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Control of Expression of the Arginine Deiminase Operon of *Streptococcus gordonii* by CcpA and Flp

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In *Streptococcus gordonii* DL1, inactivation of the *ccpA* gene and a gene encoding an Fnr-like protein (Flp) demonstrated that CcpA was essential for carbohydrate catabolite repression and that Flp was required for optimal expression and anaerobic induction of the arginine deiminase system.

The arginine deiminase system (ADS) (encoded by the *arc* operon) is a multienzyme pathway that catalyzes the conversion of arginine to ornithine, ammonia, and CO_2, with the concomitant production of ATP. The ADS is widely distributed among prokaryotes, and the primary structures of the enzymes involved in the AD pathway have been reasonably conserved throughout evolution. However, the genetic regulation characteristics of ADS differ among organisms. For instance, both *Pseudomonas aeruginosa* (17) and *Bacillus licheniformis* (26) utilize the ADS exclusively under anaerobic conditions through a transcriptional regulator belonging to the Anr (for "anaerobic regulation of arginine catabolism and nitrate reduction")/Fnr (for "fumarate nitrate reductase") family. Induction by Anr/Fnr can be further enhanced in the presence of arginine by ArgR, the transcriptional regulator. In some lactic acid bacteria, such as *Streptococcus sanguis* (8) and *Lactobacillus sakei* (31), the expression of the operon is under the control of carbon catabolite repression (CCR) and is inducible by arginine; however, the mechanisms for CCR and arginine induction are not fully understood. Furthermore, in some oral bacteria (such as *S. sanguis* NCTC10904 and *Streptococcus rattus* FA-1) the ADS can be repressed by aeration through an as-yet-undefined pathway (2). In the oral cavity, the ADS is one of two primary pathways for ammonia generation. Ammonia produced by the ADS can neutralize acids generated by bacterial glycolysis, thereby increasing dental biofilm pH. The elevated pH induced by arginine catabolism is thought to be important in inhibiting the development of tooth decay and in modulating the composition of plaque (1, 3). Among the comparatively few ADS-positive species that colonize the mouth, *Streptococcus gordonii* is one of the more abundant organisms in tooth biofilms and makes up a significant portion of healthy human supragingival dental plaque (3, 19). Thus, the ADS of *S. gordonii* may play a critical role in the prevention of dental caries.

Dong et al. previously reported that the *arc* operon in *S. gordonii* is arranged as *arcABCDT*. In addition to the genes encoding enzymes involved in the AD pathway, *arcR*, a gene encoding an activator for the *arc* operon, is located 3′ to *arcT* and transcribed in the opposite orientation. We also showed that the expression of the *S. gordonii arc* operon is inducible by arginine and subject to CCR. Arginine induction is mediated in large part by the activity of ArcR, but the molecular basis for CCR of the *arc* operon has not been defined (7). In most gram-positive bacteria, binding of CcpA (for "catabolite control protein A") (13) to a palindromic sequence, the carbon catabolite response element, results in repression of CCR-sensitive operons (28). However, other pathways such as CcpB (4), CcpC (15), and phosphotransferase system-dependent CCR (exerted through phosphorylation of specific transcriptional regulators) are known to be involved in CCR of some gram-positive bacteria (10, 20, 27). To determine whether CcpA is the primary protein exerting CCR of *arc* operon expression in *S. gordonii*, an apparent CcpA homolog, which was identified from the partial *S. gordonii* genome database (The Institute for Genomic Research website [http://www.tigr.org]), was amplified by PCR using primers *ccpA*-S and *ccpA*-AS (Table 1), with *S. gordonii* DL1 chromosomal DNA as the template. An erythromycin resistance cassette was then cloned into the unique EcoRV site within the *ccpA* gene, and the resulting plasmid was used to transform *S. gordonii* to generate a CcpA-deficient derivative via double-crossover recombination. The *arcA* promoter (p_{arcA}) (obtained as a 337-bp fragment upstream of the *arcA* start codon by PCR with primers $p_{arcA}5'$ and $p_{arcA}3'$) (Table 1) was directly fused to a chloramphenicol acetyltransferase (CAT) gene (*cat*) in plasmid

TABLE 1. Primers used in this study

Primer	Sequence[a]	Application
*flp*5′	5′-CCAGTTTTATATGCCGTA-3′	*flp* amplification
*flp*3′	5′-GTCCAGTAGACTAACTTTCT-3′	*flp* amplification
flp SmaI-S	5′-TCTTTTTTTCTGGAGA**CCCGGG**TGATCGCCTTTTTCTTC-3′	Adding SmaI site to *flp*
flp SmaI-AS	5′-GAAGAAAAAGGCGATCA**CCCGGG**TCTCCAGAAAAAAAGA-3′	Adding SmaI site to *flp*
ccpA-S	5′-ACAGACGATACAGTAACCAT-3′	*ccpA* amplification
ccpA-AS	5′-TAGTCAACATACGCATAC-3′	*ccpA* amplification
$p_{arcA}5'$	5′-AAAAGGTTGAGAGAAGAGCTCCGTATCAGCTATGAG-3′	p_{arcA} amplification
$p_{arcA}3'$	5′-GGATGTGTAGACATGGATCCTCCTCCTTGTTCTTTG-3′	p_{arcA} amplification

[a] The introduced restriction recognition site within *flp* is indicated in boldface characters.

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TABLE 2. CAT-specific activities in wild-type and CcpA-deficient strains of *S. gordonii*/p_{arcA}-*cat* and wild-type and Flp-deficient strains of *S. gordonii*/p_{arcA}-*cat* under aerobic and anaerobic growth conditions

S. gordonii strain	CAT-specific activity under the indicated conditions[a]			
	With galactose[b]	With glucose[b]	Anaerobic[c]	Aerobic[c]
WT[d]	1,024 ± 17	60 ± 6	794 ± 28	159 ± 21
CcpA deficient	907 ± 15	882 ± 35		
Flp deficient			70 ± 14	37 ± 6

[a] The values presented are the means and standard deviations of three independent experiments and are expressed as nanomoles of chloramphenicol acetylated per minute per milligram of total protein. All CAT assays were done in triplicate.

[b] Cells were grown in 5% CO_2 and 95% air and TY medium with the carbohydrate as indicated.

[c] All cultures were grown in TY medium–10 mM galactose–50 mM arginine under anaerobic conditions in a GasPak jar or under aeration conditions with shaking at 300 rpm (see text for details).

[d] WT, wild type.

pMC286, which was constructed by insertion of the promoterless *cat* gene from pC194 (14) into pGEM-Zf3(+) at BamHI and SphI sites. The fusion was constructed such that translation was driven from the *arcA* ribosome binding site. The transcriptional fusion was then released and cloned into pMJB8 (5), an *S. gordonii* integration vector that allows insertion of foreign DNA at the *gtfG* (glucosyltransferase gene) locus with the selection of a kanamycin-resistant phenotype. The construct was then used to transform wild-type and CcpA-deficient strains of *S. gordonii*. CAT activity (optical density at 600 nm ≅ 0.6 to 0.7) was measured in mid-exponential-phase cells grown in TY medium (29) with 10 mM glucose (a repressing sugar) or 10 mM galactose (a nonrepressing sugar) by the method of Shaw (24). In the wild-type background, the level of CAT activity in cells grown in TY medium with galactose was 17-fold higher than that seen with cells grown in TY medium with glucose (Table 2). In the CcpA-deficient strain, CCR was relieved and there was no significant difference in CAT activity between cells grown in galactose or glucose. These results indicated that (excluding other possible pathways such as those of CcpB, CcpC, and phosphotransferase system-dependent CCR) CcpA is the primary regulator for CCR of the *arc* operon in *S. gordonii*.

After the incomplete *S. gordonii* genome was searched, one 684-bp open reading frame (ORF) (located 263 bp 5′ to *arcA*) was predicted to code for a homologue of known proteins of the Crp/Fnr family, which has been proven to be responsible for anaerobic versus aerobic gene regulation in many gram-negative bacteria (16, 17, 30) and in some gram-positive bacteria, including *Bacillus subtilis* and *B. licheniformis* (6, 18). The proteins with the most similarity to this ORF (*Streptococcus pyogenes* SPY1548 [43% identical residues], *B. licheniformis* ArcR [37% identical residues], *P. aeruginosa* Anr [23% identical residues], *L. sakei* ArcR [34% identical residues], and *Enterococcus faecalis* ArcE [35% identical residues]) were putative members of the Crp/Fnr family that were either linked to, or already shown to be involved in, *arc* regulation. Thus, we designated this ORF *flp* (for "Fnr-like protein") in *S. gordonii*. A multiple-sequence alignment of Flp and selected representatives of the Crp/Fnr family showed that there are conserved residues throughout the proteins (Fig. 1). Although the overall level of similarity between Flp and each of the aligned proteins is not high, the predicted helix-turn-helix motif located in the C-terminal part of the proteins is more conserved. Of note, conserved residues (such as arginine and glutamic acid) (32) shown to be involved in recognition of the DNA binding site by Crp were found in *S. gordonii* Flp at positions 212 and 213 (Fig. 1). However, Flp in *S. gordonii* has only two cysteine residues instead of the four conserved cysteine residues that are usually found in other Fnr homologues (12, 21, 25). To date, only two other known Fnr-like proteins containing two cysteine residues that regulate the redox responses in *Lactococcus lactis* and *Lactobacillus casei* have been identified (11, 23).

It was found that the *flp* gene was preceded by a putative Shine-Dalgarno sequence and began with an ATG codon. In addition, a putative *rho*-independent terminator (5′-TTTCTTTTTTAGCAAAAACAAAGATTTTATAAAAAAATAAA-3′ [the bases forming the stem-loop are underlined]) was found between *flp* and *arcA* (Fig. 2), in consistency with our finding that *arc* gene expression is driven from its own promoter (7). We also examined the two ORFs immediately 5′ to the *flp* gene in *S. gordonii*; these ORFs were revealed in BLAST searches to be conserved hypothetical proteins.

To determine the function of Flp in regulation of *arc* operon expression, *flp* was amplified by recombinant PCRs with primers (Table 1) that were designed on the basis of the *flp* se-

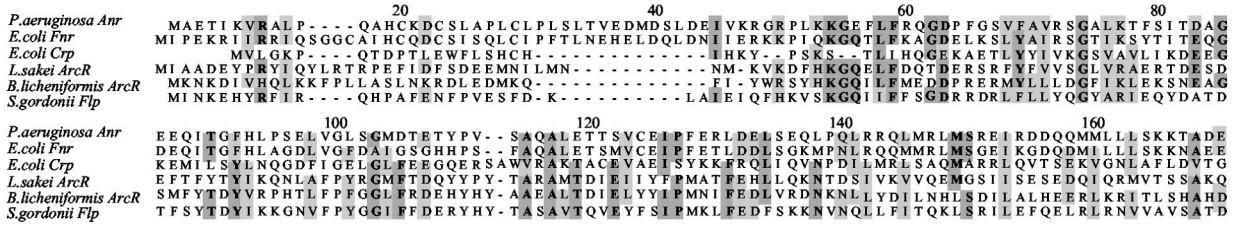

FIG. 1. Alignment of the deduced amino acid sequences of Flp and representative members of the Crp/Fnr family. The identical residues are indicated with boldface characters and gray-shaded boxes; the conserved residues Arg-212 and Glu-213 (potentially involved in protein-DNA interactions) are indicated by asterisks. This alignment was performed with ClustalW alignment and MacVector software.

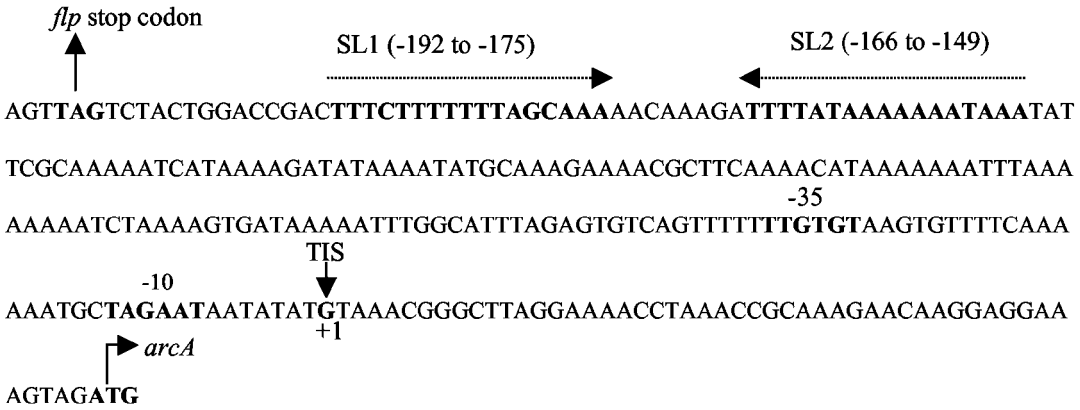

FIG. 2. Nucleotide sequence and relevant features of the 5′ region of *arcA*. The start codon of *arcA* and the stop codon of *flp* are indicated with solid arrows. The promoter that directs the transcription of *arcA* (7) is indicated with boldface characters. The two inverted repeats with the potential to function as terminators, SL1 (positions −192 to −175) and SL2 (positions −166 to −149), are between the *flp* and *arcA* genes and are indicated by boldface characters and dotted arrows. TIS, transcription initiation site.

quence identified from the partial sequence in the *S. gordonii* genome database. The introduction of a unique SmaI site 141 bp 3′ to the start codon of *flp* allowed the subsequent cloning of a spectinomycin resistance cassette into the *flp* gene, and the resulting plasmid was used to transform *S. gordonii* to generate a Flp-deficient derivative via double-crossover recombination. The p_{arcA}-*cat* fusion was then used to transform the Flp-deficient mutant and wild-type *S. gordonii*. Early-log-phase cells (optical density at 600 nm ≅ 0.25 to 0.35) grown in TY medium containing 10 mM galactose and 50 mM arginine under anaerobic conditions in a GasPak jar (BBL), or with aeration by shaking at 300 rpm with 50 ml of culture in a 250-ml flask (2), were harvested and used to measure CAT activity. Inactivation of *flp* resulted in 11- and 4.3-fold decreases in CAT activity compared to the results seen with the wild-type strain grown under anaerobic and aerobic growth conditions (Table 2), respectively, which suggested that Flp was an activator of *arc* operon expression in *S. gordonii*. In the Flp-deficient strain, furthermore, CAT activity in cells grown under anaerobic conditions was less than twofold higher than that seen in aerobically grown cells compared to a fivefold difference in the results seen with the wild-type strain, which indicated Flp might be involved in anaerobic induction of the ADS in *S. gordonii*. So far, there is no evidence to indicate that Flp is a global regulatory protein, as it is in *Escherichia coli* and *P. aeruginosa* (9, 22, 30), but the possibility cannot be excluded that Flp regulates genes other than the *arc* operon.

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