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Environmental and Growth Phase Regulation of the *Streptococcus gordonii* Arginine Deiminase Genes[▽]

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A 1,026-bp open reading frame sharing significant similarity with *queA*, which encodes a predicted *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase responsible for queosine modification of tRNAs, was found immediately 5′ of the gene for the transcriptional activator (ArcR) of the arginine deiminase system (ADS) operon of *Streptococcus gordonii*. The role of QueA in bacterial physiology is enigmatic, but loss of QueA has been shown to compromise stationary-phase survival or virulence in certain enteric bacteria. Interestingly, *S. gordonii* appears to be unique among ADS-positive bacteria in the linkage of *queA* with the ADS genes. A putative σ^{70} promoter (p_{queA}; TTGCCA-N_{21}-TATAAT) was mapped 5′ of *queA* by primer extension, and *queA* and *arcR* were shown to be cotranscribed. The expression from p_{queA} was found to be constitutive under all conditions tested, but the expression of p_{arcA}, which drives the expression of the *arc* structural genes, was enhanced in stationary phase and could be induced by low pH and arginine. QueA and CcpA acted repressively on *arc* transcription, but neither QueA-deficient strains nor CcpA-deficient strains showed significant differences in arginine deiminase enzyme activities compared with the wild-type strain. The growth rate of a QueA-deficient strain did not differ significantly from that of the parental strain, but the QueA-deficient strain did not compete well with the wild-type during serial passage. In addition to the finding that ADS expression can be regulated separately by growth phase and pH, a significant linkage between the ADS, translational efficiency modulated by QueA, and post-exponential-phase survival of *S. gordonii* was found.

The arginine deiminase system (ADS) consists of arginine deiminase (AD), ornithine carbamyltransferase, and carbamate kinase. The ADS catalyzes the conversion of arginine to ornithine, ammonia, and CO_2 with concomitant production of ATP (11). The ADS has a number of important physiological functions; it contributes to pH homeostasis, it protects cells from lethal acidification, and it provides ATP for growth and maintenance (8, 22, 30, 39). In addition, recent studies have linked the ADS with the pathogenicity of some organisms (12, 44). In the oral cavity, the ADS is one of two major ammonia-generating pathways (6), and several studies have demonstrated that there is an inverse relationship between arginine metabolism and dental caries (6, 34). A thorough analysis of the regulation and function of the ADS in oral biofilms is needed to understand the contribution of arginine catabolism to oral health and to establish a foundation for designing new strategies for caries prevention that capitalize on moderating dental plaque acidification (4, 6).

The ADS is widely distributed among prokaryotes, and the primary structures of the enzymes in the pathway have been conserved throughout evolution. In contrast, diversity in the gene organization and regulation of operons encoding the ADS have been revealed by physiologic and genetic studies (46). Most microorganisms studied so far have ADS genes that are organized in one cluster. The *arcA*, *arcB*, and *arcC* genes are conserved in the *arc* operons and encode AD, ornithine carbamyltransferase, and carbamate kinase, respectively. Additional genes are often associated with the ADS, including *arcD*, which encodes an arginine:ornithine antiporter, and *arcT*, which encodes a putative peptidase (45) that may release arginine from internalized peptides. In all cases examined thus far, regulation of the ADS in bacteria is tightly controlled, but the mode and mechanisms of control vary widely. For instance, both *Pseudomonas aeruginosa* and *Bacillus licheniformis* utilize the ADS exclusively under anaerobic conditions, and expression can be further enhanced in the presence of arginine (28, 29). In some lactic acid bacteria, such as *Streptococcus sanguis* and *Lactobacillus sakei*, the expression of the *arc* operon is under the control of carbon catabolite repression (CCR) and is inducible by arginine (18, 45). The ADS of *S. sanguis* and *Streptococcus rattus* can also be repressed by aeration (5).

We previously reported that the *arc* operon of *Streptococcus gordonii* DL1 is arranged as follows: *arcABCDT* (15). In addition to the genes encoding enzymes involved in arginine utilization, two other genes were shown to regulate *arc* operon expression. The first of these genes is *flp* (*F*nr-*l*ike *p*rotein), which is located 5′ of *arcA* (Fig. 1) and encodes an activator of *arc* transcription that responds to oxygen availability (14). The second gene, *arcR*, is located 3′ of *arcT* and is transcribed in the opposite direction (Fig. 1). ArcR is an activator of the *arc* operon that governs induction of expression by arginine (15). We also showed that the expression of the *arc* operon in *S. gordonii* was subject to CCR through the catabolite control protein A (CcpA)-catabolite response element pathway (14).

In this study, we observed that a 1,026-bp open reading frame (ORF) is located 5′ of *arcR* and is transcribed in the same direc-

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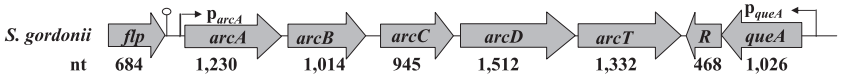

FIG. 1. Schematic diagram of the *arc* operon, *arcR*, *queA*, and *flp* of *S. gordonii* DL1, showing the gene order and arrangement. The size of each ORF (in nucleotides [nt]) is indicated. Transcription initiation sites upstream of *arcA* (14) and *queA* are indicated by arrows. A putative *rho*-independent terminator between *flp* and *arcA* is indicated by a loop.

tion, with only 15 bp separating the genes. This ORF encodes a predicted protein with significant similarity to *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase (QueA). Previous biochemical analysis of *Escherichia coli* revealed that the function of QueA was to modify tRNA with queuosine. Introduction of queuosine into tRNAs is initiated when a transglycosylase, encoded by the *tgt* gene, exchanges the guanine base at the first position of the GUN anticodon in tRNAs with $preQ_1$, a precursor of the base (queuine) and queuosine. QueA then attaches a substituted cyclopentenyl group to $preQ_1$ to produce epoxyqueuosine, which is followed by a B_{12}-dependent step in which the epoxide in epoxyqueuosine is reduced, yielding queuosine (20, 24, 25, 33, 36, 37).

Although our understanding of the biochemistry of queuosine modification of tRNAs is increasing (21, 40, 41), it is not yet clear what the impact of this modification on gene expression and cellular physiology is. A connection between queuosine modification of tRNA and the efficiency and fidelity of translation has been reported (32). However, there have been only two cases in which the effect of queuosine modification of tRNAs on a bacterial phenotype was documented. In one case, it was shown that the pathogenic potential of *Shigella flexneri* is diminished in a *queA* mutant (17). In the second case, *E. coli* mutants lacking QueA exhibited an apparently normal growth phenotype during favorable conditions, but upon entry into stationary phase the wild-type strain survived better than a queuosine-deficient strain (19, 31). QueA has been identified in a variety of other organisms by genomic and genetic studies, but functional analyses have not been undertaken yet. In this paper, a unique linkage of *queA* with genes of the ADS is described and new information implicating QueA and the ADS in post-exponential-phase homeostasis is presented.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains were grown in Luria-Bertani medium supplemented with ampicillin (100 μg ml^{-1}), kanamycin (Km) (40 μg ml^{-1}), erythromycin (Em) (300 μg ml^{-1}), spectinomycin (250 μg ml^{-1}), or chloramphenicol (20 μg ml^{-1}), where indicated. *S. gordonii* DL1 was maintained in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) at 37°C in 5% CO_2–95% air. Depending on the experiment, *S. gordonii* strains were cultured in tryptone-yeast extract (TY) medium (43) or the chemically defined medium FMC (38). All recombinant *S. gordonii* DL1 strains carrying a promoterless chloramphenicol acetyltransferase (CAT) gene (*cat*) fused to the *arcA* (p_{arcA}-*cat*) or *queA* (p_{queA}-*cat*) promoter cloned into pQY1 were selected and maintained on BHI agar supplemented with 5 μg ml^{-1} Em. The QueA-deficient strains were selected on BHI agar with Km (250 μg ml^{-1}). The CcpA-deficient strains constructed previously (14) were maintained on BHI agar with Em (10 μg ml^{-1}). Preparation of competent cells and transformation of *S. gordonii* were done as previously described (27).

RNA methods. *S. gordonii* was grown to mid-exponential phase (optical density at 600 nm [OD_{600}], ≈0.5 to 0.6) in TY medium containing 10 mM galactose and 50 mM arginine. RNA was extracted as previously described (10). For reverse transcription (RT)-PCR, the first-strand cDNA was amplified from total RNA with random hexamers. Then the first-strand cDNA was amplified by PCR at high stringency with primer SqueA (5′-TGCCTTTTCGACCAATTTCCACTT ACC-3′), which contained the sense sequence of *queA* located 151 bp 5′ of the *queA* stop codon, and primer AsarcR (5′-AACAATAACGCCATTGGCTTCA AGGAG-3′), which contained the antisense sequence of *arcR* located 110 bp 3′ of the *arcR* start codon. Primer extension was carried out as previously described (3), with primer annealing and reverse transcription performed at 42°C. An oligonucleotide designated EXTQueA (5′-AAGAGGAGTTTGGGCAATAAG CTCCTCTGGTAAGTG-3′), which contained the antisense sequence of *queA* from 30 to 65 bases 3′ of the *queA* start codon, was used in primer extension reactions. Primer extension products were analyzed alongside DNA sequencing products generated with the same primer.

Construction of wild-type and CcpA-deficient strains of *S. gordonii* carrying a p_{queA}-*cat* fusion. A 277-bp BamHI-SstI fragment immediately 5′ of the start codon of *queA* was amplified by PCR with primers $p_{queA}5'$ (5′-CTTTTCTCTA ATATTATT**GAGCTC**TATAGACCAATTTTTG-3′) and $p_{queA}3'$ (5′-ATCGGC AGTGTTCAT**GGATCC**TCCTTAAACAGTCCAT-3′). To facilitate fusion construction, SstI and BamHI recognition sequences (indicated by bold type) were included in primers $p_{queA}5'$ and $p_{queA}3'$, respectively. The product harboring p_{queA} was fused with the promoterless *cat* gene derived from pC194 (23) on pGEM-Zf3(+). The fusion was constructed so that translation was driven from the *queA* ribosome binding site. To facilitate integration of the p_{queA}-*cat* fusion, the integration vector pYQ1 was constructed. Plasmid pYQ1 is a derivative of pMJB8 (9) in which the ΩKm cassette was replaced with an Em cassette (42). Plasmid pYQ1 allows insertion of foreign DNA into the *gtfG* gene (encoding glucosyltransferase) with concomitant acquisition of an Em resistance phenotype. Plasmid pYQ1 carrying p_{queA}-*cat* was then used to transform the wild-type and CcpA-deficient strains of *S. gordonii* (14) in order to construct strains WT/p_{queA}-*cat* and $CcpA^-$/p_{queA}-*cat*.

Construction of QueA-deficient strains of *S. gordonii* carrying a p_{arcA}-*cat* fusion. A 1.26-kbp EcoRI-XbaI fragment containing the *S. gordonii queA* gene was amplified by recombinant PCR to introduce a unique BamHI site 515 bp 3′ of the start codon of *queA*. A BamHI fragment containing a nonpolar Km (26) resistance cassette was subsequently cloned into the BamHI site in the PCR product. The resulting plasmid was used to transform *S. gordonii* in order to generate nonpolar QueA-deficient mutants via double-crossover recombination. The correct configuration of integration was confirmed by PCR. A p_{arcA}-*cat* fusion, described elsewhere (14), was cloned into pYQ1 and used to transform the QueA-deficient and wild-type *S. gordonii* strains to construct $QueA^-$/p_{arcA}-*cat* and WT/p_{arcA}-*cat*, respectively.

Growth phase regulation of p_{queA} and p_{arcA} expression. The expression from p_{queA} and p_{arcA} as a function of growth phase was monitored by measuring the CAT activity in early-exponential-phase (OD_{600}, ≈0.25 to 0.3), mid-exponential-phase (OD_{600}, ≈0.5 to 0.6), early-stationary-phase (OD_{600}, ≈1.0 to 1.1), late-stationary-phase (3 h after cultures entered stationary phase), and overnight cultures grown in FMC containing 20 mM glucose with 20 mM arginine.

Relationship of p_{queA} to CCR. Expression from p_{queA} was monitored by measuring CAT activities of WT/p_{queA}-*cat* and $CcpA^-$/p_{queA}-*cat* cultured in TY medium with 20 mM arginine and 20 mM galactose or glucose. Cells were collected at mid-exponential phase (OD_{600}, ≈0.5 to 0.6).

Chemostat cultivation to monitor gene expression. To determine potential effects of pH and arginine induction without inducing changes in the growth rate or growth phase, WT/p_{queA}-*cat*, WT/p_{arcA}-*cat*, QueA-deficient strains, and CcpA-deficient strains carrying a p_{arcA}-*cat* fusion were grown in a Biostat *i* twin-controller chemostat (B. Braun Biotech, Inc., Allentown, PA) in TY medium (43) supplemented with 10 mM glucose at a dilution rate of 0.3 h^{-1}. The pH of the cultures was maintained at 5.9 or 7.0 by addition of 2 M KOH. Cultures were sampled when the cells reached steady state, which was achieved after 10 generations under particular growth conditions (43) or 1 h after pulsing with arginine.

Biochemical assays. CAT activity was measured as previously described (10). Briefly, cells were washed once with 10 mM Tris-HCl (pH 7.8), and cell pellets were quickly frozen in an ethanol-dry ice bath and stored at −80°C until assays were performed. Cells were disrupted with a Bead Beater (Biospec Products, Inc., Bartlesville, OK) for a total of 40 s at 4°C. The cell lysates were centrifuged at 18,000 × *g* for 5 min, and each soluble fraction was recovered and used to

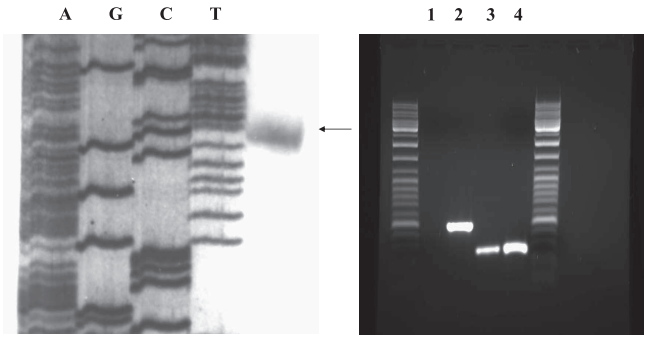

FIG. 2. Cotranscription of *queA* with *arcR* and primer extension analysis of *S. gordonii queA*. (A) Transcription initiation site of *S. gordonii queA* analyzed by primer extension. The arrow indicates the transcription initiation site at a G residue 181 bases upstream of the *queA* start codon. (B) RT-PCR analysis of mRNA from *S. gordonii* grown in TY medium containing 10 mM galactose with 50 mM arginine. Primers specific for the *arcR* and *queA* intergenic regions were used to amplify cDNA. Lane 1, negative control, in which the reaction mixture contained mRNA from *S. gordonii* but no RT was performed; lane 2, positive control, in which the control RNA from the RT reaction kit was used as the template to perform RT-PCR as recommended by the supplier; lanes 3 and 4, *arcR-queA* intergenic region, in which mRNA from *S. gordonii* and chromosomal DNA, respectively, were used as the templates for RT-PCR.

measure the CAT activity by the method of Shaw (35). The concentration of protein was determined by using a Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard. AD activity was measured by monitoring citrulline production from arginine, as previously described (2). Cells were harvested by centrifugation, washed once with 10 mM Tris-maleate buffer (pH 6.8), and resuspended using 1/10 the original culture volume in the same buffer. The cells were permeablized by vortexing them with toluene and were collected by centrifugation at 18,000 × *g*. The supernatant fluid was discarded, and the pellet was resuspended in 10 mM Tris-maleate buffer and used to measure AD activity in a reaction mixture containing 20 mM arginine, 10 mM hexanoic acid, and 50 mM Tris-maleate buffer (pH 6.0). The concentration of protein used in each assay was determined as described above.

Real-time PCR. Levels of *arcA* expression in the wild-type, QueA-deficient, and CcpA-deficient strains carrying the p_{arcA}-*cat* fusion were quantified by real-time PCR. Cells were cultured in TY media with 20 mM galactose and 20 mM arginine to mid-exponential phase. The primers used were *arcA* antisense (5′-GACCGCGAACCAATTCACTTCC-3′) and *arcA* sense (5′-CGCTCCAGGTGTTGTTGTTGTG-3′). Extraction of RNA, RT-PCR, and real-time RT-PCR were performed as previously described (1).

Competition-persistence comparison. Overnight cultures of the wild-type and Km-resistant QueA-deficient strains of *S. gordonii* were diluted 1:100 in TY medium containing 0.2% glucose or TY medium containing 0.2% galactose and 10 mM arginine. When the OD_{600} of the cultures reached 0.5 to 0.6, equal volumes of the wild-type and QueA-deficient strains were mixed, and the culture was diluted 1:100 with fresh medium (zero subculture). The cultures were grown for 4 days, and samples were removed each day to determine the number of CFU/ml of culture. The levels of the wild-type and QueA-deficient *S. gordonii* strains in the cultures were determined by plating dilutions on BHI agar and BHI agar with Km (250 μg ml^{-1}).

RESULTS AND DISCUSSION

***queA* and *arcR* are cotranscribed from a promoter 5′ of *queA*.** To locate the transcriptional initiation site of the *queA* gene, primer extension was performed. A single product, which was 181 bases 5′ of the *queA* start codon and started with a G residue, was observed. A putative σ^{70} promoter, TTGCCA-N_{21}-TATAAT, was identified 8 bases 5′ of the transcription initiation site (Fig. 2A). The results of the primer extension analysis were further confirmed by 5′ random amplification of cDNA ends (data not shown). It is unlikely that a functional promoter is present in the 310 bp 5′ of the *arcR* start codon, since when we tried to complement an ArcR-deficient strain (13) with the entire *arcR* structural gene and 310 bp of DNA upstream of the *arcR* start codon delivered on plasmid pDL278 (16), arginine-dependent activation of ADS expression could not be restored (data not shown). However, when the promoter for the urease operon of *Streptococcus salivarius*, which has been demonstrated to be an efficient promoter for gene expression in *S. gordonii* (9), was fused with the entire *arcR* gene and cloned onto pDL278, the resulting construct was able to restore the AD activity to wild-type levels (data not shown).

By searching the *S. gordonii* genome at http://ncbi.nlm.nih.gov, a 1,026-bp ORF that was 15 bp 5′ of, and was transcribed in the same orientation as, *arcR* was identified. Based on BLAST searches, this ORF was predicted to code for an *S*-adenosylmethionine:tRNA ribosyltransferase-isomerase involved in queosine modification of tRNAs. The overall similarity of the deduced amino acid sequences of the product of this ORF and QueA of other bacteria was high (Table 1). Thus, we designated this gene in *S. gordonii queA*. The ORF immediately 5′ of the *queA* gene in *S. gordonii*, whose product was revealed by BLAST searches to be a conserved hypothetical protein, was transcribed in the orientation opposite that of *queA* transcription.

The *queA* gene was preceded by a putative Shine-Dalgarno sequence and began with an ATG codon. No putative *rho*-independent terminator could be located between *queA* and *arcR*. To determine whether *queA* and *arcR* were cotranscribed, RT-PCR using primers that were internal to *queA* and *arcR* was employed, as described in Materials and Methods. A PCR product of the expected size was detected after RT-PCR, indicating that a continuous *queA-arcR* transcript was present (Fig. 2B).

We examined other known bacterial *arc* operons and their flanking regions in sequenced bacterial genomes to determine whether it was common to find *queA* and *arcR* genetically linked. We found *queA* genes in *Streptococcus pyogenes*, *Enterococcus faecalis*, *Bacillus subtilis*, *Lactococcus lactis*, *B. licheniformis*, *P. aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*, but none of them were linked to the *arc* operons (Fig. 3). We also searched for the *tgt* gene, which encodes the tRNA-guanine transglycosylase that is needed for the first step in queosine modification of tRNA. An apparent *tgt* gene was found in all cases and was linked to *queA*, except in the streptococci, *E. faecalis*, and *L. lactis* (the latter two

TABLE 1. Homologies of the deduced amino acid sequence of *S. gordonii* QueA with the QueA sequences of other bacteria

Species	*S. gordonii* QueA homology	
	% Similarity	% Identity
S. pneumoniae	92	85
S. pyogenes	90	83
E. faecalis	88	76
B. subtilis	78	65
E. coli	67	48
S. flexneri	67	48

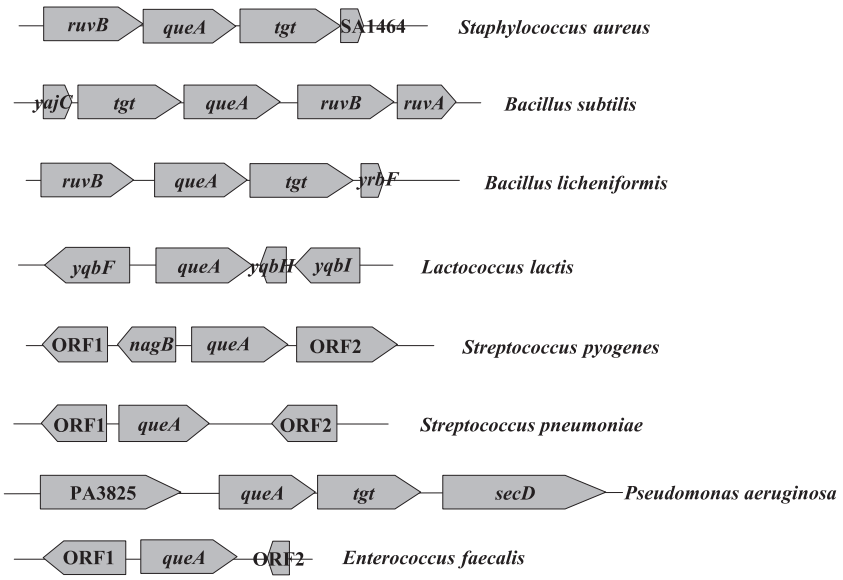

FIG. 3. Genetic arrangement of *queA* in ADS-positive bacteria. In *S. aureus*, *ruvB* encodes a holiday junction resolveasome helicase subunit and SA1464 encodes a hypothetical protein. In *B. subtilis*, *yrbF* encodes a hypothetical protein, *tgt* encodes queuine tRNA ribosyltransferase, *ruvB* encodes a holiday junction resolveasome helicase subunit, and *ruvA* encodes a holiday junction DNA helicase. In *L. lactis*, *yqbF* encodes a hypothetical protein, *yqbH* encodes a transcriptional regulator, and *yqbI* encodes a hypothetical protein. In *S. pyogenes*, ORF1 encodes a putative pseudouridylate synthetase, *nagB* encodes a putative *N*-acetylglucosamine-6-phosphate isomerase, and ORF2 encodes a hypothetical protein. In *S. pneumoniae*, ORF1 encodes glucosamine-6-phosphate isomerase and ORF2 encodes a surface protein. In *B. licheniformis*, *ruvB* encodes a holiday junction resolveasome helicase subunit, *tgt* encodes queuine tRNA ribosyltransferase, and *yrbF* encodes a preprotein translocase subunit. In *P. aeruginosa*, PA3825 encodes a hypothetical protein, *tgt* encodes queuine tRNA ribosyltransferase, and *secD* encodes a preprotein translocase subunit. In *E. faecalis*, ORF1 encodes a glyoxylase family protein and ORF2 encodes a transcriptional regulator belonging to the Cro/CI family.

organisms were previously members of the genus *Streptococcus*). In the *S. gordonii* genome, *tgt* was not linked to *queA* or the ADS genes. Thus, low-G+C-content gram-positive cocci show substantial divergence in the genomic organization of the *tgt* and *queA* genes, and *S. gordonii* is unique in the linkage of *queA* and the *arc* operon among organisms for which such sequence information is available.

Expression of p_{queA} and p_{arcA}. *S. gordonii* WT/p_{queA}-*cat* and WT/p_{arcA}-*cat* were grown in FMC containing 20 mM glucose with 20 mM arginine. The expression level of p_{queA}, which drives the expression of the *arc* operon regulator *arcR*, and the expression level of p_{arcA}, which drives the expression of the ADS genes (14, 15), were examined in different growth phases. The data showed that there was little difference in expression of p_{queA} as a function of growth phase, but p_{arcA} expression was significantly higher in cells that had entered stationary phase than in exponential-phase cells (Fig. 4A). Also, AD enzyme assays showed that ADS expression was upregulated during stationary phase (Fig. 4B). The pH of the culture medium dropped from 6.6 in early exponential phase to 4.4 in overnight cultures (Fig. 4). Consequently, enhanced ADS expression during stationary phase could benefit *S. gordonii* not only by providing ATP but also by moderating acidification of the environment. Caldelari et al. previously reported greater AD enzyme activity as cells entered stationary phase (7), although in their study, no effort was made to control pH and cells were cultivated in the presence of glucose. As a result, it was not possible to attribute increases in AD activity measured in stationary-phase cells solely to growth phase, since a decrease in the pH and alleviation of CCR in stationary phase may have contributed to the observations.

Since the expression of the *arcA* operon of *S. gordonii* is under the control of CCR (13), we examined whether the *queA-arcR* operon is also CCR sensitive. Wild-type and CcpA-deficient *S. gordonii*/p_{queA}-*cat* strains were cultured in TY medium with 20 mM arginine and 20 mM galactose (nonrepressing conditions) or glucose (repressing conditions) to mid-exponential phase, and p_{queA} expression was examined. No significant differences in CAT activities were detected between the wild type and the CcpA mutant (Table 2), indicating that there was no modulation of *queA-arcR* transcription by CCR.

To examine the effects of acidic conditions on ADS expression and to compare the expression of p_{queA} and p_{arcA} in response to pH and arginine, *S. gordonii* WT/p_{queA}-*cat* and WT/p_{arcA}-*cat* were grown in a chemostat at pH 7.0 and 5.9 to a steady state following a 10 mM arginine pulse for 60 min. The use of pH 5.9 was dictated by the observation that *S. gordonii* could not be maintained at steady state at lower pH values under the conditions tested because of washout. As noted with batch cultivation, no effects of arginine or low pH on p_{queA} expression were observed (Table 3). However, p_{arcA} expression was 1.5-fold higher at pH 5.9 than at pH 7.0 and was induced 1.3-fold by addition of arginine (Table 3). For both the WT/p_{queA}-*cat* and WT/p_{arcA}-*cat* strains, threefold upregulation of arginine deminase enzyme activity was observed under acidic conditions compared to the activity at neutral pH (Table 3). While the increase was consistent with *arcA* transcriptional data, the greater increase in AD activity than in reporter gene

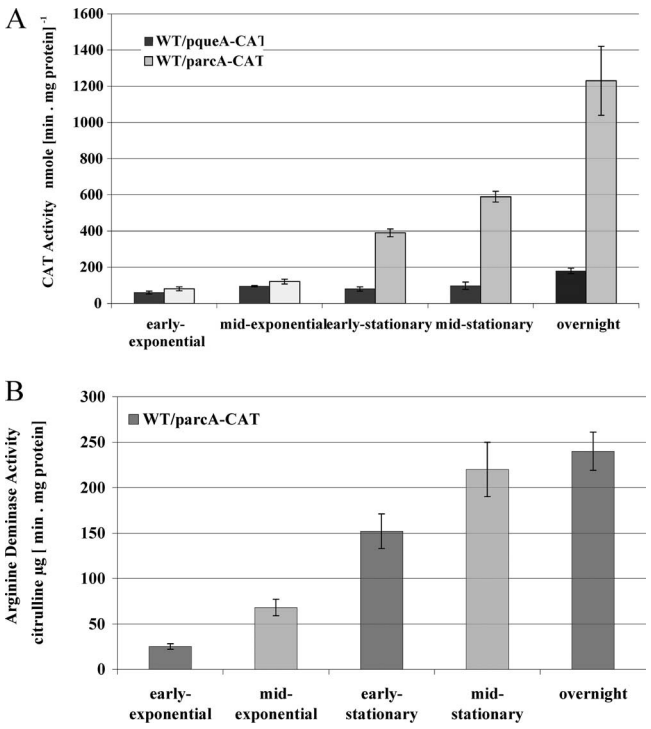

FIG. 4. CAT specific activities (A) and AD activities (B) of cells of WT/p_{queA}-*cat* and WT/p_{arcA}-*cat* in different growth phases. Cells were grown in FMC containing 20 mM glucose with 20 mM arginine to the early exponential, mid-exponential, early stationary, and late stationary phases and overnight and were used to measure the CAT specific activity and AD activity. The final pH values of the cultures were 6.6 at early exponential phase, 5.8 at mid-exponential phase, 5.2 at early stationary phase, 5.18 at stationary phase, and 4.4 in overnight cultures. The values are the means and standard deviations of three independent experiments.

activity raises the possibility that there is posttranscriptional regulation of AD expression at low pH. The expression level of p_{queA} was around 10-fold lower than that of p_{arcA}, as determined from comparisons of the levels of expression of *cat* in various culture conditions. We are presently exploring how other factors, such as lower oxygen tension and growth rate effects, could contribute to ADS upregulation in stationary phase.

Analysis of QueA effects on p_{arcA}. Very little is known about the physiological consequences of queuosine modification of tRNA in bacteria, although work with *E. coli* and *S. flexneri* suggested a possible role in modulation of translational efficiency, virulence, and post-exponential-phase survival (17, 19, 31). We posited that a possible role for QueA in ADS regulation is to modulate the translational efficiency of ArcR based on a comparison of the expression of the ADS operon promoter (p_{arcA}) of the wild-type strain and the expression of the ADS operon promoter of the QueA-deficient strain. The expression of the *arcA* promoter in the QueA-deficient strain was around threefold higher than that in the wild-type strain at both pH 7.0 and pH 5.9 in chemostat cultures (Table 3). These results were consistent with real-time PCR data showing that the *arcA* transcript was 14-fold more abundant in the QueA-deficient strain than in the wild-type strain (Fig. 5). These results support the hypothesis that QueA has a role in modulation of ADS gene expression, perhaps exerting a negative effect on the translation of *arc* regulatory proteins. Interestingly, no significant differences in AD activity were detected between the wild-type and QueA-deficient strains, in spite of the fact that transcription of *arcA* was increased in the *queA* mutant. These results imply that there is tight posttranscriptional control over the levels of AD activity in the cells, which is probably critical for maintaining appropriate arginine pools to support protein synthesis. It is also noteworthy that the *queA* transcript contains a large leader mRNA, raising the possibility that the efficiency of *queA* translation may be regulated or perhaps autoregulated. We are also investigating the possibility that there is a small protein that is expressed from the mRNA immediately upstream of *queA*.

CcpA is not required for low-pH induction of the ADS. CcpA appears to have primary control of CCR of the *arc* operon of *S. gordoniii* (14). Our chemostat studies revealed that the expression from the *arcA* promoter in the CcpA-deficient strain was about 2.5-fold higher than that in the wild-type strain at pH 7.0 and was 3-fold higher at pH 5.9. Real-time PCR demonstrated that *arcA* mRNA levels were 21-fold higher in the CcpA-deficient strain than in the wild-type background (Fig. 5), confirming that CcpA represses *arc* transcription (14). AD enzyme activity was also measured to test whether CcpA could affect the translation of *arcA*. As noted for the *queA* mutant, however, a substantial increase in *arcA* promoter activity was not accompanied by an increase in AD activity, further supporting the idea that there is tight posttranslational control over the amount of AD enzyme that can accumulate in cells, possibly as a result of differential mRNA stability and translational efficiency.

The results of the chemostat experiments clearly showed that a low pH and arginine could increase *arc* operon expression in wild-type *S. gordonii* (Table 3). Importantly, our results indicate that neither QueA nor CcpA is involved in the responses of *S. gordonii* to pH or arginine. It is fairly well established (14) that ArcR is the primary control circuit for arginine induction of the operon, but the factor(s) responsible for pH induction, as well as stationary-phase gene expression, remains undefined. While the importance of acid tolerance in persistence and virulence of oral streptococci is well documented, the molecular mechanisms of regulation of genes by pH in these organisms remain largely unexplored. The ADS will undoubtedly prove to be valuable for identifying factors responsible for activation or derepression of genes in response to acidic conditions.

TABLE 2. CAT specific activities of wild-type and CcpA-deficient p_{queA}-*cat* strains of *S. gordonii* grown in TY medium with 20 mM arginine and 20 mM galactose or glucose

Strain	CAT sp act[a]	
	With galactose	With glucose
WT/p_{queA}-*cat*	61.73 ± 11.21	64.73 ± 9.31
CcpA$^-$/p_{queA}-*cat*	70.13 ± 19.72	76.28 ± 15.74

[a] Cells were collected at mid-exponential phase (OD_{600}, 0.5 to 0.6). The data are the means ± standard deviations of three independent experiments and are expressed in nanomoles of chloramphenicol acetylated per minute per milligram of total protein. All CAT assays were done in triplicate.

TABLE 3. CAT specific activities and AD activities of wild-type, CcpA-deficient, and QueA-deficient p_{arcA}-*cat S. gordonii* strains grown in chemostats containing TY medium at pH 7.0 and 5.9 separately with 10 mM glucose at a dilution rate of $0.3h^{-1}$[a]

Strain	pH	CAT activity[b]		AD activity[c]	
		Before arginine pulse	After arginine pulse	Before arginine pulse	After arginine pulse
WT/p_{queA}-*cat*	7.0	28.98 ± 0.93[d]	26.80 ± 0.82[d]	56.10 ± 6.41	102.10 ± 8.57
	5.9	30.12 ± 0.46[d]	30.72 ± 1.60[d]	138.00 ± 27.04	190.81 ± 23.77
WT/p_{arcA}-*cat*	7.0	216.70 ± 2.12	380.99 ± 9.46	58.35 ± 6.71	116.60 ± 16.69
	5.9	301.24 ± 12.88	450.25 ± 10.24	126.95 ± 12.73	241.45 ± 44.35
CcpA$^-$/p_{arcA}-*cat*	7.0	540.96 ± 3.90[e]	532.92 ± 30.21[e]	60.95 ± 22.22	94.83 ± 12.02
	5.9	1,025.26 ± 5.12[e]	1,004.00 ± 6.70[e]	155.13 ± 27.57	160.52 ± 14.20
QueA$^-$/p_{arcA}-*cat*	7.0	680.95 ± 102.55[e]	1,041.87 ± 119.35[e]	68.81 ± 3.39	106.51 ± 4.19
	5.9	1,071.10 ± 168.17[e]	1,694.36 ± 109.52[e]	175.12 ± 33.48	168.34 ± 2.87

[a] Cells were cultured to steady state and then pulsed with 10 mM arginine for 60 min.
[b] The data are the means ± standard deviations of three independent experiments and are expressed in nanomoles of chloramphenicol acetylated per minute per milligram of total protein. All CAT assays were done in triplicate.
[c] The data are the means ± standard deviations of three independent experiments and are expressed in micrograms of citrulline per minute per milligram of total protein. All AD assays were done in triplicate.
[d] The CAT activities of WT/p_{queA}-*cat* differed significantly ($P < 0.05$) from those of WT/p_{arcA}-*cat* when cells were cells were cultured in the same environmental conditions, including pH and arginine concentration.
[e] The CAT activities of the CcpA$^-$/p_{arcA}-*cat* and QueA$^-$/p_{arcA}-*cat* strains differed significantly ($P < 0.05$) from those of WT/p_{arcA}-*cat* when cells were cultured in the same environmental conditions, including pH and arginine concentration.

Growth of *S. gordonii* and QueA-deficient strains. An *E. coli* mutant lacking QueA exhibited a decreased ability to survive in stationary phase compared to the wild-type strain (19, 31). To determine if the QueA-deficient *S. gordonii* strain has a growth deficiency compared to the wild-type strain in stationary phase, we examined the growth rates of the wild-type and *queA* mutant strains of *S. gordonii.* No significant differences were detected in the growth rates of the wild-type and *queA* mutant strains of *S. gordonii* in FMC containing glucose or galactose with arginine (data not shown). Therefore, a persistence experiment was carried out by using serial passage of a mixture of the wild-type and mutant strains for 4 days in TY medium containing 0.2% glucose or TY medium containing 0.2% galactose with 10 mM arginine (Fig. 6). Such competition experiments can sensitively detect minor changes in the fitness of a mutant strain. From day 1 to day 4 in both media, the proportion of QueA-deficient cells declined (Fig. 5), suggesting that the lack of queuosine modification in *S. gordonii* may

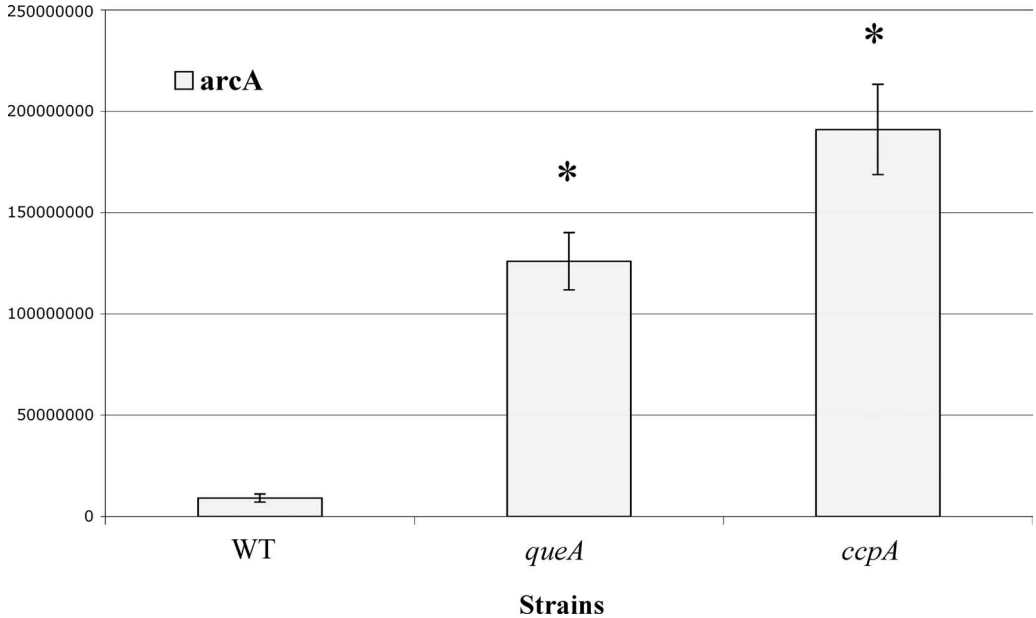

FIG. 5. Quantitative real-time PCR of *arcA* gene expression. After RT from 1 μg of total RNA from wild-type, QueA-deficient, and CcpA-deficient strains of *S. gordonii*, the amount of *arcA* gene cDNA was determined by real-time PCR using SYBR green. The data are means ± standard deviations, which were obtained using three different RNA preparations and RT reactions. An asterisk indicates that the copy number of the *arcA* mRNA of the QueA-deficient or CcpA-deficient strain differs significantly from that of the wild-type strain of *S. gordonii* ($P < 0.05$, as determined by the Student *t* test).

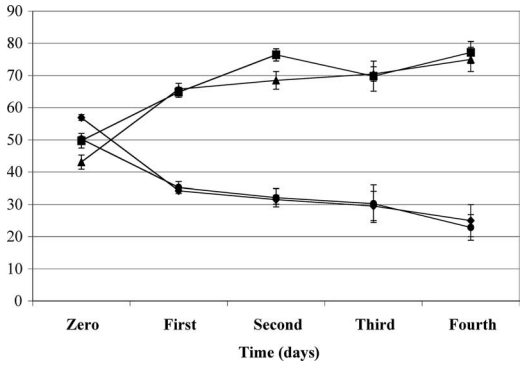

FIG. 6. Persistence experiment using a mixed inoculum containing wild-type *S. gordonii* and a nonpolar QueA-deficient strain. The proportions of the wild type (▲ and ■) and the QueA-deficient strain (◆ and ●) in TY medium containing 0.2% glucose (◆ and ▲) and TY medium containing 0.2% galactose (● and ■) with 10 mM arginine are shown. The values are the means and standard deviations (error bars) of three independent experiments.

adversely affect the competitive fitness of the organism. Although the final pH of the glucose culture was 0.5 pH unit lower than that of the galactose-arginine culture, no significant differences in the proportions in the cultures were detected (Fig. 5). Thus, the poorer competition associated with the deficiency of the QueA mutant did not correlate closely with pH and arginine metabolism, so the underlying basis for the defect remains to be determined.

Summary. In summary, the findings obtained in this study enhance our understanding of the mode and mechanisms of regulation of the ADS of *S. gordonii* and highlight the complexity underlying differential expression of *arc* genes. In addition, the results not only revealed a novel association between QueA, the *arc* operon, and post-exponential-phase gene expression but also demonstrated that ADS gene expression, in addition to being highly regulated by carbohydrate and arginine, is sensitive to pH and is upregulated in stationary phase independent of CCR or pH. Given that the nutrient source, the growth rate, pH, oxygen, and other influences and stresses that regulate ADS expression also have a profound impact on oral biofilm physiology and the expression of virulence, the ADS continues to be an excellent model for studying gene regulation and physiology in oral streptococci. In addition, there is tremendous potential to exploit the ADS and arginine catabolism in preventive strategies against dental caries and other oral diseases (6), so this exploration provided considerable practical knowledge for developing strategies to optimize ADS expression in oral biofilms.

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