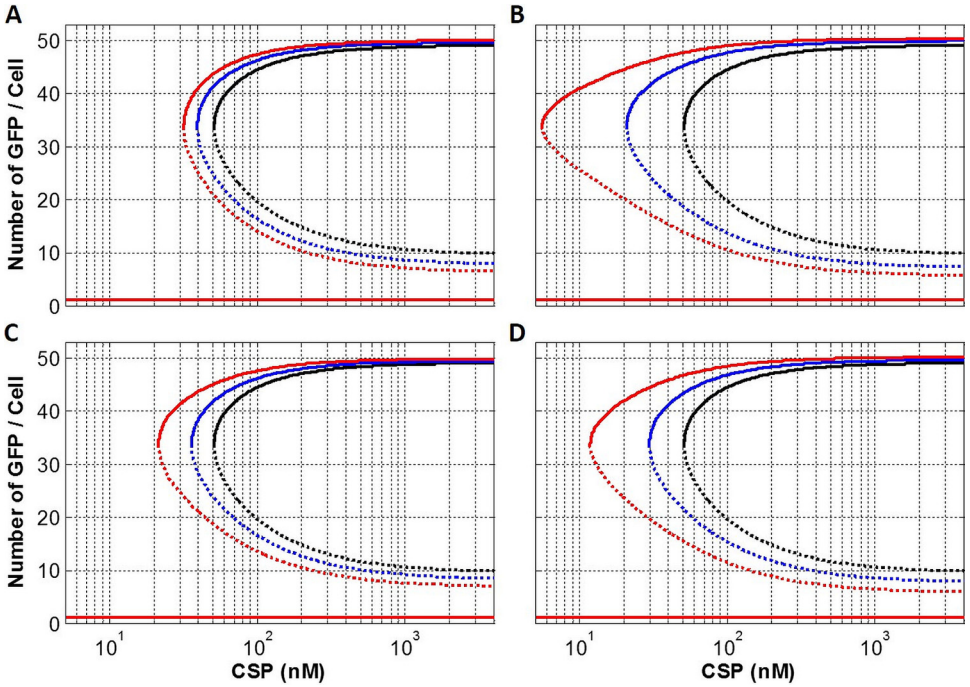

FIG 10 Effect of different carbohydrate sources on a deterministic simulation for the ComR/S circuit. Each panel shows the hysteresis (the "S" shaped curve) from the ComR/S auto-feedback circuit. Each curve is composed of two solid lines (two stable states, bimodal) and one dotted line (unstable state, a threshold to activate the bimodal circuit). To evaluate the most probable mechanism, we simulated the proposed mechanisms for which the degradation rate of ComR (A), the degradation rate of XIP (or ComS) (B), the dissociation constant for ComR/XIP (or ComR/S) complex inducing P*comS* (C), or the dissociation constant for ComR/XIP (or ComR/S) complex formation (D) is decreased by 0% (glucose [black line]), 20% (fructose [blue line]), and 40% (trehalose [red line]). See the text for more details.

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