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## MULTIPLE SUGAR:PHOSPHOTRANSFERASE SYSTEM PERMEASES PARTICIPATE IN CATABOLITE MODIFICATION OF GENE EXPRESSION IN *STREPTOCOCCUS MUTANS*

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### Summary

*Streptococcus mutans* is particularly well-adapted for high-affinity, high-capacity catabolism of multiple carbohydrate sources. *S. mutans* EII<sup>Lev</sup>, a fructose/mannose permease encoded by the *levDEFG* genes, and *fruA*, which encodes a hydrolase that releases fructose from fructan polymers, are transcriptionally regulated by the LevQRST four-component signal transduction system. Here, we demonstrate that (1) *levDEFGX* are co-transcribed and the *levE/F* intergenic region is required for optimal expression of *levFGX*; (2) D-mannose is a potent inducer of the *levD* and *fruA* operons; (3) CcpA regulates *levD* expression in a carbohydrate-specific manner; (4) deletion of the genes for the fructose/mannose-EII enzymes of *S. mutans* (*manL*, *fruI*, and *levD*) enhances *levD* expression; (5) repression of the LevQRST regulon by EII enzymes depends on the presence of their substrates and requires LevR, but not LevQST; and (6) CcpA inhibits expression of the *manL* and *fruI* genes to indirectly control the LevQRST regulon. Further, the *manL*, *ccpA*, *fruI/fruCD* and *levD* gene products differentially exert control over the cellobiose and lactose operons. Collectively, the results reveal the existence of a global regulatory network in *S. mutans* that governs the utilization of non-preferred carbohydrates in response to the availability and source of multiple preferred carbohydrates.

### Keywords

Sugar:phosphotransferase system;  $\beta$ -D-fructosidase; Catabolite repression; CcpA; Gene regulation

### Introduction

Carbohydrate metabolism by *Streptococcus mutans*, the most common etiologic agent of dental caries, is intimately linked to persistence, growth and pathogenesis by this organism. The phosphoenopyruvate (PEP):sugar phosphotransferase system (PTS) of *S. mutans* catalyzes high-affinity and high-capacity internalization of a wide variety of carbohydrates (Ajdic and Pham, 2007; Vadeboncoeur and Pelletier, 1997). In addition, the production of extracellular polysaccharides from sucrose enhances colonization, biofilm formation and virulence of the organism. *S. mutans* produces three glucosyltransferase enzymes that convert dietary sucrose into extracellular homopolymers of glucose (glucans), which function as an adhesive scaffolding for biofilm formation (Banas, 2004; Kuramitsu, 2003). The organism also produces an extracellular fructosyltransferase enzyme that converts sucrose into fructose homopolymers (fructans), which rapidly accumulate in oral biofilms and serve primarily as a storage polymer. Metabolism of fructan polymers by a secreted

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fructosidase, encoded by the *fruA* gene, enhances the amount and duration of acid production and, thus, the virulence of the organism (Burne *et al.*, 1987; Burne and Penders, 1992, 1994; Burne *et al.*, 1996; Burne *et al.*, 1999).

Expression of *fruA* is regulated by induction and is sensitive to carbohydrate catabolite repression (CCR) in the presence of rapidly catabolized sugars, such as glucose. A four-component signal transduction system regulates induction of the *fruA* gene of *S. mutans* (Zeng *et al.*, 2006). This complex includes LevQ and LevT, which are predicted to be extracellular sugar-binding proteins, and a two-component system consisting of the sensor kinase LevS and the response regulator LevR. All four gene products are required for the activation of *fruA*; and low concentrations of fructose, the end product of the enzymatic action of FruA on fructan polymers, serves as the inducing signal. We also noted that LevQRST controlled the activation of the genes for a fructose/mannose PTS Enzyme II complex, encoded by the *levD*, *levE*, *levF* and *levG* genes located immediately downstream of *levQRST*. Additionally, expression of *fruA* was enhanced in a strain lacking an intact LevD protein. Since then, our laboratory has also demonstrated that CCR of *fruA* can be exerted by an apparent homologue of catabolite control protein A (CcpA) in response to readily metabolizable hexoses via direct binding to a conserved catabolite response element (CRE) in the *fruA* promoter region (Abranches *et al.*, 2008).

A well-supported model for CCR in low-G+C Gram-positive bacteria has emerged that establishes the PTS as the central mediator of CCR through inducer exclusion and modulation of transcriptional regulation by CcpA (Postma *et al.*, 1993). The form of the general PTS protein HPr that is phosphorylated at serine residue 46 by HPr kinase can alter the activity of non-PTS permeases and function as a co-factor in the binding of CcpA to its target sites (Deutscher and Saier, 1983; Deutscher *et al.*, 1994; Fujita *et al.*, 1995). In addition to transporting sugars, the PTS, and in particular the mannose PTS permease in oral streptococci, influences multiple cellular functions; including regulation of the internalization of carbohydrates (Lieberman and Bleiweis, 1984), carbon catabolite repression (Abranches *et al.*, 2003; Abranches *et al.*, 2006; Gauthier *et al.*, 1990; Vadeboncoeur *et al.*, 1983), coordination of carbon and nitrogen metabolism (Chen *et al.*, 1998), and global control of gene expression (Abranches *et al.*, 2006). However, additional complexities in catabolite modification of gene expression have been noted in oral streptococci, such as *S. mutans* and *S. salivarius* (Gauthier *et al.*, 1997; Lieberman and Bleiweis, 1984; Vadeboncoeur and Pelletier, 1997; Ye *et al.*, 1996); including the discovery that PTS permeases other than EIIAB<sup>Man</sup> can exert negative control over gene expression in response to readily metabolizable carbohydrates (Wen and Burne, 2002; Zeng *et al.*, 2006). Results presented in this report reveal a complex regulatory network consisting of CcpA and four PTS permeases that comprise a hierarchical system for coordinating sugar catabolism by *S. mutans* in response to carbohydrate source and availability.

## Results

### Characterization of the genetic organization of the *levD* operon

RT-PCR was employed to demonstrate the co-transcription of the *levD*, *-E*, *-F*, and *-G* genes, and *Smu1776* (<http://www.oralgen.lanl.gov/>), which is designated here as *levX* because the function for this gene product has not been identified. Four pairs of specific primers (Fig. 1), designed to target DNA sequences encompassing adjacent open reading frames (ORF), were used in PCR reactions after reverse transcription with total RNA from *S. mutans* UA159. As shown in Supplementary Figure 1, these fragments were readily amplified in the test, but not the control samples, indicative that these 5 genes can be co-transcribed.

Quantitative real-time RT-PCR was utilized to measure the concentrations of the transcripts of each of the five genes. The results (Fig. S2, supplementary material) demonstrated that, for all conditions tested, the *levD-G* genes were transcribed at a similar level. Also, the amount of transcripts of the genes when fructose or mannose was used to culture the bacteria was approximately 10-fold higher than that found in cells grown in glucose, consistent with observations made using gene fusions (Table 1).

To further dissect the regulation of the *levD* operon, promoter-*cat* fusions were constructed using DNA sequences derived from the *levD* operon (Fig. 1). The intergenic region between the *levE* and *levF* genes, which is 374-bp, was used to engineer a fusion designated *PlevIGR-cat*. This construct produced no detectable CAT activity when tested in the wild-type background under conditions that activate the *levD* promoter (Table 1). However, when a larger fragment that included the *levD* promoter, the *levD* and *levE* genes, and the *levEF* intergenic region was fused to *cat* (*PlevDEF-cat*), CAT activities in the wild-type background were comparable to those expressed from *PlevD-cat* (Zeng *et al.*, 2006). Additionally, when tested in the *levR* mutant background, *PlevDEF-cat* produced minimal levels of activity. These data demonstrate that transcription of *levF*, and likely *levGX*, arises from the same promoter as *levD*, and that there is no promoter in the *levEF* intergenic region that is functional under the conditions tested.

To explore the potential role of the 374-bp *levEF* intergenic region in the expression of this operon, *PlevDEFΔIGR-cat* was constructed by deleting the intergenic region from the *PlevDEF-cat* fusion construct (Fig. 1). When assayed in the wild-type background, *PlevDEFΔIGR-cat* expressed reduced levels of CAT activity compared to the *PlevDEF-cat* fusion strain grown in TV-glucose or TV-fructose (Table 1). Future studies will explore the mechanism by which the *levEF* intergenic region affects expression of downstream genes.

### Mannose is a second induction signal for the LevQRST regulon

We previously noted that *S. mutans* cells cultivated in the presence of mannose displayed elevated mannose-PTS activity compared to cells grown in glucose, and that this enhanced activity required the presence of an intact *levR* gene (Zeng *et al.*, 2006). Further confirmation of this effect was noted in Figure S2 (supplementary material), where growth in mannose resulted in *levD* operon expression levels comparable to growth on the known inducing carbohydrate, fructose. To investigate the possibility that the LevQRST pathway was responsive to mannose, expression from *PlevD-cat* and *PlevDEF-cat* was monitored in the wild-type and *LevR*-deficient backgrounds (*levR*-/*PlevD-cat* and *levR*-/*PlevDEF-cat*, respectively) in cells grown to mid-exponential phase in TV supplemented with 0.5% of glucose, fructose or mannose (Table 1). In the wild-type background, *PlevD-cat* expression was increased by more than 100-fold in TV-mannose compared to cells grown in TV-glucose. CAT activity in the TV-mannose culture was about 2-fold higher than in cells grown in TV-fructose. The CAT activities measured from these gene fusions in the *LevR*-deficient strain were near the lower limit of detection of the assay. Similar results were obtained with strains lacking the *levQ*, *-S* or *-T* genes, which are other members of the signal transduction complex (Data not shown).

Since *fruA* expression is also under the control of the LevQRST regulon, CAT activity expressed from a *PfruA-cat* fusion (Zeng *et al.*, 2006) in the wild-type background was measured in cells grown to early exponential phase in TV-galactose medium after treatment with various concentrations of mannose for 3.5 hours. As shown in Fig. 2, mannose at concentrations as low as 0.01 mM was able to induce the expression of *PfruA-cat*, with activity plateauing at 0.5 mM and dropping at concentrations of 2 to 4 mM.

### Loss of CcpA results in altered expression of the *levD* operon

We recently reported that CcpA in *S. mutans* is a repressor of *fruA* expression in the presence of relatively high levels of glucose, fructose or mannose (Abranches *et al.*, 2008). However, the potential role of CcpA in regulating the *levD* operon has not been explored in detail. As presented in Table 1, a *PlevD-cat* fusion in a *ccpA* mutant background expressed much lower CAT activity in TV-fructose (~4-fold) or TV-mannose (~37-fold) than in the wild-type background, suggesting that CcpA is required for optimal expression of *levD-X* in the presence of inducer. Interestingly, deletion of *ccpA* also resulted in a modest up-regulation of *PlevD-cat* expression in glucose-containing medium, indicating that the effects of CcpA on the *levD* operon depend on the carbohydrate source.

### PTS EII enzymes negatively control *levD* expression

Deletion of the genes encoding the fructose-PTS enzymes II (*fruI*, *fruCD*) or mannose-PTS enzymes II (*manL*) results in derepression of *fruA* expression (Abranches *et al.*, 2006; Wen and Burne, 2002). Similarly, deletion of *levD* enhances expression from the *levD* and *fruA* promoters (Zeng *et al.*, 2006). Since *fruA* and *levD* depend on the LevQRST signal transduction system for activation, negative regulation of these two genes by EII enzymes may involve a common mechanism. As shown in Table 2, mutations in the genes for the fructose PTS permeases (*fruI*, *fruCD*), for the IIB domain of a mannose/fructose PTS (*manL*), and for the LevD mannose/fructose IIA<sup>lev</sup> domain, caused enhanced expression from *PlevD-cat* under inducing conditions.

Of particular interest, the effects of the loss of the various PTS permeases on expression of *fruA* and *levD* were specific to the carbohydrate source. Compared to the behavior of the gene fusions in the wild-type background, all mutations in the EII-encoding genes of interest resulted in elevated CAT activity expressed from the *levD* promoter in glucose-grown cultures, with the *fruI/fruCD* mutant having the greatest level of alleviation. However, when fructose was the growth carbohydrate, alleviation of *PlevD-cat* expression was seen in the *fruI/fruCD* and *levD* mutant backgrounds, but not in the *manL* mutant background. In contrast, dramatic derepression of *PlevD-cat* expression was noted in the *manL* mutant in mannose-grown cultures, whereas loss of *fruI/fruCD* elicited no derepression in mannose, and loss of *levD* caused a modest, albeit not statistically-significant, increase in expression in mannose. Importantly, mutation of the *ptsG* gene, encoding a product involved in maltose transport in *S. mutans* (Webb *et al.*, 2007), produced no derepression of *PlevD-cat*, regardless of whether maltose was present in the growth medium (Data not shown). Therefore, the effects seen with ManL, LevD, and FruI/FruCD are not observed with all PTS permeases.

### The PTS permeases exert CCR on the *fruA* operon

To demonstrate that the effects of the EII enzymes of interest on gene regulation and that the dependence on specific substrates was not a peculiarity of the *levD* operon, we tested the effects of ManL deficiency on *fruA* expression. When the *manL* mutant and the wild-type strain were compared for their response of *PfruA-cat* to fructose using a fructose-pulsing experiment (Table 3), there was much greater derepression of *fruA* associated with the loss of ManL in TV-glucose medium, compared to when the cells were grown under conditions that poorly induce CCR, i.e. in TV-galactose. When TV medium containing 0.5% fructose was used to culture these two strains, no alleviation of *fruA* expression was detected in the *manL* mutant. When TV-mannose was used to grow the cells, *fruA* expression was greatly derepressed in the *manL* mutant, compared to the wild-type background. Finally, alleviation of *fruA* expression in the *fruI/fruCD* mutant was only evident in TV-fructose cultures.

We further probed the roles of the different EII enzymes in catabolite-dependent regulation of carbohydrate utilization by comparing the growth curves of the *manL* mutant in TV-medium containing 0.5% of the fructose homopolymer inulin, which is a substrate for FruA, and 0.05% of glucose or fructose, using inocula prepared in glucose or fructose alone, respectively. As shown in Fig. S3 (supplementary material), diauxic growth was evident in the wild-type genetic background when cells were grown on a mixture of glucose and inulin. Consistent with the behavior of the gene fusion strains, deletion of *manL* diminished the extent of the diauxie. In contrast, when fructose was used instead of glucose as the repressing carbohydrate in these growth assays, the *manL* mutant presented a diauxic growth curve. Furthermore, when the *levD*, *fruI/fruCD* and *ccpA* mutants were used in the same experiment, their growth behaviors were also consistent with regulation of *fruA* by EII permeases (see Fig. S3 for details). Collectively, these observations provide further support that multiple EII enzymes, in concert with CcpA, modulate expression of the LevQRST regulon in a carbohydrate source-dependent manner.

### Derepression associated with loss of PTS permeases does not occur in a LevR-deficient strain

We examined whether the permeases of interest could exert their influence through the LevQRST signal-transduction pathway. To explore this possibility, additional mutants were engineered in the background of the strain carrying a *PlevD-cat* fusion, where mutations in *fruI/fruCD*, *levD* or *manL* were combined with deletions of the *levR*, *levS*, *levQ* or *levT* genes (Table 4). First, in the background of a *levR* mutant, introduction of mutations in *fruI/fruCD*, *manL* or *levD* failed to significantly alter the expression from the *PlevD-cat* fusion, although expression levels were very low because of the requirement for LevR for activation of the operons. However, in the *levQ* mutant, where a similar level of *PlevD-cat* expression was obtained as in the *levR* mutant, deletion of *manL* or *fruI/fruCD* resulted in apparent derepression of *PlevD-cat* expression, indicating that these EII enzymes can still exert negative control on the *levD* promoter in the absence of LevQ. Also, in the *levQ* mutant background, substantial derepression of *levD* expression was caused by mutation of *manL* only when mannose was used to culture the bacteria. Also consistent with data presented above, the *fruI/fruCD* mutation resulted in higher levels of expression when fructose was used to grow the cells, as compared with mannose. Results similar to that seen in the *levQ* mutant background were obtained when using the *levS* mutant that lacks the sensor kinase; i.e. expression of *PlevD-cat* was increased when *manL* or *fruI/fruCD* mutations were introduced into the *levS* mutant background. The results from the *levT* mutant were similar to that of the *levS* and *levQ* mutants, although an overall higher level of alleviation of expression from *PlevD-cat* expression was caused by the *manL* or *fruI/fruCD* mutations in the *levT* mutant background.

### CcpA regulates EII permease gene expression

Although CcpA appears to be required for optimal expression of *levD* (Table 1), we have been unable to demonstrate that a purified CcpA protein from *S. mutans* is able to bind the *levD* promoter region. Specifically, a recombinant His-tagged CcpA protein that binds the CRE in the *fruA* promoter (Abranches *et al.*, 2008) was used in an electrophoretic mobility shift assay with a ~0.3 kbp radio-labeled DNA fragment containing the entire *levD* promoter region, the same DNA fragment that was used to construct the *PlevD-cat* fusion. No interaction between CcpA and the probe was detected in this assay (Data not shown). Consistent with this observation, there are no CREs within 200-bp 5' or 3' of the *levD* promoter region, suggesting that direct binding of CcpA to the *levD* promoter may not occur *in vivo*.

We previously observed that deletion of *ccpA* resulted in major changes in the transcriptome and metabolic potential of *S. mutans* (Abranches *et al.*, 2008), so we explored whether CcpA was able to alter the expression of factors that govern *levD* and *fruA* expression. In particular, the expression of *fruI*, *fruCD*, *manL*, and *levQRST* was compared in the wild-type and CcpA-deficient strains. A *PmanL-cat* reporter fusion was constructed using the *manL* promoter and used to monitor the expression of *manL* in the *ccpA* mutant background and the parental strain. Cells were grown to mid-exponential phase in TV-base medium supplemented with 0.5% glucose, fructose or mannose, and harvested for CAT assays. The results (Fig. 3) indicated that *manL* expression was derepressed in the *ccpA* mutant 1.5- to 5-fold, with the highest level of increase seen in the TV-fructose cultures. This finding was confirmed when the levels of *manL* transcripts were compared in strain UA159 and the *ccpA* mutant using real-time quantitative PCR (Data not shown). Similarly, when the transcript levels of *fruI* or *fruC* were compared in UA159 and the *ccpA* mutant strain, significant increases in *fruC*, and especially *fruI* mRNA, were detected in the *ccpA* mutant grown in TV-fructose or TV-mannose (Fig. S4 of the supplementary material). For *fruI*, there was about a 4-fold increase in transcription in TV-fructose cultures due to deletion of *ccpA*, and about a 50% increase in TV-mannose cultures. However for *fruC*, the only increase, about 2-fold, came when the cells were grown in TV-mannose medium. Also noteworthy is that while transcription of both *fruI* and *fruC* was increased in cells cultured with fructose, compared to mannose-grown cells, the overall level of *fruI* transcript was about 3-logs greater than that of *fruC*. The significance of this observation in terms of carbohydrate transport remains to be determined.

The fact that very little derepression in *fruC* transcription was seen in the *ccpA* mutant prompted us to study the effects of the *fruCD* and *fruI* mutations on *levD* expression, separately. As shown in Table 2, a mutation in *fruI* alone resulted in an elevation of expression of *PlevD-cat* of a magnitude similar to that seen in the *fruI/fruCD* double mutant, whereas the *fruCD* mutation alone caused little change in *PlevD-cat* expression. The same trend was observed when *fruA* gene expression was analyzed in the individual *fruI* and *fruCD* mutants (Wen *et al.*, 2001), despite major differences in the growth media and in the conditions used to induce the operon in this study and that of Wen *et al.* (2001). Therefore, we conclude that FruCD does not play a significant role in the regulation of the *levD* or *fruA* operons under the conditions tested, and therefore the primary effectors of catabolite modification are ManL, FruI and LevD.

As FruI and ManL have been implicated in the negative regulation of *levD*, it is reasonable to interpret that reduced *PlevD-cat* expression in a *ccpA* mutant grown in fructose or mannose medium results from elevated expression of the PTS permeases, FruI and ManL. Indeed, the characteristics of expression of the *PlevD-cat* gene fusion in two additional mutants, *manL/ccpA* and *fruI/fruCD/ccpA*, support this idea (Table 5). In particular, a strain carrying a *fruI/fruCD* double mutation and lacking CcpA displayed dramatic repression of *PlevD-cat* activity in TV-mannose-grown cells, likely due to the elevated expression of *manL*. On the other hand, when the *ccpA* mutation was introduced into a *manL* mutant strain, a reduction in *PlevD-cat* expression was seen only in TV-glucose and TV-fructose cultures, presumably related to enhanced expression of *fruI*. It is also important to note that the expression of *levQRST* was not altered by the *ccpA* mutation (Data not shown), so the observed effects of loss of CcpA are not due to down regulation of the primary activator of *levD*.

### The role of the EII enzymes in catabolite repression is not restricted to the LevQRST regulon

A major question arising from these findings is whether the ability of ManL, FruI and LevD to exert catabolite control of gene expression is peculiar to the LevQRST regulon, or if this

is a general mechanism for repression of genes for the catabolism of non-preferred carbohydrates by glucose, fructose or mannose. In a previously published microarray study (Abranches *et al.*, 2006), a *manL* mutant strain (JAM1) showed markedly elevated expression of the cellobiose-PTS enzyme II component genes and *celA*, which encodes a 6-phospho- $\beta$ -glucosidase that is capable of hydrolyzing  $\beta$ -glucosides, including cellobiose.

We measured mRNA levels of *celC*, the gene encoding the domain A of the cellobiose-PTS Enzyme II, in strain UA159 and in the *manL* and *levD* mutants. As shown in Fig. 4A, expression of *celC* was elevated nearly 1000-fold in the *manL* mutant growing in glucose-cellobiose medium when compared to the wild-type background, whereas only a 3-fold increase was seen in fructose-cellobiose medium. On the other hand, loss of *levD* showed little change in the level of *celC* transcript, regardless of whether glucose or fructose was present in the medium. As the utilization of cellobiose by *S. mutans* requires both cellobiose-PTS activity and a 6-phospho- $\beta$ -glucosidase (*celA*), the level of *celA* expression was probed using a *PcelA-cat* fusion, constructed in a manner similar to that of the *PlevD-cat* fusion. As shown in Fig. 4B, inactivation of *levD* resulted in a slight, but significant, alleviation of expression of *celA* during growth in the presence of fructose and cellobiose, and only low levels of derepression of *celA* expression were noted in the strains lacking a single fructose-PTS enzyme, i.e., LevD, FruI or FruCD. However, a *levD/fruI/fruCD* triple mutant showed significantly increased *celA* expression compared to the wild-type background when fructose and cellobiose were present. Finally, the loss of ManL caused a dramatic elevation (~150-fold) in transcription of the lactose utilization operon in *S. mutans* (data not shown), as measured by quantitative RT-PCR of the mRNA for the *lacG* gene (coding for phospho- $\beta$ -galactosidase), adding further support to the idea that this permease participates in global control of CCR in *S. mutans*.

We also tested *manL* and *levD* mutants in a diauxic growth assay using TV broth supplemented with 0.05% of glucose or fructose and 0.5% of cellobiose. Results of these tests were consistent with the effect of loss of *manL* or *levD* on the expression of the *cel* locus, i.e. the *manL* mutant showed faster growth on cellobiose than the wild-type strain when cultured in TV-glucose-cellobiose, whereas the *levD* mutant displayed non-diauxic growth in TV-fructose-cellobiose (see Fig. S5, supplementary material). Similar results were obtained when cellobiose was replaced with lactose as the non-preferred sugar substrate (Fig. S6).

## Discussion

Our previous work on *fruA* and *levDEFG* (Wen and Burne, 2002; Zeng *et al.*, 2006) has established that a four-component regulatory system, encoded by the *levQRST* operon immediately upstream of *levD*, is the primary induction mechanism for the expression of both *fruA* and *levD*. In addition to the complexity of induction of these genes, *fruA* has proven to be a particularly useful model for studying CCR in *S. mutans*. One longstanding enigma associated with CCR in *S. mutans* arose from the observations that deletion of *ccpA* rarely resulted in increased expression of genes that were sensitive to CCR (Griswold *et al.*, 2006; Simpson and Russell, 1998; Wen and Burne, 2002). On the other hand, inactivation of *manL* did induce a loss of diauxic growth when *S. mutans* was cultivated on glucose-inulin medium (Abranches *et al.*, 2003). It is now established that CcpA can control CCR of *fruA* through CRE binding (Abranches *et al.*, 2003).

It was of interest that mannose was at least as effective an inducer of the LevQRST regulon as fructose. The reason why mannose would induce expression of the genes for a mannose transporter (LevDEFG) seems obvious, but the rationale for induction of the *fruA* operon by mannose is less clear. The *fruA* operon encodes a levanase/inulinase enzyme (FruA) and a

protein with strong homology to known  $\beta$ -fructosidases (FruB), although an enzymatic activity has not been ascribed to FruB. We have not detected an ability of *S. mutans* UA159 to utilize mannans, and a strain lacking FruB behaves like the parental strain in terms of mannose utilization (Zeng and Burne, Unpublished). Frequently, carbohydrate transporters that internalize fructose also internalize mannose, so it seems that the conservation of the response to mannose by LevQRST could be due to constraints that govern carbohydrate recognition by the complex. Analysis of the carbohydrate recognition capabilities of the four-component signal transduction system are ongoing.

Mannose was also able to repress *fruA* at concentrations above 4 mM, similar to what was noted for the pattern of *fruA* induction with fructose (Abranches *et al.*, 2008). Thus, it appears that CCR begins to affect expression of these operons when a readily metabolizable hexose is present at concentrations of about 3~4 mM. Repression at this concentration could involve CcpA, since mannose enters the Embden-Meyerhoff pathway after isomerization from mannose-6-phosphate to fructose-6-phosphate. Subsequent phosphorylation would yield fructose-1,6-bisphosphate, which activates HPr kinase to produce HPr-SerPO<sub>4</sub>, which in turn stimulates binding of CcpA to CRE (Deutscher *et al.*, 1995). As is demonstrated in this report, though, there are multiple CcpA-independent pathways for repression of the *fruA* and *levD* operons.

A central finding of this study is that inactivation of *manL*, *fruI* or *levD*, but not *ptsG*, relieved CCR of operons involved in use of non-preferred carbohydrate sources in a carbohydrate source-dependent manner. Based on these results, we propose that the substrate specificity of these permeases is a determining factor in their ability to negatively regulate catabolic operons. Specifically, the current evidence supports that FruI, FruCD, and LevD are mainly required for internalization of fructose, whereas ManL participates primarily in mannose and glucose transport (Abranches *et al.*, 2003; Wen *et al.*, 2001; Zeng *et al.*, 2006). Since derepression of *levD* expression was seen in glucose cultures of all mutants tested, except *ptsG*, we postulate that glucose could be a common substrate for these EII enzymes. This idea is supported by enzymatic assays that show changes in the transport of glucose in mutants defective in these particular permeases (Abranches *et al.*, 2003; Wen *et al.*, 2001; Zeng *et al.*, 2006).

A major question arising from this investigation is how the permeases elicit their effects on gene expression. The LevS sensor kinase, LevR response regulator and LevQT sugar binding proteins constitute the complete sensing complex of the LevQRST four-component system (Zeng *et al.*, 2006). One possible mechanism for negative regulation of *fruA* and *levD* operon expression could involve direct interaction of the permeases with LevR. In fact, only a LevR-deficient strain showed a lack of response to the elimination of ManL, FruI or LevD, in cells grown in the presence of the cognate substrates for these permeases. Although this finding may be due to the profound lack of activation of the operon in the absence of LevR, strains lacking LevQ, LevS or LevT showed differential expression of *fruA* and *levD* depending on whether *manL*, *levD* or *fruI* mutations were present. Thus, these results could indicate that the permeases, directly or indirectly, affect the activity or phosphorylation state of LevR. The lack of an apparent PTS regulatory domain (PRD) (van Tilbeurgh and Declerck, 2001) in LevR suggests that the effects in this particular case may not involve phosphorylation. It should also be noted that there were different degrees of derepression in the *levS*, *levT* and *levQ* mutants in response to the loss of ManL, FruI and LevD. Given that LevT and LevQ encode sugar binding proteins with substantial similarity to ABC transporters, the PTS permeases could fine tune the capacity of LevQST to efficiently sense inducers or phosphorylate LevR. Mechanisms for this modulation of activity could be similar to examples of PTS modification of the activity of non-PTS permeases with similarities to LevQ or LevT (Deutscher and Sauerwald, 1986; Romano *et*



*al.*, 1990). Thus, the simplest explanation for our data is that the dephosphorylated forms of the PTS permeases, which would be present if they are engaged in transport of their cognate sugar, act as direct effectors of CCR. In support of this idea, mutants that should have a constitutively phosphorylated ManL protein, by virtue of elimination of the EIIC<sup>man</sup> or by mutagenesis of the histidine residue in the B domain of ManL, also show alleviation of CCR (Zeng and Burne, Unpublished). Although beyond the scope of this communication, we are in the early stages of experiments to assess the potential for direct modulation of LevR activity or phosphorylation state by EII enzymes, and to assess whether the permeases could impact LevS-dependent phosphorylation of LevR through the LevQT sugar binding proteins, by purifying and reconstituting these systems for *in vitro* analysis.

The model for direct interaction of the permeases as a mechanism for exerting CCR may also apply to the cellobiose or lactose operons. For example, dephosphorylated permeases could interfere with CelR or LacR activity, or the permeases may cause inducer exclusion by negatively affecting the transport of cellobiose or lactose by the PTS permeases that are specific for these sugars, required for induction and encoded within the *cel* and *lac* operons, respectively (Sondej *et al.*, 1999). Alternatively, phosphotransfer from the permeases to the CelR or LacR regulators could influence DNA binding activity. Although CelR contains two conserved PRD domains, as well as an EIIA-like domain, LacR has no obvious potential phosphorylation sites. As above, *in vitro* reconstitution of these systems will be needed to clarify the underlying mechanisms. Given the heterogeneity of the systems involved, CCR by the permeases is likely to involve multiple mechanisms.

As an alternative to a direct influence of the permeases on regulatory proteins or transporters to effect CCR, it should be recognized that the phosphorylation state of the various permeases, which is dependent on the availability of their cognate substrates, could affect the phosphorylation state of Enzyme I or HPr. Similar to the Ser-46 phosphorylated form of HPr, HPr-His15-PO<sub>4</sub> has been documented to have multiple regulatory functions, including inducer exclusion and modulation of the phosphorylation state of transcriptional regulators and antiterminators (Deutscher, 2008; Vadeboncoeur and Pelletier, 1997). Thus, the EI-HPr circuit could be the primary pathway that integrates the information from the PTS porters examined in this study. Specifically, direct phosphorylation by EI or HPr, or allosteric interaction of certain forms of HPr with components of the regulatory proteins or transporters, could explain the ability of multiple carbohydrates and permeases to control CCR in *S. mutans*. The strongest piece of data arguing against a model where the permeases influence expression through EI or HPr is the finding that the inactivation of *ptsG*, which encodes a maltose transporter, does not affect CCR even when cells are grown on maltose. Still, more information is needed to draw firm conclusions.

In *B. subtilis* and some other organisms it has been possible to evaluate the role of EI and HPr in CCR *in vivo* by using mutants with deletions or defects in the *ptsH* (HPr) or *ptsI* (EI) genes, but such an approach may not be possible with *S. mutans*. Although an EI truncation mutant of *S. mutans* has been isolated that was unable to accumulate sugars via the PTS system (Cvitkovitch *et al.*, 1995), the mutant exhibited extremely slow growth and there were technical considerations that precluded use of such a mutant in these types of experiments. Specifically, the operons here are involved in catabolism or uptake of PTS sugars and, unlike *B. subtilis*, *S. mutans* lacks an electron transport chain for energy generation and depends entirely on sugar catabolism for growth. A variety of strategies to replace the entire coding sequence of the *ptsI* gene with an antibiotic resistance marker have yielded no viable mutants. Similar results were obtained when we tried to replace the HPr coding sequence using an antibiotic resistance gene cassette or to introduce a single copy of mutated versions of the *ptsH* gene. Therefore, if HPr or EI of *S. mutans* is directly involved

in CCR, it may be necessary to demonstrate their involvement using *in vitro* systems or a heterologous host.

In conclusion, this study has begun to fill critical gaps in our understanding of the complex regulation of carbohydrate catabolism in *S. mutans*, a major human pathogen responsible for what is perhaps the most common infectious disease of man. The results presented herein have provided substantial insights into the molecular basis for CcpA-independent CCR and revealed that multiple PTS permeases are utilized by *S. mutans* in a complex network that appears to be structured to allow the organism to monitor the availability of specific carbohydrates using PTS permeases, while monitoring overall carbohydrate flow through the glycolytic pathway via HPr kinase and CcpA (Warner and Lolkema, 2003). Recently, studies with Gram-positive pathogens have revealed the involvement of some catabolic regulators in control of major virulence factors; for example the link between HPr-Ser-PO<sub>4</sub> and virulence in *Listeria monocytogenes* and *Streptococcus pyogenes* (Deutscher *et al.*, 2005), and a demonstration of a role for CcpA in virulence regulation in both *Streptococcus pneumoniae* (Giammarinaro and Paton, 2002) and *S. pyogenes* (Almengor *et al.*, 2007; Shelburne *et al.*, 2008). Thus, detailed analysis of CCR in *S. mutans* may have broad relevance to carbon-source dependent virulence regulation in other important human pathogens.

## Experimental procedures

### Bacterial strains, growth conditions, and reagents

*E. coli* strains were grown in Luria-Bertani (LB) medium supplemented, when needed, with antibiotics at the following concentrations: ampicillin (Ap, 100 µg ml<sup>-1</sup>), kanamycin (Km, 40 µg ml<sup>-1</sup>), erythromycin (Em, 500 µg ml<sup>-1</sup>), spectinomycin (Sp, 50 µg ml<sup>-1</sup>), and tetracycline (Tc, 10 µg ml<sup>-1</sup>). *S. mutans* UA159 and its derivatives were maintained in brain heart infusion broth (BHI, Difco Laboratories, Detroit, Mich.) at 37°C in 5% CO<sub>2</sub> and 95% air with antibiotics added at the following concentrations: Km (700 µg ml<sup>-1</sup>), Em (5 µg ml<sup>-1</sup>), Sp (500 µg ml<sup>-1</sup>), and Tc (10 µg ml<sup>-1</sup>), when necessary. *S. mutans* cultures for CAT assay and growth rate tests were done in tryptone-vitamin-base (TV) medium (Burne *et al.*, 1999) with 0.5% of glucose, fructose, or mannose as the carbohydrate source. All chemical reagents and antibiotics were obtained from Sigma Chemical Co. Growth curves of *S. mutans* UA159 and mutant strains were generated using a Bioscreen C (Oy Growth Curves AB Ltd., Helsinki, Finland), with readings taken every 30 minutes.

### DNA manipulation

Standard recombinant DNA techniques were performed to engineer plasmids. All restriction and modifying enzymes were purchased from Invitrogen (Carlsbad, CA) or New England Biolabs (Beverly, Mass.) and used as recommended by the supplier. DNA purification was carried out using Qiaquick DNA purification kits purchased from Qiagen, Inc. (Valencia, CA). All primers (Supplementary Table S1) were synthesized by Integrated DNA Technologies (IDT), Inc. (Coralville, IA). The majority of the gene inactivations were carried out using the PCR-ligation-transformation technique, according to previously published protocols (Lau *et al.*, 2002). Alternatively, constructs for gene inactivation were created by inserting antibiotic-resistance markers into *S. mutans* DNA cloned on an appropriate vector plasmid, and then used in transformation of *S. mutans* strains. All mutants were confirmed by PCR followed by DNA sequencing, including determining the sequence of the chromosomal content of the arms used for recombination to ensure no undesired mutations were inserted during PCR or cloning. In some cases, strains carrying multiple mutations were generated by transformation with chromosomal DNA isolated from other

mutant strains of *S. mutans*, followed by selection for the appropriate antibiotic resistance and PCR confirmation.

Promoter-reporter gene fusions were constructed by inserting a promoter-containing sequence, including the cognate ribosome-binding site (RBS), into the integration vector plasmid pJL84 in front of a *cat* gene from *Staphylococcus aureus* that lacks a promoter and RBS (Zeng *et al.*, 2006). DNA sequences flanking the promoter-*cat* fusion were derived from the *mtlA-phnA* locus, which serves as the integration site after transformation into *S. mutans*. All gene fusions were confirmed by sequencing before being used to transform *S. mutans* and the correct conformation of the integration cassette was confirmed by PCR.

### CAT assay

Bacterial cells with various *cat* fusions were grown in TV-base medium, supplemented with various carbohydrates, to exponential phase ( $OD_{600} = 0.3$  to  $0.4$  for fructose or glucose cultures,  $OD_{600} = 0.1$  to  $0.2$  for mannose cultures), washed once with the same volume of 10 mM Tris buffer (pH 7.8), and then disrupted in a Bead Beater for 30 sec, twice, with a 2-min interval on ice. The cell lysates were centrifuged at  $18,000 \times g$  for 10 min and the supernates were recovered and used for measuring CAT activity by the method of Shaw (Shaw, 1979). The concentration of protein in the lysates was determined by the bicinchoninic acid (BCA) assay (Sigma). CAT activity was expressed as nmoles of chloramphenicol acetylated  $\text{minute}^{-1} \times (\text{mg protein})^{-1}$ .

### RNA isolation, RT-PCR and quantitative real-time RT-PCR

Total RNA was extracted from *S. mutans* cultures by using RNeasy mini kit (Qiagen) according to a protocol adapted for small scale purification (Ahn *et al.*, 2005). Briefly, 10 ml of a mid-exponential phase bacterial culture was harvested, treated with RNAProtect reagent at room temperature for 5 min, and then resuspended in 250  $\mu\text{l}$  of TE (50/10) buffer. A solution containing 5  $\mu\text{l}$  of 20% SDS, 300  $\mu\text{l}$  of acid phenol and 250  $\mu\text{l}$  of glass beads was added to the cells, followed by 30 seconds of bead beating, twice, with a 5 min interval on ice. After 10 min of centrifugation at 14,000 rpm at  $4^{\circ}\text{C}$ , 200  $\mu\text{l}$  of the supernatant fluid was removed from the tube and processed with the RNeasy mini kit, including an on-column DNase I (Qiagen) treatment, according to protocols provided with the kit.

For RT-PCR and quantitative real-time PCR, cDNA templates were generated from 2  $\mu\text{g}$  of total RNA with random hexamers using the Superscript III first-strand synthesis system (Invitrogen) according to instructions from the supplier. At same time, a cDNA control was generated for each sample by omitting the reverse transcriptase in the RT reaction. For RT-PCR assays, specific primers were designed to amplify each DNA fragment overlapping two adjacent open-reading frames, with UA159 chromosomal DNA, cDNA control and cDNA test samples serving as the PCR templates. Real-time PCR reactions were carried out using an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) and iQSYBR green supermix (Bio-Rad), according to the standard protocol provided by the supplier. Three individual cultures from each strain were used to prepare total RNA and cDNA, and triplicates were included in subsequent reactions for each cDNA sample, along with cDNA control and standard DNA generated with a PCR reaction using the specific primers for each target gene.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

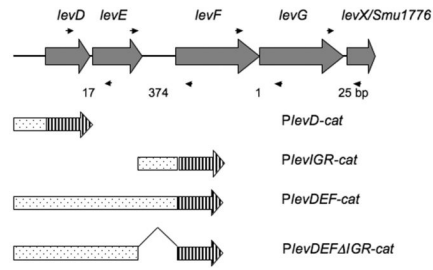
This work was supported by DE12236 from the National Institute of Dental and Craniofacial Research. Lin Zeng is supported by T32DE007200 training grant from Department of Health and Human Services.

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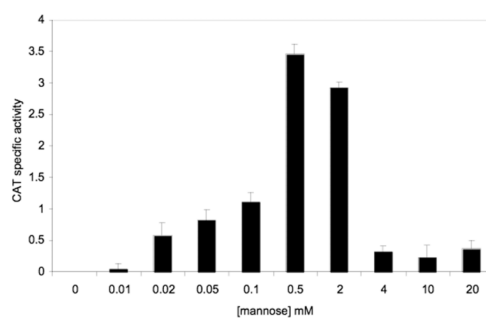
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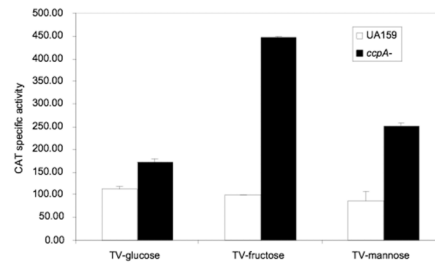
**Figure 1.**

Schematic representation of the *levD* operon in *S. mutans* UA159. The *cat* gene is represented by a barred arrow while the fused fragments are shown as dotted bars aligned with their locations in the *levD* operon. The size of each intergenic region is indicated by numbers (bp) below the diagram. Small arrows below and above the ORFs denote the primers used in RT-PCR reactions.



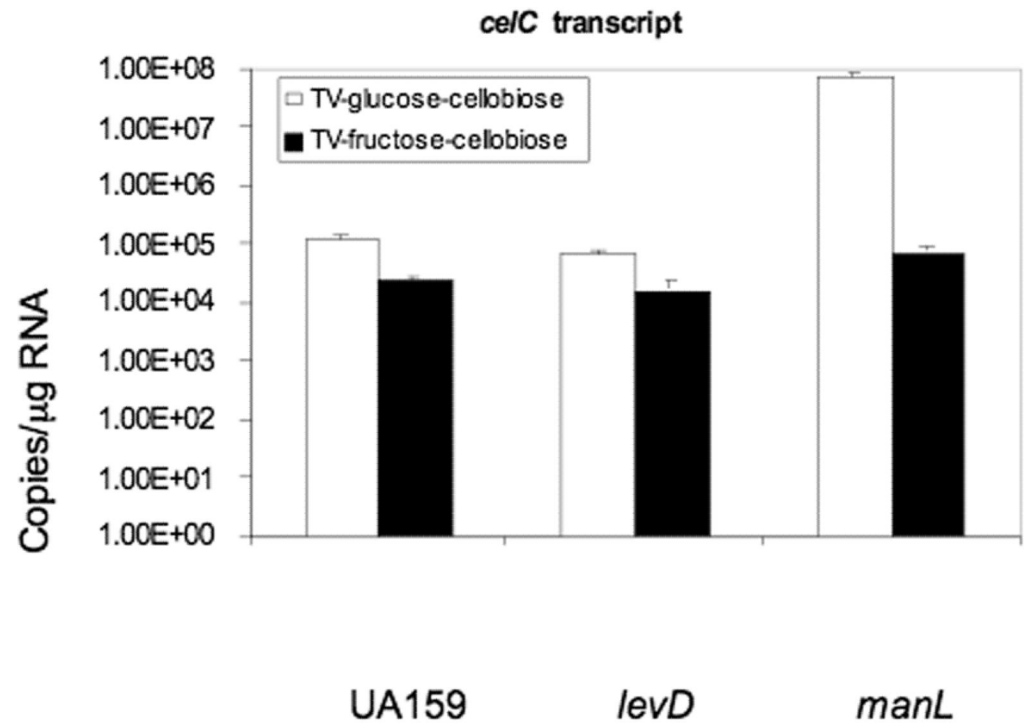
**Figure 2.** Induction of *PfruA-cat* by D-mannose. *S. mutans* strain UA159/*PfruA-cat* was grown to  $OD_{600} = 0.2$  in TV supplemented with 0.5% of galactose. Mannose was then added to final concentrations ranging from 0.01 mM to 20 mM and the cultures were incubated for an additional 3.5 hours before harvesting for CAT assays. The results represent the average of three independent cultures and are expressed as  $\text{nmole (mg protein)}^{-1} (\text{min}^{-1})$ .



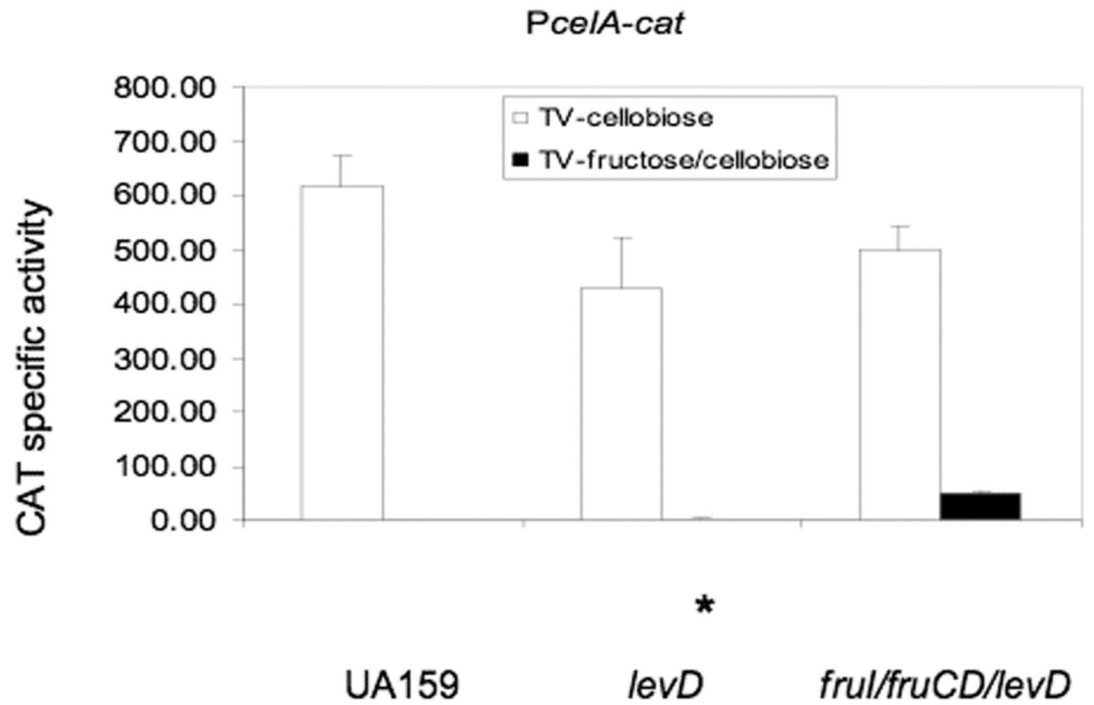


**Figure 3.**

Expression of *P<sub>manL</sub>-cat* in the wild-type (UA159) and *ccpA* mutant backgrounds. Cells were grown to mid-exponential phase in the specified media before being subjected to CAT assays. See experimental procedures for detail.

**A**

B



**Figure 4.** The transcript level of *celC* measured by qRT-PCR (A) and the CAT activity of *PcelA-cat* (B) in strain UA159 and various mutants. The asterisk indicates P-values of less than 0.05.

Table 1

Expression of *PlevD-cat* and its derivative fusions.

Strains	CAT specific activity <sup>a</sup>					
	TV-glucose	SD	TV-fructose	SD	TV-mannose	SD
UA159/ <i>PlevD-cat</i>	6.32	1.58	474.04	44.68	870.43	85.15
<i>levR-βPlevD-cat</i>	0.18	0.04	0.03	0.05	0.24	0.07
<i>ccpA-βPlevD-cat</i>	10.46	1.51	107.50	11.49	23.02	2.67
UA159/ <i>PlevDEF-cat</i>	2.35	0.32	545.71	28.54	1292.88	161.59
<i>levR-βPlevDEF-cat</i>	0.19	0.05	0.16	0.09	0.31	0.11
UA159/ <i>PlevIGR-cat</i>	0	0	0	0	0	0
UA159/ <i>PlevDEFΔIGR-cat</i>	1.54	0.84	235.65	9.90	849.03	74.12

SD, standard deviation.

<sup>a</sup> values (in the unit of (nmole (mg protein)<sup>-1</sup> min<sup>-1</sup>) represent averages of at least three separate experiments.

Table 2

Expression of *PlevD-cat* in various EII mutants.

Background	TV-glucose		PlevD-cat CAT specific activity <sup>a</sup>		TV-mannose		SD
	TV-glucose	SD	TV-fructose	SD	TV-fructose	SD	
UA159	6.32	1.58	474.04	44.68	870.43	85.15	
<i>levD-</i>	10.19	0.29	749.91	54.38	950.69	51.11	
<i>fruI-</i>	48.71	7.65	814.99	36.05	679.91	42.18	
<i>fruCD-</i>	1.01	0.40	455.67	8.05	698.23	64.28	
<i>fruI/fruCD-</i>	79.09	0.40	787.48	21.84	681.99	11.12	
<i>manL-</i>	21.92	1.60	458.59	5.26	2199.88	85.68	
<i>ptsG-</i>	6.65	0.39	394.39	9.36	698.07	53.13	

In this case, mutations in *fruI* and *fruCD* were studied in combination, since our previous work found that loss of either gene resulted in elevated *fruA* expression, while deletion of both had additive effects on derepression of *fruA* (Wen and Burne, 2002).

SD, standard deviation.

<sup>a</sup> values represent averages of at least three separate experiments.

Table 3

Expression of *PfruA-cat* fusion.

	Pulsed for 3.5 h with fructose at concentration (mM)				
	0 mM	0.05 mM	3 mM	5 mM	10 mM
0.5% glucose	0	0	0.157 (0.068)	0.079 (0.040)	0.180 (0.102)
<i>manL-</i>	0	0.189 (0.067)	3.017 (0.557)	0.313 (0.256)	0.195 (0.168)
0.5% galactose	0	2.490 (0.033)	2.484 (0.403)	1.147 (0.153)	1.083 (0.028)
<i>manL-</i>	1.102 (0.086)	2.615 (0.290)	2.684 (0.157)	1.227 (0.025)	1.074 (0.110)
	Grown to mid-exponential phase without fructose pulsing				
0.5% fructose			0.439 (0.056)		
<i>manL-</i>			0.458 (0.053)		
<i>fruL-fruCD-</i>			2.336 (0.123)		
0.5% mannose			0.253 (0.112)		
<i>manL-</i>			26.73 (2.59)		
<i>fruL-fruCD-</i>			0.206 (0.277)		

As CCR of *fruA* has been shown to be triggered at sugar concentrations at around 2 to 4 mM, we studied the expression of *PfruA-cat* in both the wild type (UA159) and the *manL* mutant backgrounds by adding fructose, at concentrations ranging from 50  $\mu$ M to 10 mM, to early-exponential phase cultures prepared using TV supplemented with 0.5% of glucose or the poorly-repressing substrate galactose, followed by 3.5 h of incubation at 37°C. A similar strategy was utilized to study CCR of *fruA* by CcpA in *S. mutans* (Abranches *et al.*, 2008). Also presented are CAT activities of *PfruA-cat* expressed in the wild type, the *manL*, and the *fruL/fruCD* mutant backgrounds, when cells were grown to mid-exponential phase with TV supplemented with 0.5% of fructose or mannose. Values are averages followed by standard deviations in parentheses of at least three independent experiments.

Table 4

Repression of *levD* expression by EII enzymes in strains lacking components of the LevQRST signal transduction complex.

Background	<i>PlevD-cat</i> CAT specific activity <sup>a</sup>					
	TV-glucose	SD	TV-fructose	SD	TV-mannose	SD
<i>levR-</i>	0.18	0.04	0.03	0.05	0.24	0.07
<i>levR-/levD-</i>	0.13	0.04	0.01	0.01	0.09	0.07
<i>levR-/manL-</i>	0.17	0.06	0.03	0.03	0.00	0.00
<i>levR-/fruL-/fruCD-</i>	0.15	0.12	0.13	0.11	0.02	0.01
<i>levQ-</i>	0.12	0.11	0.00	0.00	0.29	0.29
<i>levQ-/manL-</i>	0.90	0.06	0.19	0.06	12.47	2.61
<i>levQ-/fruL-/fruCD-</i>	1.14	0.34	2.54	1.17	0.56	0.15
<i>levT-</i>	3.06	0.02	0.14	0.03	1.18	0.63
<i>levT-/manL-</i>	32.24	1.91	0.45	0.08	536.93	19.80
<i>levT-/fruL-/fruCD-</i>	5.54	1.41	44.71	9.61	1.17	0.18
<i>levS-</i>	3.78	1.22	0.49	0.06	1.30	0.08
<i>levS-/manL-</i>	12.28	1.75	1.41	0.27	8.12	1.66
<i>levS-/fruL-/fruCD-</i>	9.34	0.43	8.38	0.61	1.61	0.04

SD, standard deviation.

<sup>a</sup> values represent averages of at least three separate experiments.

**Table 5**

Effect of loss of CcpA on expression of *PlevD-cat* in the background of *manL* or *fruL/fruCD* mutants.

Background	<i>PlevD-cat</i> CAT specific activity <sup>a</sup>					
	TV-glucose	SD	TV-fructose	SD	TV-mannose	SD
UA159	6.32	1.58	474.04	44.68	870.43	85.15
<i>fruL/fruCD-</i>	79.09	0.40	787.48	21.84	681.99	11.12
<i>fruL/fruCD-/ccpA-</i>	26.93	1.31	595.65	30.78	7.02	3.11
<i>manL-</i>	21.92	1.60	458.59	5.26	2199.88	85.68
<i>manL-/ccpA-</i>	1.52	0.25	34.21	9.18	1706.42	105.49
SD, standard deviation.						

<sup>a</sup> values represent averages of at least three separate experiments.