Analysis of cis- and trans-Acting Factors Involved in Regulation of the *Streptococcus mutans* Fructanase Gene (*fruA*)

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There are two primary levels of control of the expression of the fructanase gene (*fruA*) of *Streptococcus mutans*: induction by levan, inulin, or sucrose and repression in the presence of glucose and other readily metabolized sugars. The goals of this study were to assess the functionality of putative cis-acting regulatory elements and to begin to identify the trans-acting factors involved in induction and catabolite repression of *fruA*. The *fruA* promoter and its derivatives generated by deletions and/or site-directed mutagenesis were fused to a promoterless chloramphenicol acetyltransferase (CAT) gene as a reporter, and strains carrying the transcriptional fusions were then analyzed for CAT activities in response to growth on various carbon sources. A dyadic sequence, ATGACA(TC)/TGTCAATT, located at −72 to −59 relative to the transcription initiation site was shown to be essential for expression of *fruA*. Inactivation of the genes that encode fructose-specific enzymes II resulted in elevated expression from the *fruA* promoter, suggesting negative regulation of *fruA* expression by the fructose phosphotransferase system. Mutagenesis of a terminator-like structure located in the 165-base 5′ untranslated region of the *fruA* mRNA or insertional inactivation of antiterminator genes revealed that antitermination was not a mechanism controlling induction or repression of *fruA*, although the untranslated leader mRNA may play a role in optimal expression of fructanase. Deletion or mutation of a consensus catabolite response element alleviated glucose repression of *fruA*, but interestingly, inactivation of the *cpaA* gene had no discernible effect on catabolite repression of *fruA*. Accumulating data suggest that expression of *fruA* is regulated by a mechanism that has several unique features that distinguish it from archetypal polysaccharide catabolic operons of other gram-positive bacteria.

*Streptococcus mutans*, the principal etiologic agent of human dental caries, is able to synthesize and hydrolyze polymers of D-fructose, commonly known as fructans. The ability of this cariogenic bacterium to produce and degrade fructans enhances the survival of the organism during periods of nutrient starvation, results in prolonged production of organic acids through glycolysis, and has been shown to contribute directly to caries formation (4). Fructanase (FruA) is apparently the sole enzyme involved in utilization of β(2,1)- and β(2,6)-linked extracellular fructose polymers by *S. mutans* (5, 6). FruA is enzymatically similar to the levanase of *Bacillus subtilis* (11), attacking levens and inulins to liberate fructose as the only end product and cleaving sucrose into glucose and fructose (6). In *S. mutans*, *fruA* is cotranscribed with *fruB*, which lies immediately downstream of *fruA* and is predicted to encode a protein with strong similarity to bacterial β-fructosidases, although no function has yet been assigned to FruB (7). Previously, we reported that expression of *fruA* was inducible by growth on the homopolymers of fructose, levan, and inulin and was also exquisitely sensitive to carbon catabolite repression (CCR) (7; Z. T. Wen and R. A. Burne, Abstr. 100th Gen. Meet. Am. Soc. Microbiol., abstr. H66, p. 362, 2000).

In most cases, the genes for catabolism of nonpreferred carbohydrates and most polysaccharides are inducible by growth on the cognate substrate(s), and induction is frequently controlled by transcriptional activation or antitermination (35). For example, the levanase gene of *B. subtilis* requires the action of a sigma 54-like regulator that activates transcription when cells are growing with levens or low levels of fructose (22). Similarly, a variety of transcriptional antiterminator proteins with homology to BglG, the transcriptional antiterminator of the *Escherichia coli* bglGFB operon, are present in *Bacillus* (SacY, SacT, LicT, and GctT), *Lactobacillus casei* (LacT), *Staphylococcus aureus*, *Lactococcus lactis*, and *S. mutans* (BglG) (9, 10, 12, 14, 17, 19, 31, 32, 37), where they have been shown to control the expression of carbohydrate catabolism by allowing transcription of various operons. The binding site of antiterminator proteins, a ribonucleic antiterminator (RAT) sequence, has been well characterized in *B. subtilis* (2, 20). In many cases, the activity of these antiterminators is modified by both the general and sugar-specific components of the phosphotransferase system (PTS) (36). CCR of genes for catabolism of secondary carbohydrate sources in *Bacillus* and other gram-positive bacteria is mediated primarily through catabolite control protein A (CcpA), a member of the LacI-GalR family of bacterial transcriptional regulators (15). A palindromic sequence, the carbon catabolite response element (CRE), located in the promoter regions or coding sequences, is the target site for repression of genes exerted through CcpA (41). The histidine-containing phosphocarrier protein (HPr) of the phosphoenolpyruvate-dependent sugar:PTS, when phosphorylated by a fructose-1,6-bisphosphate-activated HPr kinase at serine residue 46, allosterically stimulates the binding of CcpA to CREs (25). Recently, CcpA-independent mechanisms for CCR that involve PTS-dependent phosphorylation of transcriptional activators and antiterminators have been reported for the levanase and licBCAH...
FIG. 1. Relevant nucleotide sequences and features of the 5′ region of fruA (positions −80 to 152 relative to the TIS). In boldface is the extended −10-like promoter that directs transcription of fruA. Boxed and labeled are two CREs, CRE-S (positions 2 to 15 relative to the TIS) and CRE-W (−26 to −14). Two inverted repeats, SL1 (positions 80 to 106) and SL2 (94 to 116), with potential to function as Rho-independent transcriptional terminators, are indicated with dashed arrows. Overlapping SL1 and SL2 and underlined is a sequence with 50% similarity to a RAT sequence (positions 76 to 104). A dyad sequence (DS) located at −72 to −59 relative to the TIS is a putative binding site of a positive regulatory protein. The start codon of fruA is indicated with an arrow.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All E. coli strains were grown in Luria-Bertani medium with or without inclusion of ampicillin (100 μg/ml), kanamycin (40 μg/ml), tetracycline (10 μg/ml), erythromycin (500 μg/ml), chloramphenicol (20 μg/ml), kanamycin and chloramphenicol (16 μg/ml each), or erythromycin and tetracycline (10 μg/ml each), when necessary. S. mutans UA159 strains were maintained on brain heart infusion medium, and when necessary, kanamycin (1 mg/ml), tetracycline (10 μg/ml), and/or erythromycin (10 μg/ml) was added. Preparation of competent cells and transformation of S. mutans were done as previously described (7). For enzyme assays and growth studies, S. mutans strains were grown on tryptone-vitamin-based (TV) medium (7) with similarly, but agar (Difco Laboratories, Detroit, Mich.) was added at a concentration of 1.5% (wt/vol).

CAT assay. For assay of chloramphenicol acetyltransferase (CAT) activity, S. mutans strains were grown in TV medium with the indicated carbohydrates, and early-log-phase cells (optical density at 600 nm = 0.2 to 0.35) were harvested by centrifugation at 3,800 × g at 4°C for 10 min. The cells were then washed once with 10 mM Tris buffer (pH 7.8), and cell pellets either were quickly frozen in an ethanol-dry ice bath and stored at −80°C until assays were to be performed or were immediately homogenized as described previously (7). The cell lysates were assayed for CAT activity using the method of Shaw (33). CAT activity was expressed as nanomoles minute−1 milligram of protein−1.

DNA manipulations. Unless otherwise stated, standard recombinant DNA techniques were performed as described previously (7, 30). All restriction and modifying enzymes and reagents were purchased from Gibco BRL Life Technologies, Inc. (Rockville, Md.), New England Biolabs (Beverly, Mass.), or MBI Fermentas (Amherst, N.Y.) and used as recommended by the supplier. For Southern hybridization analysis, 12 μg of genomic DNA was digested with enzymes and transferred to NewBond nylon membranes (NEN Life Science Products, Inc., Boston, Mass.). Probes were labeled with [α-32P]dATP using the Random Primers DNA Labeling System of Gibco BRL.

Strategy for dissecting cis-acting elements. Mutations or deletions were generated by using regular PCR and recombinant PCR (16). All primers (Table 1) were designed in such a way as to introduce a BamHI site at the 5′ ends and a PstI site at the 3′ ends of the PCR products for subsequent cloning purpose. The fruA promoter (PfruA), obtained as a 232-bp fragment upstream of the fruA start codon, or its derivatives generated by site-directed mutagenesis and/or deletions were directly fused with a promoterless CAT gene (cat) in plasmid pU17 (7). The correct nucleotide sequences of all PCR products were confirmed by sequencing. The transcriptional fusions were then released by partial Smal and HindIII digestions and inserted into EcoRV- and HindIII-digested pHBlueScript KS(+)+. The resulting plasmids were then digested with Smal and HindIII, and the DNA fragments with the gene fusions were gel purified and ligated into the integration vector pBGK (42) at the unique Smal site. Plasmid pBGK is an insertion vector constructed from the S. mutans gtfA gene and allows insertion of foreign DNA immediately downstream, with respect to the direction of transcription of gtfA, of a kanamycin marker flanked by strong transcriptional terminators. Clones carrying the fusions were selected on kanamycin- and chloramphenicol-containing medium and analyzed by BamHI and EcoRI digestions to determine the orientations of the transcriptional fusions with respect to that of gtfA gene on the vector. Although we have previously shown that orientation of the fusion had little effect on measured transcriptional activity, only those clones with orientations opposite to that of gtfA were selected for further study to ensure that readthrough from gtfA would not affect the results (42). The constructs were then used to transform S. mutans UA159 using procedures described previously (7). Transformants of S. mutans were selected on brain heart infusion containing kanamycin, and integration of the individual fusions into the chromosome as a result of double-crossover recombination was confirmed by PCRs (Table 1).
the membranes were then hybridized with radioactively labeled probes. Standard procedures (30). RNAs were UV cross-linked to the membranes, and nylon membranes (GeneScreen Plus; NEN Life Science Products, Inc.) by using concentration of 0.5% (wt/vol). Total RNAs were denatured and transferred to 0.4) in TV medium supplemented with the carbohydrate(s) of interest at a

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Application</th>
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<tr>
<td>ccpA5</td>
<td>5'-GACAGGATCTGAAAGGACACAGC-3'</td>
<td>ccpA amplification</td>
</tr>
<tr>
<td>ccpA3</td>
<td>5'-CTGGGATTACCTCAGATGAAATG-3'</td>
<td>ccpA amplification</td>
</tr>
<tr>
<td>FruA5</td>
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</tr>
<tr>
<td>FruA3</td>
<td>5'-TTTTCAATATATGAACTGACAAACTC-3'</td>
<td>FruA amplification</td>
</tr>
<tr>
<td>cat3</td>
<td>5'-CGGAAATCTGCTGATTCTACCTC-3'</td>
<td>Fusion confirmation</td>
</tr>
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<td>mtaA</td>
<td>5'-CTGCTGAAACCTGCTTTCTCAGCTTACCT-3'</td>
<td>Mutations of SL1</td>
</tr>
<tr>
<td>mtB</td>
<td>5'-GAACTGGAAGAGGATTACCTCAGATGAAATG-3'</td>
<td>Mutations of SL1</td>
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<tr>
<td>eW1</td>
<td>5'-GGGAAATCTGCTGATTCTACCTC-3'</td>
<td>Mutations of CRE-W</td>
</tr>
<tr>
<td>eW2</td>
<td>5'-CTCAGTATCCTGTTACCTCAGATGAAATG-3'</td>
<td>Mutations of CRE-W</td>
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<tr>
<td>DCs</td>
<td>5'-CTATCTTATATGAACTGACAAACTC-3'</td>
<td>Deletion of CRE-S</td>
</tr>
<tr>
<td>DT</td>
<td>5'-CTATTAGTTGCTCAGTATTTAAC-3'</td>
<td>Deletion of SL1 and SL2 and their 3' sequences</td>
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<tr>
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<td>Mutations of CRE-S</td>
</tr>
<tr>
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<td>Mutations of SL1 and SL2</td>
</tr>
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<td>Deletion of DS</td>
</tr>
<tr>
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<td>Amplification of PgtFC</td>
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<td>5'-GAGGATTACCTCAGATGAAATG-3'</td>
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</tbody>
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Quantitative RNA analysis. For slot blot analysis, total RNAs were prepared using a previously described procedure (7). Briefly, S. mutans UA159 and its derivatives were grown to mid-exponential phase (optical density at 600 nm = 0.2 to 0.4) in TV medium supplemented with the carbohydrate(s) of interest at a concentration of 0.5% (wt/vol). Total RNAs were denatured and transferred to nylon membranes (GeneScreen Plus; NEN Life Science Products, Inc.) by using standard procedures (30). RNAs were UV cross-linked to the membranes, and the membranes were then hybridized with radioactively labeled probes.

RESULTS

Analysis of the 5' UTR. Substrate induction by transcriptional antitermination, in which the antiterminator binds to the upstream region of the RNA hairpin stem of the intrinsic terminator transcript, protecting the elongation complex from destabilization (43), is well documented in gram-positive bacteria (23, 27, 43). Previously, we identified in the 5' UTR of the fruA transcript two overlapping stem-loops, designated SL1 at positions 80 to 106 (relative to the transcription initiation site [TIS]) and SL2 at positions 94 to 116, with the potential to function as transcriptional terminators (Fig. 1). Overlapping with SL1 (bases 76 to 104) was another dyadic sequence with 50% similarity to the binding site of the BglG family of transcriptional antiterminators, known as RAT sequences (2). CAT activity of the strain that carried the intact promoter-reporter fusion, Pfrua-CAT, was 34.56 ± 4.89 nmol min⁻¹ mg of protein⁻¹ in cells grown on inulin, 1.37 ± 0.65 nmol min⁻¹ mg of protein⁻¹ in cells grown on glucose plus inulin, and 0.43 ± 0.14 nmol min⁻¹ mg of protein⁻¹ in cells grown on glucose alone (Fig. 2). Deletion of the two stem-loops (SL1 and SL2) and the 3' region of the UTR (bases 78 to 152) did not affect the pattern of expression from the fruA promoter (DT-1 [Fig. 2]). When SL1 was destabilized by mutating the sequence of the stem region from TAAAAATTTCGCAGCAGCCAATTTTTA to acAcAgTTTTCGCAGCAGCCAATTTTTA (MP-I), no differences in expression from the fruA promoter were observed in cells grown on inulin, although there was a slight increase of CAT activity in cells grown on glucose plus inulin (Fig. 2) compared to the wild-type fusion. However, strain DP-1, carrying an internal deletion of SL1 and SL2 (bases 78 to 116), yielded only about one-third of the CAT activity of the strain that carried the Pfrua-CAT fusion (10.64 versus 34.56 nmol min⁻¹ mg of protein⁻¹ for the strain with Pfrua-CAT [Fig. 2]) when cells were growing on inulin as the sole carbohydrate source. CAT activity was barely detectable when glucose or glucose plus inulin was used to support growth.

As demonstrated by enzyme assays and reporter gene studies, FruA is strongly repressed in response to availability of rapidly metabolizable carbohydrates, including fructose and glucose (7) (Fig. 2). Consequently, effects of alterations in the DNA sequence of the 5' UTR on substrate induction may not have been apparent because of the dominant effect of CCR on fruA expression. To eliminate the effects of CCR on expression, the DNA for the 5'UTR beginning just 3' to CRE-S, and with the same 3' end as in the aforementioned constructs, was amplified by PCR and cloned behind the S. mutans gtfC promoter, which appears to be constitutively expressed (13). Plasmids carrying the intact fruA promoter Pfrua and the constructs MP-I and DP-1 (Fig. 2) were used as the templates in separate reactions, generating constructs that had deletions of the 5' portion of the 5' UTR (46 to ~80, relative to the TIS), including CRE-S and the fruA promoter, and that had the 3' region of this 5' UTR intact (G19), SL1 mutated (G20), or SL1 and SL2 deleted (G21), respectively (Fig. 2). These constructs were then fused with the gtfC promoter (13), which was amplified from S. mutans UA159 by PCR. In general, CAT activities of the strains carrying the truncated 5' UTR or its derivatives were lower than those of strains carrying the fusions that had the 5' region of the 5' UTR intact (data not shown), although the patterns of expression of cat were similar in all cases. Thus, the results obtained using fusions with mutations and deletions in the 5' UTR indicated that antitermination is not a primary mechanism for induction or repression of fruA. The significant decrease in CAT activity associated with dele-
tion of SL1 and SL2 indicates that these structures may be involved in stability of the fruA transcript.

A dyadic sequence upstream of the fruA promoter is required for fruA expression. Expression of fruA is inducible by substrate (7) (Fig. 2), with the highest activities measured when levans or inulins are the carbohydrate sources for growth. However, the 152-base 5'-UTR of the fruA transcript does not seem to have any major role in induction of fruA in response to growth on inulins or levans, as demonstrated by use of deletions and/or base substitutions of the putative transcriptional terminators and RAT-like sequence (Fig. 1). Consequently, we examined the region 5' to the promoter and identified an inverted repeat, ATGACA(TC)TGTCAT, designated DS (for dyadic sequence) and located at positions -72 to -59 relative to the initiation site of transcription. Deletion of this region (from -80 to -55) resulted in complete loss of expression of cat from the fruA promoter regardless of the sugar on which cells were grown (UP1-I and UP2-I [Fig. 2]). To verify the functionality of the dyadic sequence in fruA expression, DS was mutated to gatcgA(TC)TGTCAT by recombinant PCR. For that purpose, the intact fruA promoter, PfruA, was used as the template and oligonucleotide primers mDS and fruA3, or the primers mDS and dCS, were used in PCRs to generate two constructs: one with the full-length fruA promoter and a mutated DS and the other with a mutated DS and deletion of CRE-S and regions 3' to CRE-S (Fig. 1 and 2). These constructs were then fused with the promoterless cat gene and examined for CAT activity. No CAT activity could be measured with strains that carried the reporter gene fused with either one of the promoters containing mutations of DS (18-I
with CRE-S and its 3′ UTR deleted) integrated in the chromosome were grown on glucose-containing medium. However, CAT activity in the wild-type background was barely detectable when the cells were grown on glucose plus inulin as the sole carbohydrate source. CAT activity in the wild-type background was elevated by 50% when inulin was used as the sole carbohydrate source, but no significant differences were observed between the wild type and the fruCD mutant when the cells were grown on glucose or glucose plus inulin. Clearly, the sensitivity of the operon to repression by glucose exerted through CRE-S makes it difficult to distinguish between induction and alleviation of CCR. Therefore, to exclude the impact of catabolite repression exerted through CRE-S, the fruI, fruCD, and fruI fruCD mutations were introduced into a strain carrying the PfruACRE-′cat fusion, which had CRE-S and the 5′ UTR deleted (DC-I) (Fig. 2). Similar to what was seen with the wild type (Fig. 2) following inactivation of fruI or fruCD, expression of the PfruACRE-′cat fusion was increased by as much as fourfold when the cells were grown on inulin, compared to the PfruA-′cat fusion in the wild-type background (Table 2). Furthermore, CAT expression under the direction of PfruACRE-′cat in the fru and fruCD mutants was increased by 7- and 25-fold, respectively, when the cells were grown on glucose plus inulin and glucose alone, compared to CAT expressed from the same promoter in a wild-type background. In the fruCD fruI mutant background, CAT activity expressed from PfruACRE-′cat was further increased by 30 and 10% when the cells were grown on inulin-containing medium and glucose alone, respectively. Therefore, we can conclude that IPRi proteins act as negative regulators of fruA expression and likely control, at least in part, the induction of fruA by its substrates.

**Involvement of CRE sequences in CCR of fruA.** Two sequences, called CRE-S and CRE-W, for strong and weaker homology to the consensus sequence for CREs, were identified at positions 2 to 15 and 26 to 13 with respect to the TIS, respectively (7) (Fig. 1). Previously, by using plasmid-borne transcriptional fusions, we found that deletion of CRE-S and the entire 5′ UTR largely alleviated glucose repression of fruA expression (7). To confirm the potential role of CRE-S in CCR and to determine if CRE-W was also involved, the fusion PfruACRE-′cat was cloned into the integration vector pBGK (42) and integrated into the S. mutans chromosome in single copy. As shown in Fig. 2, deletion of CRE-S and the 5′ UTR (DC-I) resulted in about a 6-fold increase of CAT activity when the cells were grown on glucose, a 12-fold increase when they were grown on glucose plus inulin, and a 2.5-fold increase when they were grown on inulin, compared to the strain that carried the intact PfruA-′cat fusion grown under identical conditions, which further supports the notion that CRE-S is at least partially responsible for glucose-induced CCR of fruA.

According to Weickert and Chambliss (41), a palindromic with the consensus sequence TGWNNACGNTNWCA is the target site for CCR of many genes in B. subtilis. Some bases are more important than others, but it is the dyad symmetry that determines the strength of this element. The nucleotide sequence of CRE-S, which differs from the consensus at a single base pair, was mutated from AGATAGCGCTTACA to AGAGTCTACTAA; base changes in the selected positions have been shown to give maximal derepression of amyE in B. subtilis (41). CAT activity of the strain that had the construct with mutations of both CRE-S and SL1 (MM-I) was increased by sevenfold when the strain was grown on glucose, eightfold
when it was on glucose plus inulin, and twofold when it was grown on inulin, but mutations of SL1 alone (MP-I) had no major impact on cat expression (Fig. 2). These data further support the notion that CRE-S is a dominant element involved in CCR of *fruA*.

CRE-W, positioned at −26 to −13 relative to the TIS, differs from the CRE consensus sequence at positions 1, 13, and 14. Because it overlaps with the probable extended −10 promoter for *fruA* (7), the feasibility of assessing the functionality of this element without perturbing promoter recognition was uncertain. Notwithstanding this, specific primers were designed for recombinant PCR to introduce point mutations of the CRE-W element without altering key bases in the extended −10 promoter (28) (Fig. 1; Table 1). The resulting PCR product had the CRE-W sequence altered from AGATAGCGAT TTGG to AGAcgatacTTTGG. Compared to that in the strain carrying the *PfruA*-cat fusion, CAT activity in the strain carrying the mutated CRE-W was decreased by 50% when the cells were grown on glucose-containing medium (data not shown). Although base substitutions were designed so as not to physically alter the putative promoter sequence, it seems that the imposed mutations did in fact interfere with transcription, resulting in a decrease in CAT activity. Importantly, though, no alleviation of CCR was observed, indicating that CRE-W has either a secondary role or no role in CCR of *fruA*.

**Inactivation of ccpA** and its impact on glucose repression of *fruA*. In *Bacillus*, CcpA is found to bind CREs and to be primarily responsible for CCR (15, 41). We previously cloned a gene from *S. mutans* UA159 of 999 bp (Wen and Burne, Abstr. 100th Gen. Meet. Am. Soc. Microbiol.) and with a deduced amino acid sequence that had 52, 59, and 55% identity to the CcpA proteins of *B. subtilis*, *Lactobacillus pentosus*, and *L. lactis*, respectively (3, 15, 21). To assess the impact of the CcpA protein on expression of *fruA*, mutants were generated by using a spectinomycin resistance element to replace a small portion (nucleotides 56 to 687 relative to the TIS) of the *fruA* gene, and successful inactivation of the wild-type gene was achieved by double-crossover integration of the intact *fruA* promoter (*PfruA*-1) and its derivatives PfruAACRE (DC-I, PfruA with CRE-S and its 3′UTR deleted) and PfruAΔSL1-2′-cat (DT-I, PfruA with SL1 and SL2 and their 3′UTR deleted) (see Fig. 1 and 2 for more detail).

### Table 3. CAT activity of CCPA mutant strains of S. mutans UA159

<table>
<thead>
<tr>
<th>Strain</th>
<th>CAT activity (nmol min⁻¹ mg of protein⁻¹)</th>
<th>of strain grown on TV medium with:</th>
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<tr>
<td></td>
<td>Inulin</td>
<td>Inulin and glucose</td>
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<tr>
<td><em>S. mutans ccpAΔ/PfruA-I</em></td>
<td>31.23 ± 1.42</td>
<td>0.96 ± 0.41</td>
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<tr>
<td><em>S. mutans ccpA/PfruA-I</em></td>
<td>32.45 ± 3.01</td>
<td>1.25 ± 0.45</td>
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<tr>
<td><em>S. mutans ccpA/DC-I</em></td>
<td>82.06 ± 6.78</td>
<td>17.62 ± 3.02</td>
</tr>
<tr>
<td><em>S. mutans ccpA/DT-I</em></td>
<td>28.73 ± 2.48</td>
<td>0.94 ± 0.53</td>
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* The *ccpA* mutant strain was introduced with transcriptional fusions of the intact *fruA* promoter (*PfruA*-1) and its derivatives PfruAACRE (DC-I, *PfruA* with CRE-S and its 3′UTR deleted) and PfruAΔSL1-2′-cat (DT-I, PfruA with SL1 and SL2 and their 3′UTR deleted) (see Fig. 1 and 2 for more detail).

**DISCUSSION**

Substrate induction of carbohydrate catabolism in *B. subtilis* is mediated primarily by transcriptional activators and antitermination (35). Neither the putative transcriptional terminators (SL1 and SL2) nor the RAT-like sequence appears to play a significant role in regulation of *fruA* in response to carbohydrate source or availability. Further supporting this idea, in a search of the *S. mutans* UA159 genome database (www.genome.ou.edu/smutans.html) we have identified a gene that we have designated *smaT*. The *smaT* gene is predicted to encode a polypeptide of 281 amino acid residues with 44, 33, 30, and 30% identity to LicT of *B. subtilis*, BglG of *E. coli*, and SacY and SacT of *B. subtilis*, respectively. Inactivation of this gene by allelic exchange had no significant effect on growth on fructans or expression of PfruA'-cat fusion under all conditions tested (Wen and Burne, unpublished data). Inactivation of other transcriptional antiterminators in the genome also had no influence on *fruA* expression, further confirming that the role of the 5′UTR in the *fruA* mRNA is unrelated to attenuating transcription in response to carbohydrate source or availability.

Considering the fact that the 5′UTR does not have any role in regulation of *fruA*, it was logical to explore whether induction of *fruA* could be mediated through a target site located 5′ to the promoter. The data presented in this communication clearly show that a dyadic sequence positioned at −72 to −59 relative to the TIS is required for *fruA* expression and is probably the target for a transcriptional activator. In some ways, then, the *fruA* operon of *S. mutans* is regulated similarly to the levanase (*sacC*) operon of *B. subtilis* (22), albeit with some important differences. First, *sacC* induction requires LevR, which is a sigma 54-like regulator that is genetically linked to
sacC. No regulatory genes are linked to the fruA operon, nor is fruA transcribed from a −12/−24-like promoter. Also, there are no genes encoding PTS-like components in the fruA operon, as there are in the B. subtilis levansan operon, and there is no gene in S. mutans that appears to be homologous to levR by use of computer algorithms or Southern hybridization.

Further, the target sequence for binding of LevR and the dyadic sequence required for fruA show no similarity. On the other hand, the transcriptional activator LicR of the B. subtilis licBCAH operon binds to an inverted repeat just upstream of the promoter (38), similar to fruA. However, there is no apparent homology between the binding site of LicR and the region upstream of the fruA promoter (data not shown).

Inactivation of the fruI and/or fruCD genes resulted in constitutive elevation of expression of the PfruA−cat fusion. In some aspects, control of fruA expression via the fructose PTS makes it again similar to levansan expression in B. subtilis (22). Levansan induction in B. subtilis is controlled by a phosphoryl-circuit involving four gene products encoded in the levansan operon that phosphorylate LevR when fructose is absent from the growth medium. When low levels of fructose are added, a preferential transfer of phosphate to the incoming fructose leaves the PRD domain of LevR in an unphosphorylated state, which renders the protein competent for activation of sacC transcription. Unlike for the B. subtilis levansan operon, there are no genes in the fruA operon for EI-like gene products that regulate transcription of the operon, yet an apparently similar phosphoryl-circuit involving IIfru proteins exists for induction and repression of fructanase in S. mutans. Notably, growth with fructose alone does not induce fruA expression, but we believe that this is a technical anomaly and that the genetic evidence for fructose induction through fructose EIIs provides a more credible picture of the fruA regulatory pathway. Specifically, the reason that there is no apparent induction of fruA in cells grown on fructose is that for the cells to grow well, it is necessary to provide relatively high concentrations of the hexose, which results in catabolite repression of fruA. In contrast, cells growing on inulin or levan, which optimally induces fruA, expression, are exposed to much lower steady-state levels of fructose because the hexose is liberated from the fructans at a rate lower than the optimal rate for fructose transport.

Utilization of secondary carbon sources in B. subtilis and other gram-positive bacteria is governed primarily by CcpA, which binds to CREs (1, 15, 39), although CcpA-independent mechanisms have also been reported (14, 22, 38). As observed with other catabolite-repressible systems, deletion or mutation of a promoter-proximal CRE, in this case CRE-S, resulted in a dramatic decrease in CCR of fruA, indicative of the central role of this element in CCR of fruA. Interestingly, inactivation of ccpA in S. mutans had little impact on CCR of fruA (Table 3), consistent with other studies that explored the role of CcpA in CCR in streptococci. For example, in S. mutans GS-5, disruption of the ccpA homologue regM had no effect on diauxic growth when the strain was grown on a variety of nonpreferred carbohydrate sources and glucose. In fact, ccpA inactivation resulted in increased glucose repression of α-galactosidase, mannotol-1-phosphate dehydrogenase, and phospho-β-galactosidase (34). Similarly, sucrose-mediated repression of α-galactosidase (aga) expression by Streptococcus pneumoniae was not affected by mutation of a gene that encodes an apparent CcpA homologue (26). Nevertheless, to our knowledge, this communication provides the first evidence that a putative CRE actually functions in CCR of an operon and yet a CcpA deficiency does not have an impact on expression of the operon containing that CRE. Thus, there is the distinct possibility that CcpA, which we have shown is in fact expressed in UA159, fulfills some other function in the cells and that some other repressor(s) acts at the CRE to exert CCR.

In attempting to reconcile the lack of involvement of CcpA in catabolite repression of an operon with a functional CRE, we identified an open reading frame corresponding to a product with a significant degree of similarity to the CcpB protein of B. subtilis (8). The B. subtilis CcpB protein has 30% identity to CcpA of the same organism and is responsible for CCR when cells are grown on solid medium or in liquid medium with little agitation (8). A homologue of CcpB was identified in S. mutans UA159, and the gene was inactivated using a strategy similar to that for ccpA, but mutation of ccpB alone, or of both ccpA and ccpB, had no effect on expression of the PfruA−cat fusion (Wen and Burne, unpublished data). Yet another protein in B. subtilis, CcpC, is a member of the LysR family of transcriptional regulators that mediates repression of citB expression by glucose and sources of 2-ketoglutarate (18). However, we could not identify a CcpC paralogue in the genome database. Therefore, if there is another repressor controlling CCR of fruA, and possibly other catabolite-sensitive operons in S. mutans, it is probably not a CcpA, -B, or -C homologue.

We also believe that, like for lev, licBCAH, and some other catabolic operons, EI and HPt may modulate the transcription of the fruA operon by influencing the activity of the transcriptional activator, which could explain the residual CCR in strains carrying gene fusions to the fruA promoter with mutations or deletion of CRE-S (22, 38) (Fig. 2) or in the ccpA mutant of S. mutans. Such a phenomenon, called CepC-indepen- dent CCR, occurs in regulation of licS, licBCAH, and lev operons of B. subtilis and the lac operon of L. casei (14, 31). Isolation of the fruA regulator of transcription will allow us to investigate this hypothesis in more detail.

In summary, expression of fruA in S. mutans UA159 is inducible by inulin and subject to catabolite repression. We present evidence here that expression of fruA requires a transcriptional activator that is probably negatively regulated by components of a fructose-specific PTS, and that CCR of fruA occurs through a CRE, as well as through yet-undisclosed mechanisms. Thus, there are fundamental differences between substrate induction and catabolite repression of fruA of S. mutans and other genes encoding polysaccharide-degrading enzymes of eubacteria.

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REFERENCES


