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The *Streptococcus mutans* Aminotransferase Encoded by *ilvE* Is Regulated by CodY and CcpA

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The aminotransferase IlvE was implicated in the acid tolerance response of *Streptococcus mutans* when a mutation in its gene resulted in an acid-sensitive phenotype (B. Santiago, M. MacGilvray, R. C. Faustoferri, and R. G. Quivey, Jr., J. Bacteriol. 194: 2010–2019, 2012). The phenotype suggested that amino acid metabolism is important for acid adaptation, as turnover of branched-chain amino acids (bcAAs) could provide important signals to modulate expression of genes involved in the adaptive process. Previous studies have demonstrated that *ilvE* is regulated in response to the external pH, though the mechanism is not yet established. CodY and CcpA have been shown to regulate expression of branched-chain amino acid biosynthetic genes, suggesting that the ability to sense carbon flow and the nutritional state of the cell also plays a role in the regulation of *ilvE*. Electrophoretic mobility shift assays using the *ilvE* promoter and a purified recombinant CodY protein provided evidence of the physical interaction between CodY and *ilvE*. In order to elucidate the signals that contribute to *ilvE* regulation, *cat* reporter fusions were utilized. Transcriptional assays demonstrated that bcAAs are signaling molecules involved in the repression of *ilvE* through regulation of CodY. In a *codY* deletion background, *ilvE* transcription was elevated, indicating that CodY acts a repressor of *ilvE* transcription. Conversely, in a *ccpA* deletion background, *ilvE* transcription was reduced, showing that CcpA activated *ilvE* transcription. The effects of both regulators were directly relevant for transcription of *ilvE* under conditions of acid stress, demonstrating that both regulators play a role in acid adaptation.

Streptococcus mutans, the major causative agent of dental caries, represents a challenge for effective treatment because of its acidogenic and aciduric nature. Survival under environmentally stressful conditions is possible due to an adaptive response which allows the organism to activate pathways that aid in coping with an acid challenge. Several contributing mechanisms have been identified for their roles in the acid adaptation process (1–4). However, reports have suggested other, uncharacterized routes that could contribute to the acid tolerance of *S. mutans* (5). Analyses of the metabolic profile of *S. mutans* have identified increased expression of genes involved in branched-chain amino acid (bcAA) biosynthesis under conditions of acid stress.

A strain of *S. mutans* GS-5 carrying an insertional mutation in a homolog of a branched-chain amino acid aminotransferase (ATase) referred to as AP-185 was impaired in both growth and sensitivity to acid challenge (6). When transcription of AP-185 was examined under various environmental stresses, expression was elevated under conditions of acid stress (6). Amino acid metabolism has been suggested as an alternative method for *S. mutans* to cope with acidic end products, whereby branched-chain amino acid synthesis, as an overflow pathway for pyruvate, reduces the generation of acidic end products and alleviates the acidification of the intracellular milieu (7). While potentially important for autoacidification as a result of carbohydrate metabolism, branched-chain amino acids are the most abundant amino acids in proteins and function as the precursors for several components necessary for effective cellular function. Branched-chain amino acids are also important as intracellular signals for the overall homeostasis of bacteria. The accumulation of bcAAs depends on many essential precursors, and hence, their relative abundance reflects the physiological state of the cell. Thus, as coeffectors for regulatory proteins, bcAAs act as metabolic sensors, allowing for the activation and/or repression of target genes at appropriate times (8).

CodY is a GTP-binding global transcriptional repressor that has been characterized extensively in several bacterial pathogens (9–11). CodY was first identified as a repressor in *Bacillus subtilis*, where an insertional mutation within the open reading frame (ORF) carrying *codY* resulted in derepression of its target gene, *dpp*, encoding a dipeptide permease (12, 13). Changes caused by nutritional limitation allow CodY to exert its regulatory function by sensing intracellular pools of branched-chain amino acids and GTP, which fluctuate when the organism enters stationary phase (14).

The *ilvBHC* and *leuABCD* operons, involved in biosynthesis of branched-chain amino acids in *B. subtilis*, are known to be regulated by CodY (15, 16). In *Staphylococcus aureus*, the *ilv-leu* operon has been identified as a target for CodY binding (17). CodY activation in *Bacillus subtilis* is dependent on direct interaction with branched-chain amino acids, which cause a conformational change in CodY upon binding that increases the affinity for its cognate motif (18). The DNA-binding region for CodY is localized upstream of promoters of the genes that it regulates, and in some cases, such as in *Lactococcus lactis*, a consensus sequence has been found that has a high binding affinity for CodY (19, 20). Branched-chain amino acid aminotransferases in *L. lactis* (*bcaT*) and *B. subtilis* (*ybgE*) (15, 21) have been identified as CodY targets, supporting the role of CodY in the regulation of the biosynthetic operon for amino acid synthesis. In turn, aminotransferases reg-

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ulate CodY action by modulating intracellular pools of branched-chain amino acids (22).

Studies of operons encoding branched-chain amino acid synthesis components in *B. subtilis* have elucidated a complex regulatory scheme. It is common for transcriptional regulation of biosynthetic genes to be mediated by several proteins not limited to CodY (23). Catabolite response protein A (CcpA) is a well-known regulator of carbon metabolism involved in carbon catabolite repression (CCR) (24). In the presence of a preferred carbon source, CcpA binds to and represses expression of genes involved with secondary carbon sources while activating carbon overflow pathways (8, 25). CcpA binds to *cre* (catabolite-responsive element) sequences in the promoter regions of its target genes. The activity of CcpA is responsive to intermediates from glycolysis, which activate HPr, a histidine protein that functions as a binding partner for regulation. CcpA is both an activator and a repressor of gene expression, and *cre* site positioning can indicate function, with upstream promoter sites being important for activation and downstream promoter sites being important for repression (24).

Other than regulating genes involved with carbon catabolite repression, CcpA has also been shown to regulate branched-chain amino acid biosynthetic operons, such as the *ilv-leu* operon (23). Insufficient expression of these genes in a *ccpA* deletion mutant leads to a nutritional requirement for branched-chain amino acids, indicating that CcpA functions as an activator (26). The expression of *ilv* genes is stimulated in the presence of glucose, a preferred carbon source, a condition where CcpA is the most active (27). In *S. mutans*, CcpA has been shown to be important for central metabolism and the regulation of virulence. Loss of CcpA results in increased acidogenicity and faster growth at low pH, due to dysregulation of metabolic pathways (25).

The regulation of branched-chain amino acid biosynthetic genes by CcpA and CodY has been shown to be antagonistic, with CcpA as a positive regulator and CodY as a repressor. Interplay between CcpA and CodY enables the organism to respond to its nutritional needs and to modulate gene expression per its requirements. CcpA activates gene expression for branched-chain amino acid biosynthetic genes, which eventually results in increased intracellular branched-chain amino acid pools. Amino acid accumulation enhances the DNA-binding capacity of CodY and eventually results in the repression of the target genes. Activation by CcpA has been described as self-limiting, since CodY repression becomes stronger as more branched-chain amino acids are available (16). Positive regulation by CcpA is suggested to be due to direct interaction with the RNA polymerase as well, by preventing CodY binding via blockage of access to the Cod box (23). In most cases, positive regulation of CcpA cannot overcome negative regulation by CodY, and hence, changes in amino acid pools are the signals that alter the balance between the effects these proteins have on gene expression.

SMU.1203c encodes a branched-chain amino acid ATase, IlvE, in *S. mutans* UA159. Our work has shown that IlvE is involved with branched-chain amino acid metabolism and plays a role in acid adaptation. Loss of *ilvE* results in an acid-sensitive phenotype and reduced levels of F-ATPase activity when the organism is grown at low pH (28). In several organisms, such as *Streptococcus pneumoniae* (11), *L. lactis* (29), and *S. aureus* (17), *ilvE* has been identified as a CodY-regulated gene. Hence, the acid-sensitive phenotype of the *ilvE* mutant and the possibility that *ilvE* is a target for CodY regulation led us to explore the mechanism of expression of this bcAA ATase.

In this study, we investigated the regulatory mechanisms for *ilvE* and the interaction of *ilvE* with CodY. The binding of CodY to its cognate regulatory element in the *ilvE* promoter was demonstrated by electrophoretic mobility shift assays (EMSAs), in which branched-chain amino acids were identified as coeffectors of binding for CodY. Similar to results for *L. lactis*, CodY in *S. mutans* did not require GTP for enhanced binding. In addition, loss of the CodY consensus binding domain in target DNA led to an inability of the protein to bind. Transcriptional reporter fusions revealed that *ilvE* transcription is modulated by the presence of branched-chain amino acids at acidic pH, suggesting a response to intracellular pools of bcAAs, consistent with the role of *ilvE* as a biosynthetic gene. Under nutrient-limiting conditions, however, the *ilvE* transcript was induced regardless of growth conditions, indicating that the nutritional requirements of the cell act as important signals, in combination with environmental pH. Transcription of *ilvE* was also examined in strains of *S. mutans* deficient in CcpA or CodY. The results demonstrated reduced expression of *ilvE* in a Δ*ccpA* background and elevated expression in a Δ*codY* background, thereby supporting the roles of the corresponding regulators as activators and repressors of transcription, respectively. Collectively, the data presented show that transcriptional regulation of *ilvE* is responsive to carbon flow and the nutritional state of the organism.

MATERIALS AND METHODS

Bacterial strains. Strains used in this study are detailed in Table 1. *Streptococcus mutans* UA159, the genomic type strain, was the parent strain used in this study (30, 31). Mutant strains of *S. mutans* UA159 were created by deleting the entire coding regions of *codY* (SMU.1824c) and *ccpA* (SMU.1591c), using a PCR-based, ligation-independent cloning method, LIC mutagenesis, as previously described (32–34). Flanking regions of approximately 400 bp upstream and downstream were used to mediate replacement of genomic copies of genes with a nonpolar erythromycin cassette, resulting in *S. mutans* Δ*codY* (SMU.1824c) and Δ*ccpA* (SMU.1591c) strains. Appropriate constructs were verified using two pairs of specific primers for each strain (Table 2).

Generation of CodY-complemented strain. A genetic complement strain of *S. mutans* was created using primers (Table 2) to amplify the *codY* coding and preceding intergenic regions from UA159 chromosomal DNA. A *codY* complement plasmid, pSUGB*cod*Y, was constructed as previously described (28, 35). The Δ*codY* mutant strain was transformed with the integration vector pSUGB*cod*Y, and colonies were selected on brain heart infusion (BHI) agar containing kanamycin (1,000 μg/ml). Positive transformants were identified by colony PCR. One strain, containing the *codY* coding region and promoter integrated into the *gtfA* locus (SMU.0881), was named UR314.

Growth conditions. *Escherichia coli* strains were maintained on Luria-Bertani (LB) agar plates with appropriate selection, where necessary, as detailed below. *S. mutans* strains were maintained on BHI (Becton, Dickinson/Difco, Franklin Lakes, NJ) agar plates with appropriate antibiotic selection (see below). For chloramphenicol acetyltransferase (CAT) assays (described below), *S. mutans* batch cultures were grown in BHI broth or FMC minimal medium (36) plus 1% (wt/vol) glucose in a 95% (vol/vol) air–5% CO_2 atmosphere. Nutritional supplements were exogenously supplied amino acids or fatty acids. Final concentrations of additives were as follows: 1 mM leucine (L), 1 mM isoleucine (I), and 1 mM valine (V), individually or mixed (at a 1 mM concentration of each) (ILV). Overnight cultures were diluted 1:20 (in triplicate) in 50 ml of fresh medium titrated to pH 5 or buffered to pH 7, with or without additives. The optical density at 600 nm (OD_{600}) was followed until the cell density was between 0.4 and

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant feature	Source or reference
Strains		
E. coli strains		
DH10B		Invitrogen
BL21(λDE3)		Novagen
BL21(λDE3)(pET19bCodY)	CodY expression construct; Amp^r	This study
BL21(λDE3)(pET19bVC)	pET19b vector control; Amp^r	This study
S. mutans strains		
UA159	Genomic type strain of *S. mutans*	30, 31
Δ*ilvE* strain	SMU.1203c (Δ*ilvE*) mutant strain of UA159; Erm^r	28
Δ*codY* strain	SMU.1824c (Δ*codY*) mutant strain of UA159; Erm^r	This study
Δ*ccpA* strain	SMU.1591c (Δ*ccpA*) mutant strain of UA159; Erm^r	(R. C. Faustoferri and C. Hubbard, unpublished data)
UR314	*codY*-complemented strain (*codY*$_c$); Kan^r Erm^r	This study
UR212	UA159 carrying pJL84 (promoterless *cat*); Kan^r	28
UR213	UA159 carrying pJL1099 (*ilvE* promoter-*cat*); Kan^r	28
UR214	Δ*codY* strain carrying pJL84; Kan^r Erm^r	This study
UR217	Δ*ilvE* strain carrying pJL1099; Kan^r Erm^r	This study
UR220	Δ*codY* strain carrying pJL1099; Kan^r Erm^r	This study
UR268	Δ*ccpA* strain carrying pJL1099; Kan^r Erm^r	This study
UR301	Δ*ccpA* strain carrying pJL1099CRE; Kan^r Erm^r	This study
UR302	UA159 carrying pJL109CRE; Kan^r	This study
UR304	Δ*codY* strain carrying pJL1099CRE; Kan^r Erm^r	This study
UR317	Δ*codY* complemented strain with pJL1099; Kan^r Erm^r	This study
Plasmids		
pET19b	T7lac, N-terminal 6× His; Amp^r	Novagen
pET19bCodY	pET19b with *codY* coding region; Amp^r	This study
pJL84	Chloramphenicol acetyltransferase gene (*cat*) reporter plasmid; Kan^r	28
pJL1099	pJL84 with *ilvE* promoter region; Kan^r	28
pJlL1099NCB	pJL84 with truncated *ilvE* promoter, no CodY box; Kan^r	This study
pSUGBcodY	*codY* complement construct used to create UR314	This study

0.6. Cells were harvested, washed with 10 mM Tris-Cl, pH 7.8, pelleted, and stored frozen until assayed for chloramphenicol acetyltransferase activity.

***ilvE* promoter-*cat* fusion construction.** For *cat* transcriptional fusions, full-length *ilvE* promoter-*cat* fusions were cloned as previously described (28). In addition, a truncated *ilvE* promoter-*cat* construct (NCB [no Cod box]) in which the predicted native CodY-binding domain was absent was created (Fig. 1). Briefly, truncated promoters were cloned into a promoterless chloramphenicol acetyltransferase reporter plasmid, pJL84 (28), using primers described in Table 2. Transformants were selected on LB agar plates containing kanamycin (50 μg/ml) and were screened by colony PCR. Correct constructs were named pJL1099CRE and pJL1099NCB (Table 1). The *S. mutans* parent strain (UA159) and the Δ*codY* and Δ*ccpA* mutant strains were transformed with pJL1099 to generate single-copy, integrated reporter fusions.

TABLE 2 Oligonucleotide primers used in this study

Primer application and name	Nucleotide sequence (5′–3′)[a]	Restriction site
Primers for cloning		
1662P8DN (*codY*)	ATTGATGATTTTCTTGACTTAGGG	
1662P7UP (*codY*)	ATCACAGCGGTAAAACCTCAGGCG	
1442P8DN (*ccpA*)	AACCAAGCGGTACCATAGATAC	
1442P7UP (*ccpA*)	AAATGGGCTATTGCTAGTGATG	
1662BglUp[b]	CATAGATCTAAGGGCTTTCATGAAAATTGTCGA	BglII
1662BglDown[b]	CATAGATCTGGTATAATCAATTGAAATCAGTGCTTTACTCATT	BglII
Primers for EMSAs and CAT constructs		
1099Fwd[c]	CATGAGCTCGATAACATTTCTTACAAAAAGACAGG	SacI
1099Rev[c]	CATGGATCCTTTAATACCTCTTTCCTCG	BamHI
1099RevNCB[c,d]	CATGGATCCTTTAAAAGATAAAATGGAGCAG	BamHI
BglIIATP[e]	ATTCAACATAGATCTTTCTCCTTC	BglII
BRLSMp567>[e]	ACCTAATATTTATAAGATAAG	
Primers for protein expression		
CodYFwd	GAAAGCCCATATGGCTAATTTATTATCG	NdeI
CodYRev	GAGTGACTTGGATCCTTAACTATAGTC	BamHI

[a] Restriction sites are underlined.
[b] Primer used for cloning of *codY* complement.
[c] Primer used for *ilvE* target.
[d] Primer used to construct a truncated *ilvE* promoter without the CodY consensus binding sequence.
[e] Primer used for ATPase control probe SMU.1534c (3).

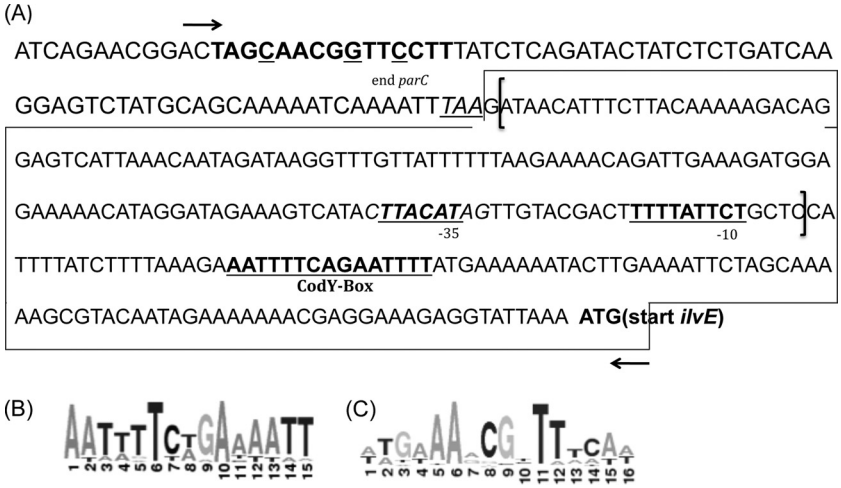

FIG 1 Intergenic region preceding *ilvE*. (A) Putative −10 and −35 sequences for *ilvE* determined by BPROM (Softberry) promoter prediction. The boxed region represents the *ilvE* promoter region. Brackets represent the truncated *ilvE* promoter region (NCB; no CodY box). Arrows indicate an extended *ilvE* promoter region that contains a *cre* site. The CodY-binding recognition site is shown in bold and underlined. The hypothetical *cre* site is shown in bold, with mismatched bases underlined. Primer binding sites are denoted by arrows. (B) Streptococcal CodY consensus sequence. (C) CcpA consensus sequence. The consensus sequences were predicted by using the RegPrecise (http://regprecise.lbl.gov/RegPrecise/) database.

UR314, the *codY*-complemented strain, was transformed with pJL1099 (33), and transformants were selected on BHI plates containing both kanamycin (1,000 μg/ml) and erythromycin (5 μg/ml). Clones were screened for proper integration into the chromosome by PCR. Integration of the promoter-*cat* construct occurs within the intergenic regions between *mtlA* (SMU.1185c)-*glmS* (SMU.1187c) and *mtlD* (SMU.1182c)-*phnA* (SMU.1180c). Transformants for each construct are described in Table 1.

CodY protein expression. CodY protein expression and purification were performed as previously described (11, 18), with modifications. The *S. mutans codY* gene was PCR amplified from UA159 chromosomal DNA by using the primer pair CodYFwd and CodYRev (Table 2) and then cloned into the Zero Blunt vector (Invitrogen, Carlsbad, CA). The *codY* coding sequence was confirmed by sequencing, and a positive clone was digested at engineered NdeI and BamHI sites to release the fragment. The gel-purified insert was cloned into NdeI/BamHI sites of pET19b (Novagen, Rockland, MA) to generate an N-terminally His-tagged recombinant CodY protein, and a correct construct was named pET19CodY. *E. coli* BL21(λDE3) was transformed with pET19b (as a negative expression control) or pET19CodY for protein expression. The BL21 strain carrying pET19b or pET19CodY was grown in LB medium containing ampicillin at 100 μg/ml, at room temperature, until the OD_{600} reached approximately 0.4 to 0.6. IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) was then added to induce protein expression, and growth was continued for 3 h. Cells were harvested by centrifugation, and cell pellets were lysed using a mini-bead beater (BioSpec Products, Bartlesville, OK). His-tagged CodY protein was purified under native conditions, using Ni-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Valencia, CA). Purified CodY protein was analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) (UR Proteomics Core Facility) and verified to be *S. mutans* CodY. Total protein concentration was estimated by the method of Bradford (Bio-Rad, Hercules, CA) (37). Protein samples were mixed with 5× SDS-PAGE loading buffer, boiled for 5 min, and loaded onto a NuPAGE Novex 4 to 12% Bis-Tris gel (Invitrogen). Proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane for Western blotting, using a Milliblot (Millipore, Billerica, MA) system, and then were immunoblotted (38). The presence of the 6×-His tag was verified by Western blot analysis using an anti-His IgG primary antibody (GE Healthcare, Piscataway, NJ).

CAT assay. CAT assays were performed following a previously described protocol (3, 28, 39). Briefly, 50-ml cell pellets were resuspended in 1 ml 10 mM Tris-Cl, pH 7.8. Cells were lysed using 0.1-mm glass beads in a mini-bead beater (BioSpec Products, Bartlesville, OK). Cell debris was removed by centrifugation at 10,000 rpm for 10 min. Lysates were removed and used for total protein quantification (37) and CAT assays. Each CAT assay reaction mixture consisted of 50 μl whole-cell lysate, 100 mM Tris-Cl, pH 7.8, 0.1 mM acetyl-coenzyme A (acetyl-CoA), and 0.4 mg/ml 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) in a total volume of 1 ml. Reactions were initiated by addition of 0.1 mM chloramphenicol. Optical density measurements at 412 nm were monitored over 3 min. Reaction rate and total protein concentration were used to determine CAT activity, and values are presented as μmol chloramphenicol acetylated/min/mg of total protein.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays were performed as described previously (11, 40). Briefly, DNA fragments containing *ilvE* (SMU.1203c) and ATPase (SMU.1534c/*c* subunit ATPase) promoter regions were amplified by PCR using primers shown in Table 2. DNA products were purified and end labeled with T4 polynucleotide kinase (PNK) and [γ-^{32}P]ATP (PerkinElmer, Waltham, MA) for 10 min at 37°C. Quantification of labeled DNA was performed by scintillation counting. A representative binding reaction mixture (25 μl) consisted of the following: binding buffer (20 mM Tris-HCl, pH 8; 8.7% [vol/vol] glycerol; 1 mM EDTA; 5 mM $MgCl_2$; 250 mM KCl; 0.5 mM dithiothreitol [DTT]; 2 μg bovine serum albumin [BSA]), radiolabeled DNA probe (3,000 cpm), purified CodY (1,500 nM), and the branched-chain amino acids isoleucine, leucine, and valine, separately or in combination, to a final concentration of 3 mM or 10 mM, as indicated. GTP was added to a final concentration of 3 mM. Binding reaction mixtures were incubated at 37°C for 45 min. CodY-DNA binding was assessed using an 8% nondenaturing gel in 1× Tris-glycine buffer or a 6% DNA retardation gel (Invitrogen). DnaK protein (1,500 nM) and the ATPase promoter were used as negative controls for protein binding and probe specificity, respectively. The gels were exposed to a phosphorimager screen, and binding was detected with a Molecular FX phosphorimager and Bio-Rad Quantity One software (Bio-Rad, Hercules, CA).

RESULTS

CodY interacts with the *ilvE* promoter region. A consensus CodY-binding sequence in the putative promoter region of *ilvE* in *S. mutans* was previously identified by Lemos et al. (20), suggesting that *ilvE* could be regulated by CodY. To determine whether

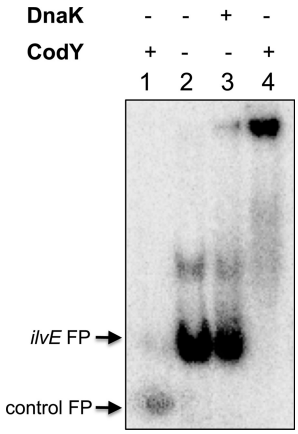

FIG 2 CodY interaction with *ilvE* promoter. Radiolabeled DNA probes were incubated with 1,500 nM CodY or DnaK, as indicated. Promoter regions used for binding assays were the ATPase promoter (lane 1) and the *ilvE* promoter region (lanes 2 to 4). No branched-chain amino acids were added to binding reaction mixtures. Arrows at left indicate migration of free probes (FP).

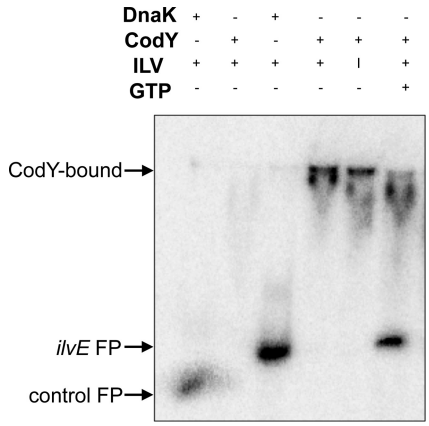

FIG 4 CodY binding is not enhanced by GTP in *S. mutans*. Radiolabeled DNA probes were incubated with 1,500 nM purified recombinant DnaK (control) or CodY. Branched-chain amino acids added were isoleucine, leucine, and valine (ILV) or isoleucine alone (I), to a final concentration of 10 mM (each). GTP was added to a final concentration of 3 mM. Promoter regions used for binding assays were the ATPase promoter (lanes 1 and 2) and the *ilvE* promoter (lanes 3 to 6).

the CodY protein was capable of direct interaction with the *ilvE* promoter, we evaluated DNA-protein binding via EMSAs. A recombinant CodY protein was purified and verified by mass spectrometry prior to performing the binding assays (data not shown). The *ilvE* promoter region containing the CodY consensus sequence (Fig. 1) was used as the target. In the presence of CodY, migration of the *ilvE* promoter target was hindered, indicating DNA-protein complex formation (Fig. 2, lane 4, and Fig. 3, lane 6). No shift was detected in the presence of control DNA (Fig. 2, lane 1, and Fig. 3, lanes 1 and 2) or with the use of a control protein, DnaK (Fig. 2, lane 3), indicating that the interaction between *ilvE* and CodY was specific. The EMSA data demonstrated that the *ilvE* promoter region, containing a predicted CodY consensus sequence, is capable of binding CodY.

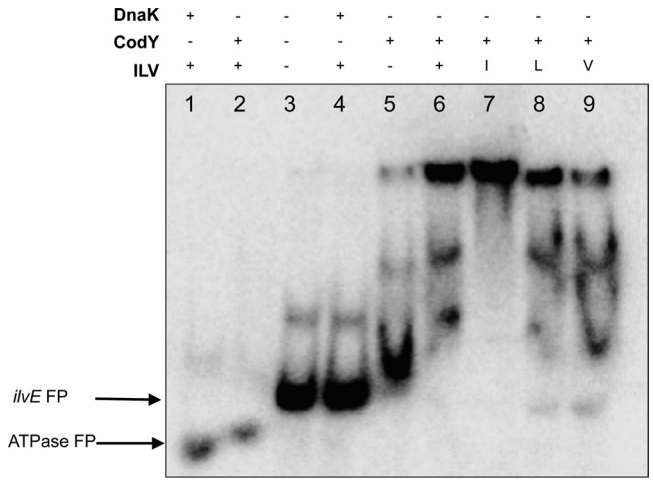

FIG 3 CodY interaction with the *ilvE* promoter is enhanced in the presence of isoleucine. Radiolabeled DNA probes were incubated with 1,500 nM purified recombinant DnaK (control) or CodY. Branched-chain amino acids added were isoleucine (I), leucine (L), and valine (V), individually or in combination (ILV), to a final concentration of 3 mM (each). Promoter regions used for binding assays were the ATPase promoter (lanes 1 and 2) and the *ilvE* promoter (lanes 3 to 9). Arrows at left indicate migration of free probes (FP).

Interaction between CodY and the *ilvE* promoter occurs in the presence of bcAAs and not GTP. The interaction between a target DNA and the CodY protein has been shown to be mediated by either GTP in conjunction with bcAAs or bcAAs alone (41). In *S. mutans*, the use of decoyinine, an analogue of adenosine that inhibits GMP synthase and causes a decrease in GTP pools, did not affect CodY-mediated repression of *ilvE*, indicating that in *S. mutans*, CodY activity is not responsive to GTP (20). To confirm these findings and to demonstrate that bcAAs are coeffectors of CodY, we tested CodY binding to the *ilvE* promoter in the presence of bcAAs as well as GTP. When 3 mM GTP was added to the binding reaction mixture, CodY binding to the *ilvE* promoter was not enhanced (Fig. 4, lane 6) and appeared to be similar to protein binding without coeffectors. However, in the presence of bcAAs, a significant shift was observed (Fig. 3, lane 5 versus lane 6), indicating that CodY binding to the *ilvE* promoter was not dependent on GTP but on bcAAs alone.

CodY binding to the *ilvE* promoter is enhanced in the presence of isoleucine. The branched-chain amino acids isoleucine, leucine, and valine are metabolite effector molecules for CodY binding in other systems (42). In *L. lactis*, isoleucine has been shown to be the stronger of the signals that enhance binding affinity for CodY (29). To determine if CodY binding in *S. mutans* exhibited a similar bias for specific bcAAs, we performed binding assays in the presence of individual bcAAs. Although CodY was able to bind the *ilvE* promoter without added bcAAs, the presence of bcAAs enhanced the binding of CodY, as shown in Fig. 3, lanes 6 to 9. In the presence of isoleucine, CodY was able to bind and completely shift the *ilvE* target in a manner similar to that seen when a mixture of all three amino acids was present (Fig. 3, lanes 6 and 7), indicating that isoleucine increased binding to a greater extent than that with any of the other amino acids added individually. These data demonstrate that of all the bcAAs tested, isoleucine is the most effective coeffector for CodY binding to the *ilvE* promoter in *S. mutans*.

Absence of the CodY consensus binding sequence abrogates the ability of CodY to bind the *ilvE* promoter. To test whether the

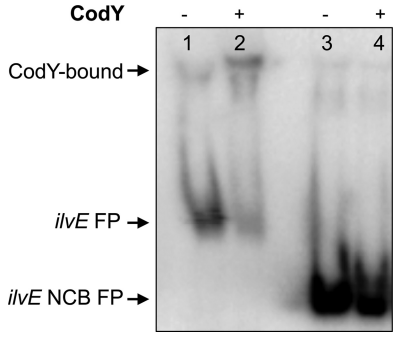

FIG 5 The CodY box is required for binding of CodY to the *ilvE* promoter in *S. mutans*. CodY binding assays for full-length and truncated (NCB) *ilvE* promoter regions (described in Materials and Methods) were performed with isoleucine (10 mM) and purified recombinant CodY (1,500 nM). Lanes 1 and 2, *ilvE* promoter; lanes 3 and 4, truncated *ilvE* promoter (NCB).

absence of a CodY consensus binding sequence would inhibit protein-DNA binding and to demonstrate that this interaction was CodY specific, an *ilvE* promoter sequence lacking the CodY consensus motif was used as a target in EMSAs (Fig. 1). Unlike our control reaction, where a visible shift occurred with the full-length *ilvE* probe, in the absence of a CodY-binding sequence, the protein was unable to bind the *ilvE* promoter, as shown in Fig. 5, lane 4. In the presence of an anti-His IgG antibody, no shift was detected using the *ilvE* promoter lacking the Cod box (data not shown), in contrast to results with the full-length *ilvE* promoter, containing a Cod box and CodY (data not shown). Taken together, these data indicate that the CodY consensus sequence is necessary for protein-DNA interaction between CodY and *ilvE*.

Transcription of *ilvE* is responsive to bcAAs. Our binding assays provided evidence that CodY binding to *ilvE* was enhanced in the presence of branched-chain amino acids. Branched-chain amino acids have also been shown to be important in the regulation of biosynthetic genes as end products of the biosynthetic pathway. To assess the effects of the presence of branched-chain amino acids on expression of *ilvE in vivo*, an *ilvE* promoter-*cat* fusion strain, UR213, was created. When the fusion-bearing strain was grown in the presence of isoleucine or leucine, the transcription of *ilvE* was reduced in comparison to expression in unsupplemented medium (Fig. 6A). The reduced expression in the presence of leucine also suggests that, like other biosynthetic genes, *ilvE* could be subject to regulation by the level of leucine. The observable effect on the transcriptional activity occurred at a low pH, which we had previously shown is a condition that triggers an increase in *ilvE* transcription (28). The results suggest that signaling by bcAAs can modulate transcription of *ilvE*, demonstrating that both pH and the nutritional state of the cell are signals that regulate *ilvE* transcription.

Transcription of *ilvE* is downregulated in the absence of *ilvE*. bcAAs, the end products of branched-chain amino acid synthesis, are the signaling molecules that allow CodY to repress transcription of target genes, which themselves encode enzymes that synthesize bcAAs. Since the Δ*ilvE* strain displayed a requirement for bcAAs during growth in minimal medium and a reduction in bcAA catabolism, we thus showed the contribution of IlvE to both processes of amino acid metabolism: synthesis and degradation (28). Aminotransferases (ATases) are enzymes of dual function, whose enzymatic activity can participate in both amino acid biosynthesis and degradation (43). Therefore, since *ilvE* encodes an ATase, the possibility existed that the gene product could contribute, via a feedback mechanism, to the expression of the cognate gene. To determine the effects that the loss of IlvE would have on its own transcription, we explored the transcriptional activity of the *ilvE* promoter in an *ilvE* deletion strain (UR217). In accordance with its role in branched-chain amino acid metabolism, the *ilvE* transcript level, as measured by a reporter fusion assay, was reduced in the absence of IlvE, indicating that the absence of IlvE affects transcription of the cognate gene (Fig. 7). Similar to data presented above, the most pronounced effects on the transcriptional activity occurred under low-pH growth conditions. The decreased *ilvE* transcript level might have been due to changes in bcAA turnover because of the loss of both enzymatic functions that IlvE provides. These data support the important role of this gene and its product in its own regulation, as well as in transcriptional regulation required for acid adaptation.

Growth under nutrient-limiting conditions induces *ilvE* transcription. Following exponential-phase growth, the availability of nutrients becomes reduced as cells enter stationary phase. During stationary phase, bcAAs become limited, and as a result,

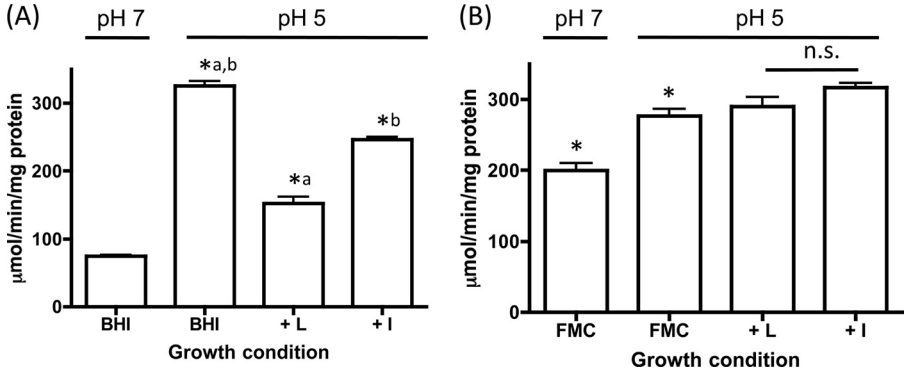

FIG 6 *ilvE* promoter activity is downregulated by bcAAs and induced under nutrient-limiting conditions. CAT activity was determined for *S. mutans* UR213 (*ilvE* promoter-*cat* fusion in the UA159 parent strain) grown in BHI medium (A) or FMC minimal medium (B) supplemented with bcAAs. Transcriptional activity was determined as described in Materials and Methods. The growth medium was supplemented with the branched-chain amino acid isoleucine (I) or leucine (L), to a final concentration of 1 mM. Statistical significance was determined by Student's *t* test. *, $P \leq 0.00001$ ($n = 3$). Pairwise comparisons are represented by lowercase letters. n.s., not statistically significant.

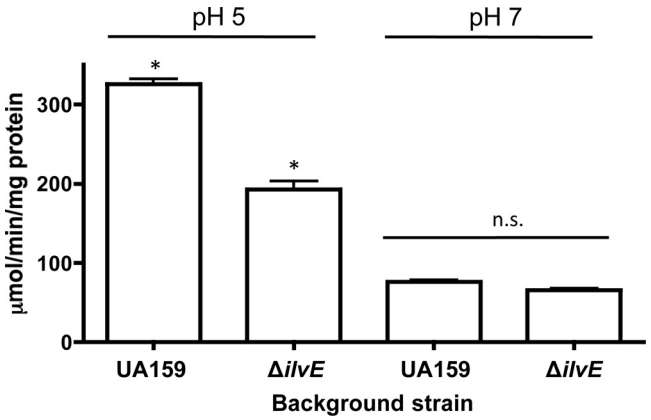

FIG 7 The *ilvE* mutant displays decreased transcriptional activity from the *ilvE* promoter. CAT activities are shown for *ilvE* promoter-*cat* fusions in the UA159 parent strain and Δ*ilvE* backgrounds, grown in BHI medium. Transcriptional activity was determined for three independent cultures in duplicate for each growth condition, as described in Materials and Methods. Statistical significance was determined by Student's *t* test. *, $P \leq 0.00001$ ($n = 3$). n.s., not statistically significant.

genes that are under the control of CodY can be derepressed (14, 21). To examine the effect that nutritional limitation would have on *ilvE* transcription, UR213 was grown in FMC minimal medium (44) in the presence or absence of bcAAs. It was expected that under nutrient-limiting conditions, CodY repression would be partially alleviated, allowing for increased transcriptional activity of *ilvE*. When UR213 was grown in FMC minimal medium at low pH, *ilvE* transcriptional activity was not enhanced (Fig. 6B) compared to the results observed for cultures grown in BHI medium (Fig. 6A). However, the pH-mediated response differed between the two growth conditions (Fig. 6B). In rich medium, the *ilvE* transcript was responsive to environmental pH, such that an increase in transcriptional activity was observed in cultures grown at pH 5 (28). Under nutrient-limiting conditions, although the pH-dependent increase in transcription was still detectable, *ilvE* transcription was elevated 2-fold in cultures grown at a pH of 7 compared to cultures grown under nutrient-rich conditions (Fig. 6B). These results suggest that *ilvE* transcription can be affected by the availability of basic metabolites in growth medium, in addition to growth at acidic pH values, although the transcriptional response to acidic conditions is still maintained in both growth environments.

CodY represses *ilvE* transcription. In an *S. mutans codY* deletion strain, a substantial majority of the genome was upregulated, supporting the role of CodY as a global repressor of gene expression (20). In previous work, we used an *ilvE* promoter-chloramphenicol acetyltransferase gene (*cat*) reporter fusion to study *ilvE* transcription, demonstrating that *ilvE* transcription was affected by extracellular pH (28). The presence of a CodY-binding domain, as well as the physical interaction between CodY and the *ilvE* promoter (Fig. 2 and 3), strongly implicated CodY as a regulator of *ilvE* transcription. To determine if CodY is a repressor of *ilvE*, a Δ*codY* strain carrying the *ilvE-cat* reporter fusion (UR220) was used in transcriptional studies. In the absence of CodY, *ilvE* transcription was upregulated approximately 2-fold at pH 5 and approximately 7-fold at pH 7 (Fig. 8A). Although a pH-dependent response was still observed, the *ilvE* message was induced to a greater extent at neutral pH in the Δ*codY* strain, indicating that in the absence of CodY, derepression had occurred. In order to confirm the role of CodY as a repressor of *ilvE* transcription, the pJL1099 promoter fusion was integrated into the *codY*-complemented strain (UR317). CAT assays performed on UR317 showed that introduction of a functional copy of CodY reestablished repression of *ilvE* transcription (Fig. 8B). Collectively, these data demonstrate that CodY acts as a repressor of *ilvE* transcription and that, in the absence of CodY, the effect of environmental pH on transcription is enhanced.

CcpA activates transcription of *ilvE*. The *ilv-leu* operon in *B. subtilis* is regulated not only by CodY but also by CcpA (26). The operon contains the prototypical *cre* site in its promoter region, which is characteristic of genes regulated by CcpA. Unlike the *ilv-leu* genes in other bacteria, which are arranged in operons, *ilvE* in *S. mutans* is located independently from other branched-chain amino acid biosynthetic genes in the genome, suggesting that regulation of *ilvE* could be distinct from that of the other genes involved in bcAA metabolism.

The *ilvE* promoter region used in transcriptional assays in this study did not contain a strong *cre* site; however, given data from studies performed on other organisms showing a link between carbon catabolite repression and bcAA synthesis (26), we explored this relationship in *S. mutans*. Transcription of *ilvE* was determined via a *cat* reporter fusion construct in a *ccpA* deletion strain (UR268). In the absence of CcpA, transcription of *ilvE* was reduced compared to the expression observed in the parent strain or in the Δ*codY* strain (Fig. 8A). The data support the role of CcpA as an activator of branched-chain amino acid biosynthetic genes. Despite the absence of a well-defined *cre* site, the loss of CcpA had an effect on *ilvE* expression, suggesting that CcpA indirectly plays a role in regulation of *ilvE* or that a less-conserved binding site or binding site location is present within the intergenic region preceding the gene (Fig. 1). Similar to results seen for expression of *ilvE* in a Δ*codY* strain, the effect of pH on transcription was not obvious, as *ilvE* transcription in UR268 was diminished in an equivalent manner in both strains, independent of the pH value of the growth media.

Transcription of *ilvE* under nutrient-limiting conditions is independent of CcpA but enhanced in the absence of CodY. The data presented thus far show that transcription of *ilvE* is affected by the loss of either the CodY or CcpA regulator. Since both of these regulators of gene expression are, in turn, influenced by nutrient levels in the growth medium, we explored the effect on *ilvE* transcription under nutrient-limiting conditions in the absence of either regulator. In FMC minimal medium, similar levels of transcription were observed in both the parent strain background and the Δ*ccpA* background when cultures were grown under identical conditions (Fig. 8C). The data show that under conditions of nutritional limitation, *ilvE* transcription was induced independently of CcpA. In the absence of CodY and under nutrient-limited growth conditions, *ilvE* transcription was upregulated approximately 5-fold at pH 5 and pH 7 compared to levels seen in the parent strain background (Fig. 8B). The results support the role of CodY in regulation of *ilvE* transcription in response to both nutritional availability and environmental pH.

DISCUSSION

Previous reports from our group and many others have focused on the nature and extent of the acid response pathways in oral

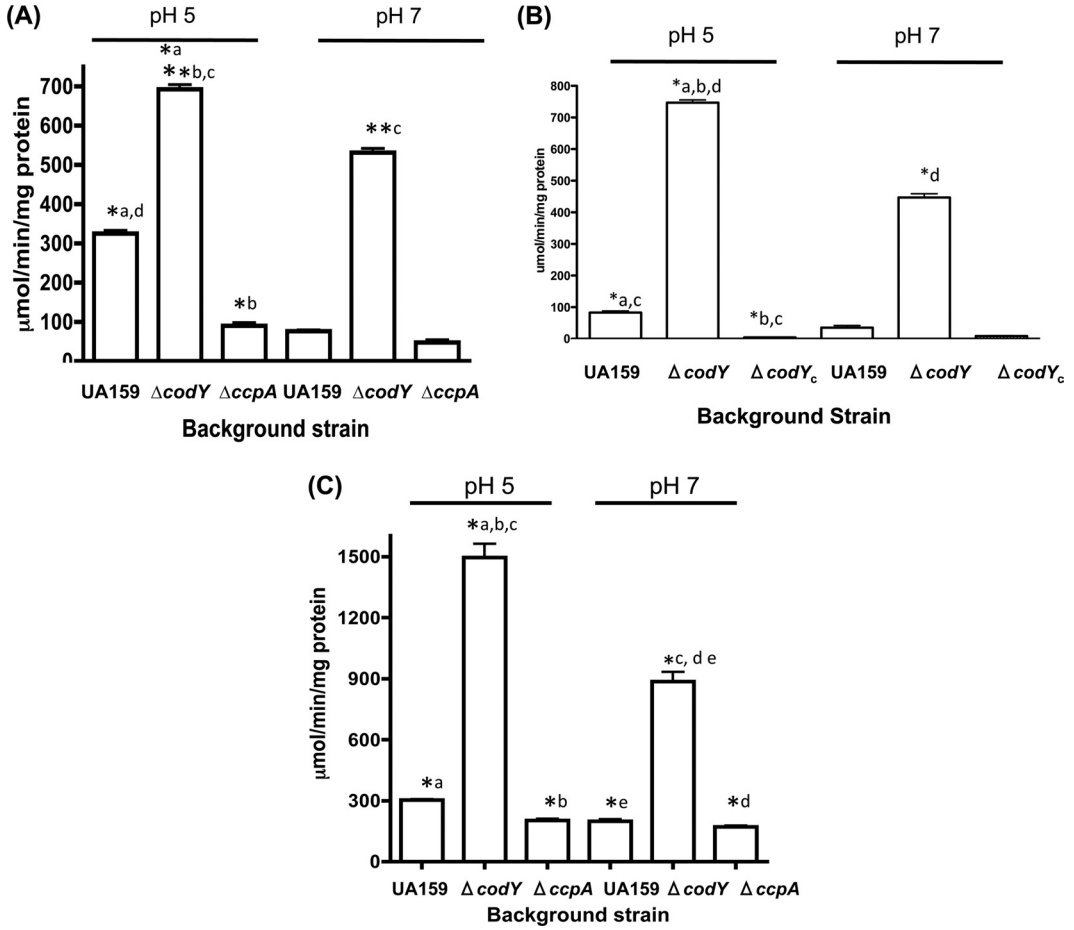

FIG 8 (A) Transcription of *ilvE* is regulated by CodY and CcpA. CAT activities are shown for a full-length *ilvE* promoter-*cat* fusion in the following background strains: UA159, Δ*codY* strain, and Δ*ccpA* strain. Cultures were grown in BHI medium as described in Materials and Methods. Statistical significance was determined by Student's *t* test. *, $P \leq 0.00001$; **, $P \leq 0.0000001$ ($n = 3$). Pairwise comparisons are represented by lowercase letters. (B) Genetic complementation of CodY restores transcriptional repression of *ilvE*. CAT activities are shown for a full-length *ilvE* promoter-*cat* fusion in the following background strains: UA159, Δ*codY* strain, and complemented Δ*codY* strain (Δ*codY*$_c$). Cultures were grown in BHI medium as described in Materials and Methods. Statistical significance was determined by Student's *t* test. *, $P \leq 0.00001$ ($n = 3$). Pairwise comparisons are represented by lowercase letters. (C) Nutritional limitation and the absence of CodY enhance transcription of *ilvE*. CAT activities are shown for a full-length *ilvE* promoter-*cat* fusion in the following background strains: UA159, Δ*codY* strain, and Δ*ccpA* strain. Cultures were grown in FMC minimal medium as described in Materials and Methods. Statistical significance was determined by Student's *t* test. *, $P \leq 0.0000001$ ($n = 3$). Pairwise comparisons are represented by lowercase letters.

streptococci (7). One mechanism of protection from acidification is the rerouting of carbon away from lactic acid. Rerouting carbon away from pyruvate would have the effect of reducing organic acid production from fermentation and would generate molecules important for bacterial homeostasis. An overflow pathway directly linked to acidic end product rerouting is provided by branched-chain amino acid biosynthesis. Proteins responsible for branched-chain amino acid biosynthesis are upregulated during acid stress and, due to their link in rerouting pyruvate, are important for acid tolerance and amino acid metabolism (Fig. 9) (7). Aminotransferases, which are key metabolic enzymes for bcAA biosynthesis, are central players in the modulation of acidic end products. The importance of IlvE, a branched-chain amino acid aminotransferase, was demonstrated when a strain carrying a deletion in the gene exhibited reduced acid tolerance (28). These findings indicated that altering the metabolic profile of *S. mutans* toward production of bcAAs affected the acid-adaptive capabilities of the organism and cemented the link between amino acid metabolism and acid resistance. Expression of *ilvE* was responsive to acidification of the growth medium, which also implied that transcription of the gene was either required or, at minimum, important during acid adaptation.

As a branched-chain amino acid biosynthetic gene, it was expected that *ilvE* could also be controlled by a combination of regulatory signals. Chief among these is regulation by CodY, a global regulator of genes in *S. mutans* known to be important for acid tolerance as well as virulence. The function of CodY as a repressor is dependent upon branched-chain amino acid levels, to which IlvE could contribute via its role as an aminotransferase. The importance of *ilvE* in acid tolerance and the identification of the gene as a target for CodY regulation led us to explore transcriptional regulation of *ilvE* by CodY in combination with the role of acidification of the extracellular environment.

The presence of a CodY consensus binding sequence and previous studies (11, 17, 20, 22) identifying *ilvE* as a target for CodY regulation prompted an investigation to determine if CodY was

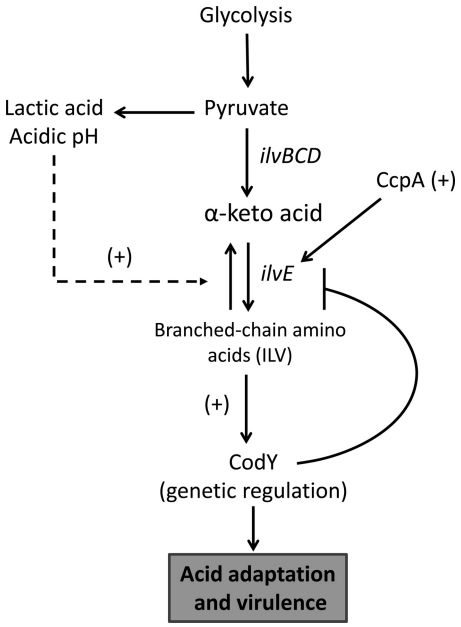

FIG 9 Pyruvate overflow pathway through branched-chain amino acid metabolism. The metabolism of carbohydrates leads to the generation of pyruvate, which results in the generation of lactic acid, the major acidic end product responsible for tooth demineralization and caries formation. Pyruvate can be rerouted to the synthesis of branched-chain amino acids by the action of the *ilv* biosynthetic pathway. CcpA aids in the activation of *ilv* when cells are exposed to an acidic environment. Branched-chain amino acids, in turn, are important molecules for genetic regulation via their role as coeffectors for CodY transcriptional regulation. As branched-chain amino acid synthesis increases, CodY-dependent repression of the *ilv* genes also increases, resulting in repression of these gene transcripts when bcAAs are in excess.

able to interact physically with the *ilvE* promoter region in *S. mutans*. EMSAs showed that CodY bound the *ilvE* promoter region in a specific manner, since the removal of the CodY consensus sequence caused an abrogation of the interaction (Fig. 5). The interaction was also shown to be similar to CodY binding in *L. lactis*, where isoleucine enhanced the binding of CodY to *ilvE* without a further requirement for GTP. These data show that in *S. mutans*, branched-chain amino acids are the predominant coeffectors in mediating an interaction between CodY and *ilvE* and that CodY repression responds to changes in amino acid levels, not to changes in the levels of GTP (Fig. 3 and 4). More importantly, when transcriptional reporter assays were performed, branched-chain amino acids were shown to repress *ilvE* transcription. The role of these metabolites as effector molecules would be expected, since biosynthetic genes usually exhibit a response to their cognate biosynthetic end products. In this case, the bcAA products of *ilvE* have been shown to enhance binding of CodY to the ATase encoded by *ilvE* (Fig. 6). Although *ilvE* transcription was not dramatically elevated in the parent strain under nutrient-limiting conditions, bcAAs were unable to repress transcription as they did in rich medium, thus demonstrating the requirement of these amino acids for growth in minimal medium and, in part, explaining the derepression of *ilvE* transcription (Fig. 6B). A characteristic of *ilvE* transcription in minimal medium was the 2-fold increase in transcriptional activity at pH 7 compared to expression in rich medium. This may have been caused by nutrient limitation conditions, where bcAA-mediated repression of *ilvE* transcription would not occur, potentially due to the fact that bcAA levels cannot accumulate and therefore CodY is unable to exert its repressive action on *ilvE*. In support of the increased transcriptional activity observed under nutrient-limiting conditions, experiments performed using FMC minimal medium showed that ATase activity for all three branched-chain amino acids was also increased under these conditions (data not shown). These findings suggest that there is a greater requirement for bcAA synthesis under nutrient-limiting conditions that outpaces the increased expression resulting from the low pH of the growth environment (Fig. 6B).

Enzymatic characterization of the Δ*ilvE* strain demonstrated that IlvE degrades branched-chain amino acids, as evidenced by the reduced catabolic properties of the deletion strain compared to the parent strain. It was also shown by our group that deletion of the gene causes a nutritional requirement for bcAAs, thereby establishing a contribution to bcAA synthesis (28). In the Δ*ilvE* strain, *ilvE* transcription was reduced approximately 2-fold (Fig. 7). In our previous work, we demonstrated that isoleucine catabolism in a Δ*ilvE* strain was reduced approximately 75% compared to that in the parent strain. Therefore, larger intracellular amounts of isoleucine in the absence of IlvE could enhance CodY repression and account, in part, for the reduced levels of *ilvE* transcriptional activity in this background.

As suggested by transcriptional and binding assays, CodY was shown to be a regulator of *ilvE* transcription. In the absence of CodY, *ilvE* expression is derepressed, as illustrated by the 2-fold increase in *ilvE-cat* specific activity compared to the levels seen in the parent strain background (Fig. 8B). The absence of CodY also had an effect on the transcriptional activity assayed at neutral pH, where a 5-fold increase in transcription of *ilvE* was detected in the *codY* deletion background compared to that in the parent strain. A similar, nearly 2-fold increase in *ilvE* transcription was also observed when activity was assayed in minimal medium, a condition that could mimic the absence of CodY, since bcAAs are limited. The data indicate that CodY is also important for pH-dependent regulation of *ilvE* transcription and that, in its absence, derepression occurs at nonacidic pH values.

Strong repression by CodY led us to explore the roles of other regulatory proteins implicated in regulation of biosynthetic genes, such as CcpA, in regulation of *ilvE*. In a Δ*ccpA* strain, *ilvE* transcription was decreased compared to that in the parent strain, demonstrating that CcpA activates *ilvE* transcription (Fig. 8A), despite the presence of a consensus *cre* sequence within the intergenic region preceding *ilvE*. This could indicate that a lower-affinity binding site may exist within the *ilvE* promoter that functions as a binding site for CcpA or that CcpA is indirectly involved with the activation of *ilvE* transcription. In the absence of an activator, CodY repression is the predominant regulatory effector and may explain why there is no pH-dependent effect on *ilvE* transcriptional activity in the *ccpA* deletion strain. Under nutrient-limiting conditions, the Δ*ccpA* strain behaved similarly to the parent strain, displaying derepression independent of the pH of the growth medium.

The mechanism of coordinated regulation by these two proteins is based on our current understanding of *ilvE* regulation. Under rich, nonacidic growth conditions, the "basal" level of *ilvE* transcription (Fig. 10A) is maintained by CodY, which plays a major role in repression of the gene. When conditions change, due to rapid sugar metabolism and consequent acidification of the

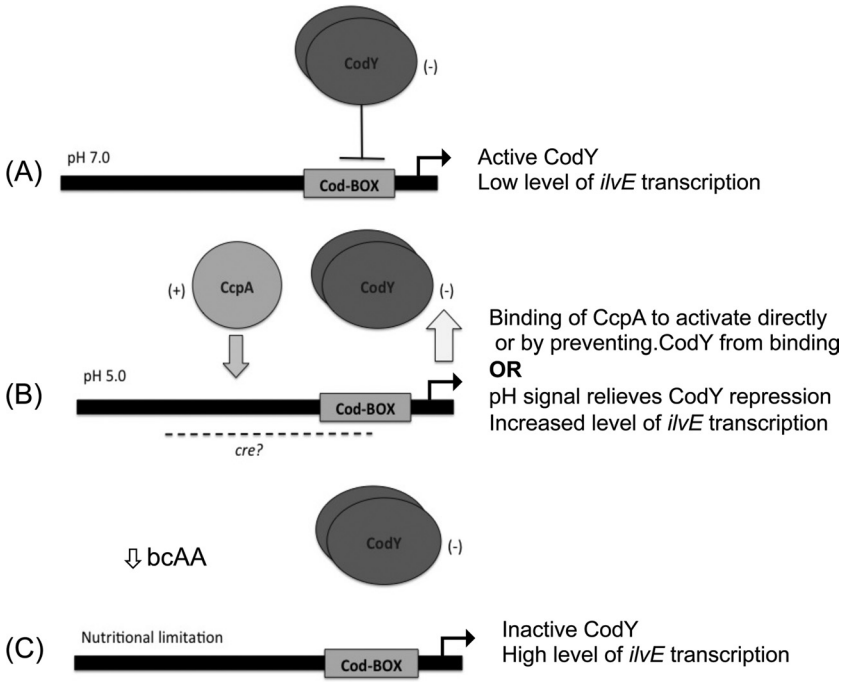

FIG 10 Proposed model for coordinated regulation of *ilvE* by CcpA and CodY. (A) The regulation of *ilvE* in *S. mutans* combines multiple signals, depending on the growth conditions and extracellular environment. Under nonacidic, nutrient-rich environmental conditions (pH 7.0), *ilvE* transcription is repressed by the action of CodY. (B) Under acidic conditions (pH 5.0), resulting from the metabolism of carbohydrates and subsequent depletion of nutrients, CodY derepression in combination with CcpA activation allows for increased transcription of *ilvE*. The CodY and CcpA proteins regulate *ilvE* by binding to domains within the *ilvE* intergenic region. The CodY-binding domain is required for interaction. The CcpA-binding domain has yet to be determined. (C) Nutritional regulation is solely dependent on CodY. CodY senses the levels of intracellular amino acids, which drop under nutrient-limiting conditions. Binding of CodY to its target genes is enhanced in the presence of isoleucine; hence, as the levels of bcAAs decrease, CodY repression is alleviated.

environment, CodY repression is partially alleviated, possibly by pH sensing in combination with CcpA activation, which results in higher transcript levels of *ilvE* at pH 5 (Fig. 10B). Activation seems to be self-limiting, as in many other organisms, since transcript levels of *ilvE* in the UA159 parent strain background at pH 5, where increased transcription of *ilvE* occurs, never reach those obtained in a *codY* deletion background. Positive regulation cannot overcome the negative regulation of *ilvE* unless CodY is either absent or induced by nutrient limitation, allowing for higher levels of transcription in response to the nutritional needs of the organism (Fig. 10C). This regulatory control allows for expression of *ilvE* under acid and nutritional stress.

A recent publication demonstrating an interaction between CcpA and CodY in *Bacillus subtilis* (45) encourages us to investigate the possibility of a direct interaction between these regulatory proteins as a mechanism for regulation of *ilvE*. Other genes involved in the acid tolerance response of *S. mutans* are currently being examined as potential targets for CodY and CcpA control. Determining whether regulation is due to complex formation between the two regulatory partners is of interest and may be important for the linkage of nutrition, acid adaptation, and virulence in *S. mutans*.

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