Global Regulation by (p)ppGpp and CodY in *Streptococcus mutans*^{▽†}

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The RelA, RelP, and RelQ enzymes are responsible for the production of the alarmone (p)ppGpp in *Streptococcus mutans*. A strain lacking all three synthetases ($\Delta relAPQ$) does not grow in minimal medium lacking the branched-chain amino acids (BCAA) leucine or valine but grows well if isoleucine is also omitted. Here, we investigated whether there was a correlation between growth in the absence of leucine and valine with (p)ppGpp pools and the activation of CodY. By using a combination of single, double, and triple mutants lacking the (p)ppGpp synthetase enzymes, we demonstrated that the ability to grow in the absence of leucine or valine required basal levels of (p)ppGpp production by RelP and RelQ. The introduction of a *codY* mutation into the $\Delta relAPQ$ strain fully restored growth in medium lacking leucine or valine, revealing that the growth-defective phenotype of $\Delta relAPQ$ was directly linked to CodY. Lowering GTP levels through the addition of decoyinine did not alleviate CodY repression or affect the expression of genes involved in BCAA biosynthesis, suggesting that *S. mutans* CodY is not activated by GTP. The results of phenotypic studies revealed that the *codY* mutant had a reduced capacity to form biofilms and that its growth was more sensitive to low pH, showing a role for CodY in two key virulence properties of *S. mutans*. Microarray results revealed the extent of the CodY regulon. Notably, the identification of putative CodY-binding boxes upstream of genes that were downregulated in the *codY* mutant indicates that CodY may also function as a transcriptional activator in *S. mutans*.

Streptococcus mutans is a gram-positive bacterium (GPB) that has frequently been associated with human dental caries and is occasionally involved in nonoral infections, particularly subacute infective endocarditis. The ability to form biofilms on hard surfaces and the capacity to survive and rapidly adapt to environmental fluctuations contribute to the pathogenic potential of *S. mutans* (18). Of the many environmental stresses to which oral bacteria are exposed, low pH and fluctuations in nutrient source and availability are considered to have the greatest impact on dental biofilm ecology and the formation of carious lesions (18).

The “stringent response” is a widely distributed bacterial adaptation system that allows for global adjustments in gene expression in response to nutrient limitation and certain environmental stresses. The stringent response is mediated by the nutritional alarmones guanosine 3′-diphosphate 5′-triphosphate and guanosine 3′,5′-bisphosphate, generally referred to as (p)ppGpp (8). During nutrient starvation, (p)ppGpp is produced at higher levels by enzymatic phosphorylation of GDP and GTP by ATP, resulting in the downregulation of genes for macromolecular biosynthesis and upregulation of genes involved in amino acid biosynthesis and stress tolerance (8, 22).

Until recently, the results of functional studies supported the idea that only two closely related enzymes were responsible for (p)ppGpp production and degradation in bacteria. In the paradigmatic gram-negative bacterium *Escherichia coli* and related

enteric bacteria, *relA* encodes a potent (p)ppGpp synthetase and *spoT* encodes a bifunctional enzyme with both hydrolase and (weak) synthetase activities (8). In GPB, a bifunctional Rel/Spo ortholog, herein designated RelA, was shown to harbor both degradation and synthesis activities (25, 27, 46). More recently, we identified two novel (p)ppGpp synthetases in *S. mutans*, designated RelP and RelQ, that shared very limited homology with Rel/Spo enzymes (20). In *S. mutans*, RelA has retained the functions generally ascribed to Rel/Spo-like enzymes and is the major enzyme controlling the stringent response (30), whereas RelP appears to be the major source of (p)ppGpp during growth under nonstressed conditions (20). The contribution of RelQ to (p)ppGpp pools and stress tolerance has not yet been established. Phenotypic characterization and transcription profiling of an RelA-deletion strain revealed that RelA coordinates the expression of genes and phenotypes that contribute to the pathogenic potential of *S. mutans*, such as biofilm formation, acid tolerance, and sugar metabolism (19, 20). More recently, apparent homologues of RelP and RelQ have been discovered and shown to be functional in *Bacillus subtilis* (29). Multiple (p)ppGpp synthetases have also been found in rice (44), suggesting that the original findings with *S. mutans* have broad biological relevance.

Based on what is currently known about the genetic and physiologic behaviors of strains of *S. mutans* lacking one or more of the *relAPQ* genes, our working hypothesis is that RelA synthesizes (p)ppGpp as part of a classic stringent response to severe nutrient limitation and perhaps other stresses and RelP and RelQ synthesize (p)ppGpp in response to yet-to-be-determined stimuli to optimize the balance between growth and persistence. In support of this idea, an RelA-deficient strain has growth requirements similar to those of wild-type *S. mu-*

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TABLE 1. *S. mutans* strains used in this study

Strain	Description	Relevant genotype	Reference or source
UA159	Wild type		Lab collection
JLrelA	<i>relA::erm</i>	$\Delta relA$	19
JLrelP	<i>relP::kan</i>	$\Delta relP$	20
JLrelQ	<i>relQ::kan</i>	$\Delta relQ$	20
JLD1	<i>relA::erm relP::kan</i>	$\Delta relA \Delta relP$	20
JLD2	<i>relA::erm relQ::kan</i>	$\Delta relA \Delta relQ$	20
JLD3	<i>relP::spc relQ::kan</i>	$\Delta relP \Delta relQ$	20
JLT1	<i>relA::erm relP::spc relQ::kan</i>	$\Delta relA \Delta relP \Delta relQ$	20
JLcodY	<i>codY::tet</i>	$\Delta codY$	This study
JLQ1	<i>relA::erm relP::spc relQ::kan codY::tet</i>	$\Delta relA \Delta relP \Delta relQ \Delta codY$	This study

tans, but an $\Delta relAPQ$ strain, which lacks all three synthetases, cannot grow in the absence of leucine or valine (20). The triple-deletion strain can, however, grow well in the absence of isoleucine or when the lack of leucine and/or valine is coupled with the removal of isoleucine. In *S. mutans*, the biosynthesis of branched-chain amino acids (BCAA; isoleucine, leucine, and valine) appears to occur via the *ilv-leu* pathway (4) and is apparently regulated by the DNA binding protein CodY.

CodY is a global nutritional repressor that is highly conserved in low-G+C GPB, including *S. mutans* (4, 41). In *Bacillus subtilis*, the GTP-sensing protein CodY regulates BCAA operon expression (41), and the results presented in recent reports have demonstrated a connection between (p)ppGpp levels and CodY activity (7, 16, 24). DNA binding by CodY is activated by two different effectors, GTP and isoleucine, and this activation was shown to be independent and additive (38). However, the results of several studies indicate that DNA binding by the CodY proteins of *Streptococcus pneumoniae* and *Lactococcus lactis* is unresponsive to GTP pools but strongly activated by isoleucine (10, 14, 34). Given our previous observations showing that the removal of isoleucine restored the growth of an $\Delta relAPQ$ mutant of *S. mutans* in medium lacking leucine and/or valine (20) and that the production of (p)ppGpp results in a marked reduction of GTP levels (20), we speculated that the inability of the $\Delta relAPQ$ strain to grow in the absence of leucine or valine was related to the repression of the *ilv* and *leu* genes by CodY (20). In this report, we demonstrate that growth in the absence of leucine or valine is linked to basal levels of (p)ppGpp that are synthesized by the newly discovered RelP and RelQ enzymes. Strains lacking CodY and various alarmone synthetases were constructed and used to establish a relationship of (p)ppGpp pools with CodY activity. Microarray results proved that CodY does indeed function as a global regulator of gene expression in *S. mutans*, and the results of phenotypic studies revealed that CodY plays a significant role in biofilm maturation and acid tolerance, two major virulence attributes of *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. *S. mutans* strains were grown in brain heart infusion (BHI) medium at 37°C in a 5% CO₂ atmosphere. To select for *codY* mutant strains, the growth medium was supplemented with tetracycline (10 µg ml⁻¹). To measure cell growth in BHI or the chemically defined medium FMC (43),

mid-exponential-phase cultures were diluted 1:100 into fresh medium and growth was monitored by using a Bioscreen C growth monitor (Oy Growth Curves AB Ltd., Helsinki, Finland). To investigate growth in the absence of particular amino acids, cultures were grown to mid-exponential phase in complete FMC and diluted 1:100 in FMC lacking selected amino acids. To lower intracellular GTP levels, decoyinine (Sigma, St. Louis, MO) was added to a final concentration of 500 µg ml⁻¹. To induce (p)ppGpp accumulation, cells were treated with 500 ng ml⁻¹ mupirocin (Sigma) as previously described (20). For microarray analysis, cells were grown in complete FMC to an optical density at 600 nm (OD₆₀₀) of 0.3, collected by centrifugation, quick-frozen in a dry-ice-ethanol bath, and stored at -80°C for RNA isolation. The ability to form stable biofilms was assessed by growing cells in wells of polystyrene plates using a semidefined biofilm medium (BM) (21) containing glucose or sucrose as the carbohydrate source. To test the capacity of the mutant strains to grow at low pH, cells were grown in BHI to an OD₆₀₀ of 0.3 and diluted up to 10⁶-fold and 5 µl of each dilution was spotted on BHI agar plates that had been adjusted to pH 5.0 with HCl. To evaluate the capacity of the strains to form colonies in the presence of H₂O₂ or NaCl, a uniform layer of exponentially grown cells was spread onto BHI agar plates and paper filter discs (1-mm diameter) saturated with 0.5% H₂O₂ or 0.5 M NaCl were placed onto the agar. All plates were incubated at 37°C in a CO₂ incubator for 72 h before growth data were recorded.

Decoyinine and mupirocin treatment. *S. mutans* strain UA159 and the $\Delta codY$ strain were grown in FMC (complete or lacking leucine or valine) in the presence of 500 µg ml⁻¹ decoyinine (dissolved in dimethyl sulfoxide) and treated with 500 µg ml⁻¹ or 1.5 mg ml⁻¹ decoyinine or 500 ng ml⁻¹ mupirocin for different times. An equal amount of dimethyl sulfoxide was added to control cultures. At various time intervals, samples were harvested and assayed for gene expression and to determine the intracellular concentrations of GTP and (p)ppGpp by thin-layer chromatography (TLC). Nucleotide pool extractions and TLC were carried out as described elsewhere (20).

Construction of *codY* and $\Delta relAPQ \Delta codY$ mutants. To evaluate the role of CodY in the impaired growth of the $\Delta relAPQ$ strain in the absence of BCAAs, the entire *codY* gene was replaced by a tetracycline resistance (Tc^r) marker in *S. mutans* UA159 by using a PCR ligation mutagenesis approach (17). To make the deletion, the Tc^r marker was obtained as a BamHI fragment and ligated to two PCR fragments flanking *codY* that were also digested with BamHI. The PCR fragments flanking *codY* were obtained by using the following primers: 5'*codY* arm1 (CCAAGATGTATTAGAAG), 3'*codY* arm1 (ATTAGCCATGGGATC CAATCGTATTAT), 5'*codY* arm2 (CTATAGTTAAGATCCTAAAGCAC TG), and 3'*codY* arm2 (CCAATCCATAATTTC). The underlined bases correspond to the BamHI restriction site included to aid in the subsequent ligation. The ligation mix was used to transform *S. mutans* UA159, and putative *codY* mutants were selected on BHI agar containing 10 µg ml⁻¹ of tetracycline. The deletion was confirmed as correct by PCR sequencing of the insertion site and flanking sequences. Subsequently, a quadruple mutant was constructed by transforming the $\Delta relAPQ$ strain (20) with chromosomal DNA isolated from the $\Delta codY$ strain (JLcodY) to give an $\Delta relAPQ \Delta codY$ strain (JLQ1).

Microarray experiments. Transcriptome analysis was performed as previously described (1, 3, 30) using *S. mutans* UA159 microarrays provided by The Institute for Genomic Research (TIGR). RNA was isolated from exponential-phase cells (OD₆₀₀ = 0.3) grown in FMC medium, as described previously (1). All samples were digested with DNase I (Ambion, Austin, TX) and purified by using an RNeasy mini kit column (Qiagen, Inc., Chatsworth, CA). The RNA concentrations were determined spectrophotometrically in triplicate, and 1 µg of RNA was run in a formaldehyde gel to verify RNA quality. Reverse-transcription (RT) reactions were performed with 10 µg of RNA as described elsewhere (1). The microarrays consisted of 1,948 70-mer oligonucleotides representing 1,960 open reading frames printed four times on the surface of each microarray slide. A reference RNA prepared from a large-scale culture of *S. mutans* UA159 cells that had been grown in BHI broth to an OD₆₀₀ of 0.5 was used in every experiment (1). The benefits and analytical methods associated with the utilization of a reference RNA as a normalization tool in microarray experiments are discussed elsewhere (36). Labeling of cDNA was performed according to TIGR protocols (<http://pfgcr.tigr.org/protocols/protocols.shtml>), with minor modifications (1). Four cDNA samples originating from four independent cultures of *S. mutans* strains UA159 and JLcodY were hybridized to the arrays along with the reference cDNA, generating a total of eight slides. Hybridizations were performed in a Maui hybridization chamber (BioMicro Systems, Salt Lake City, UT). Additional details regarding array protocols are available at <http://pfgcr.tigr.org/protocols/protocols.shtml>. Data from all individual experiments were analyzed by using Spotfinder software and normalized by using Midas according to TIGR specifications (<http://www.tigr.org/software/>). Statistical analysis was

TABLE 2. Amino acid requirements of *S. mutans* UA159 and its derivatives

Strain or mutation(s)	Requirement for amino acid(s) for growth is:	
	Strong ^a	Weak ^b
UA159	Phe	
$\Delta relA$	Phe	
$\Delta relAPQ$	Phe, Leu, Val	Asp, Gly, Pro, Ser

^a No growth after 48 h in medium lacking the indicated amino acid(s).

^b Growth after overextended adaptation (lag) phase in medium lacking the indicated amino acid(s).

carried out by using BRB-Array Tools (<http://linus.nci.nih.gov/BRB-ArrayTools.html>) with a *P* value of 0.005.

Real-time quantitative PCR. A subset of genes was selected to validate the microarray data with real-time quantitative PCR. Also, real-time quantitative PCR was used to assess the effects of decoyinine and mupirocin on the expression of *ilvB* and *ilvE* and to compare *pncA* mRNA levels in the wild type and the $\Delta codY$ strain. Gene-specific primers were designed by using Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, CA). To obtain cDNA, 1 μ g of three independent RNA samples and an iScript kit containing random primers (Bio-Rad, Hercules, CA) were used. RT and quantitative real-time RT-PCR were carried out by following protocols described elsewhere (2). Student's *t* test was performed to verify the significance of the real-time RT-PCR quantifications.

Microarray data accession number. DNA microarray data have been deposited in NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo>) and are accessible through GEO Series accession number GSE9641.

RESULTS AND DISCUSSION

Amino acid requirements of the *S. mutans* $\Delta relAPQ$ strain.

Strains of *E. coli* that produce no (p)ppGpp require multiple amino acids for growth, including arginine, glycine, histidine, leucine, methionine, phenylalanine, serine, threonine, and valine (47). This multiple auxotrophy has been linked directly to the role played by (p)ppGpp in the activation of genes for amino acid biosynthesis (47). Previously, we showed that an $\Delta relAPQ$ strain, which lacks all three *S. mutans* (p)ppGpp synthetases and produces no detectable (p)ppGpp, was unable to grow in the absence of leucine or valine but grew in the absence of the third BCAA, isoleucine (20). Here, we assessed the requirements of UA159 and the $\Delta relAPQ$ strain for single amino acids. For this purpose, growth curves were conducted in the chemically defined medium FMC, removing 1 of the 20 essential amino acids at a time. To discriminate *relA*-dependent from *relA*-independent phenotypes, growth curves were also conducted using an otherwise-isogenic $\Delta relA$ mutant (19). As demonstrated previously, the $\Delta relA$ strain grew more slowly than the parent in complete FMC, with doubling times of 100 and 60 min, respectively, but the growth rate of the $\Delta relAPQ$ strain was nearly identical to that of *S. mutans* UA159 (20). Because $\Delta relAPQ$ lacks all (p)ppGpp synthetases and since RelA is the only enzyme with predicted (p)ppGpp hydrolase activity, we attributed the slow-growth phenotype of the $\Delta relA$ single mutant to an inability to convert (p)ppGpp, produced by RelP and RelQ, into GDP and GTP. Therefore, the ability of strains to grow in FMC in the absence of a particular amino acid was compared with the growth behavior of the same strains in complete FMC.

Although all amino acid biosynthetic pathways are present in the genome of *S. mutans* UA159 (4) and despite previously

TABLE 3. Growth of *S. mutans* UA159 and its derivatives in FMC lacking leucine or valine

Strain or mutation(s)	Growth in medium lacking: ^a	
	Leucine	Valine
UA159	+++	+++
$\Delta relA$	+++	+++
$\Delta relP$	+++	+++
$\Delta relQ$	+++	+++
$\Delta relAP$	+	+++
$\Delta relAQ$	+++	++
$\Delta relPQ$	+++	+++
$\Delta relAPQ$	NG	NG

^a +++, wild-type-like growth; ++, intermediate growth; +, poor growth; NG, no growth.

reported results showing that most *S. mutans* strains can grow in vitro on chemically defined medium supplemented with few amino acids, the wild-type UA159 strain and its derivatives could not grow in FMC lacking phenylalanine (Table 2). In the original study describing FMC medium, Terleckyj and Shockman (42) found that all *S. mutans* strains that were tested required cystine (or cysteine) as a nutrient but failed to identify a strain that required phenylalanine for growth. However, the work by Terleckyj and Shockman, as well as the results of a study using another chemically defined medium, have revealed that amino acid requirements for *S. mutans* are strain specific and can be influenced by the incubation atmosphere (9, 42).

None of the strains had their growth affected by the omission of alanine, arginine, asparagine, cysteine, glutamic acid, glutamine, histidine, lysine, methionine, threonine, tryptophan, or tyrosine. The $\Delta relAPQ$ strain showed a weak requirement for aspartic acid, glycine, proline, or serine; it displayed an extended lag period (12 to 16 h) before entering logarithmic growth phase and had final growth yields comparable to those achieved by UA159 and the $\Delta relA$ strain (Table 2).

The ability to grow in the absence of leucine or valine is linked to basal levels of (p)ppGpp produced by RelP and RelQ. In order to better assess the specific contributions of RelA, RelP, and RelQ to growth in medium lacking leucine or valine, we tested the capacity of the $\Delta relP$ and $\Delta relQ$ single mutants, as well as the capacity of the $\Delta relAP$, $\Delta relAQ$, and $\Delta relPQ$ double mutants, to grow in the absence of BCAA. Here we found that, similar to the results for the $\Delta relA$ strain, the growth of the $\Delta relP$ and $\Delta relQ$ single mutants was not altered when leucine or valine was removed from the medium (data not shown), indicating that the growth defect of the $\Delta relAPQ$ triple mutant was, in fact, due to the loss of all capacity for (p)ppGpp synthesis. The results of growth curves performed using the $\Delta relAP$, $\Delta relAQ$, and $\Delta relPQ$ double mutants revealed that the growth of the $\Delta relAP$ strain was severely impaired, but not completely abolished, in FMC medium lacking leucine, whereas the $\Delta relAQ$ and $\Delta relPQ$ strains grew well (Table 3). Similar to our observations of the $\Delta relAPQ$ triple mutant strain, the removal of isoleucine allowed the $\Delta relAP$ mutant to grow in the absence of leucine (data not shown). Interestingly, the $\Delta relAP$ mutant grew fairly well in the absence of valine, and only the $\Delta relAQ$ mutant grew more slowly than the parent under this particular condition (Table 3), albeit the growth rates of the double mutants were comparable to those previ-

FIG. 1. Growth curves of *S. mutans* UA159 (wild type), JLT1 ($\Delta relAPQ$), and JLQ1 ($\Delta relAPQ \Delta codY$) in FMC lacking leucine (A) or valine (B). The results are representative of those of at least four independent experiments.

ously observed in the $\Delta relA$ single mutant grown under the same condition (20). The results of our previous work indicated that the (p)ppGpp-mediated stringent response is primarily governed by RelA and that RelP and RelQ act by maintaining low basal levels of (p)ppGpp, required for optimal growth (20, 30). The growth characteristics and amino acid auxotrophies of the mutants analyzed here confirm the importance of basal levels of (p)ppGpp for growth in the absence of leucine or valine. Based on the growth behavior of the $\Delta relAPQ$ mutant and because RelP appears to contribute more significantly to (p)ppGpp pools than RelQ (20), these results also suggest a stronger requirement for (p)ppGpp in medium lacking leucine. As is demonstrated below, these growth defects are directly associated with the activity of the CodY repressor, but a role of (p)ppGpp in the induction of BCAA synthesis should not be overlooked.

Inactivation of *codY* restores growth of the $\Delta relAPQ$ strain in medium lacking leucine or valine. The transcriptional regulator CodY was first identified in *B. subtilis* as a repressor of the dipeptidase permease operon (*dpp*) (40) and was later shown to negatively regulate the expression of more than 100 genes, many of which were involved in sporulation or adaptation to stationary phase (35). Several studies have revealed that this regulatory protein is highly conserved in low-G+C GPB and functions mainly as a global nutritional regulator. Among the direct targets of CodY repression were the genes for the biosynthesis of BCAA (28, 39, 41). In *B. subtilis*, DNA binding by CodY is activated by GTP and plays a key role in physiologic homeostasis by monitoring the general nutritional state of the cell through sensing of intracellular levels of GTP (35). *B. subtilis* CodY is also activated by BCAA, particularly isoleucine, and it was demonstrated that the activation of CodY by GTP and isoleucine is independent and additive (13, 38).

Following the stringent response, the intracellular levels of GTP suffer a significant drop due to two mechanisms: the conversion of GTP into (p)ppGpp and the inhibition of IMP dehydrogenase, an enzyme for de novo guanine nucleotide biosynthesis, by (p)ppGpp (16, 31). As a result of the decrease in intracellular levels of GTP, CodY loses its repressing activity and transcription of the CodY regulon occurs (41). However, it is important to note that sensing of GTP levels may not be a general property of CodY orthologs, as the proteins from *Lactococcus lactis* and *Streptococcus pneumoniae* were unrespon-

sive to GTP (10, 14, 34). The results of gel retardation experiments with purified CodY of these organisms have indicated that, in addition to being unresponsive to GTP, isoleucine markedly enhanced the affinity of CodY for its targets, whereas the effects of adding leucine or valine were less or insignificant, respectively (12). The fact that the removal of isoleucine restored the growth of the $\Delta relAPQ$ mutant of *S. mutans* in medium lacking leucine and/or valine led us to test whether the inability of the $\Delta relAPQ$ strain to grow in the absence of leucine or valine was linked to CodY.

In the *S. mutans* UA159 genome, *codY* is followed by and apparently cotranscribed with a gene encoding a putative pyrazinamidase/nicotinamidase (*pncA*) involved in nicotinate and nicotinamide metabolism. To ensure that polar effects did not affect *pncA* expression, the *codY* mutant was obtained by using a *tet* cassette that has an outward-reading promoter (K. A. Clancy and R. A. Burne, unpublished data). Real-time PCR analysis was used to confirm that the expression of the *pncA* gene was not significantly altered in the $\Delta codY$ mutant strain (data not shown). To evaluate the role of CodY in the impaired growth of the $\Delta relAPQ$ strain in medium lacking leucine or valine, an $\Delta relAPQ \Delta codY$ quadruple mutant strain (JLQ1) was isolated. Our first step was to evaluate the growth behavior of the $\Delta codY$ and $\Delta relAPQ \Delta codY$ strains in BHI medium and chemically defined FMC medium. No significant differences in growth were observed among the mutants grown in BHI broth (data not shown). However, in complete FMC medium, the $\Delta codY$ strain grew slightly more slowly (doubling time approximately 70 min), whereas the $\Delta relAPQ \Delta codY$ mutant and the wild-type UA159 strains displayed identical doubling times (approximately 60 min) (data not shown). Next, we tested the capacity of the $\Delta relAPQ \Delta codY$ strain to grow in the absence of leucine or valine. For control purposes, UA159 and the $\Delta relAPQ$ strain were included in the growth curve experiments. As hypothesized, the inactivation of *codY* in the $\Delta relAPQ$ strain fully restored growth in medium lacking leucine or valine (Fig. 1), indicating that the growth-defective phenotype of the $\Delta relAPQ$ strain was, in fact, due to CodY repression.

Decoyinine treatment does not alleviate CodY repression in $\Delta relAPQ$ mutant. The results of studies with *L. lactis* and *S. pneumoniae* fostered the idea that the absence of response to GTP by CodY may be a general feature of this related family of low-G+C gram-positive cocci (10, 14, 34). However, be-

FIG. 2. Effects of decoyinine and mupirocin on GTP/(p)ppGpp ratios and *ilvE* gene expression. (A) TLC depicting GTP and (p)ppGpp pools in *S. mutans* UA159 (wild type) grown in the presence of decoyinine (DEC) or treated for 30 min with 500 μ g ml⁻¹ decoyinine (0.5 DEC), 1.5 mg ml⁻¹ decoyinine (1.5 DEC) or 500 ng ml⁻¹ mupirocin (MUP). (B) Real-time PCR was used to quantify *ilvE* mRNA in *S. mutans* strain UA159 and the Δ codY strain treated for 30 min with decoyinine or mupirocin (500 μ g ml⁻¹ and 500 ng ml⁻¹, respectively). The data represent the means and standard deviations (error bars) of the results of four independent experiments. Differences that were found to be statistically significant (Student's *t* test) are indicated by brackets.

cause (p)ppGpp accumulation occurs at the expense of intracellular GTP pools, the growth behavior of the Δ relAPQ and Δ relAPQ Δ codY mutants lacking (p)ppGpp in medium lacking leucine and/or valine also raises the possibility that reductions in GTP levels or increases in (p)ppGpp pools, individually or in concert, influence CodY activity in *S. mutans*.

To evaluate whether GTP pools play a role in the activation of the *S. mutans* CodY, we used decoyinine to reduce GTP levels. Decoyinine is an analog of adenosine that causes a decrease in intracellular GTP levels by inhibiting GMP synthetase (26). In *B. subtilis* and *L. lactis*, the intracellular GTP levels were significantly lowered by the addition of 500 μ g ml⁻¹ decoyinine (34, 35). Thus, we used 500 μ g ml⁻¹ as a starting point for our experiments. The addition of decoyinine to the growth medium reduced the growth rates of *S. mutans* strains (data not shown) and decreased GTP levels by approximately 70% in the wild-type strain, as assessed by TLC (Fig. 2A). The addition of inhibitory concentrations of decoyinine (1.5 mg ml⁻¹) to exponentially growing cells resulted in a slightly greater reduction in GTP levels (approximately 80%) that was comparable to the reductions in GTP pools that were observed during mupirocin treatment, a condition that strongly induces (p)ppGpp production at the expense of GTP and GDP (Fig. 2A).

Although decoyinine effectively lowered intracellular GTP levels, the results of growth curve experiments indicated that the addition of decoyinine did not restore growth of the Δ relAPQ strain when leucine or valine was removed from the growth medium (data not shown). Next, we tested whether the addition of decoyinine (500 μ g ml⁻¹ or 1.5 mg ml⁻¹) to exponentially grown cultures would alleviate the repression of *ilvE*, a well-known target of CodY repression. The results of real-time PCR analysis indicated that lowering GTP pools through decoyinine did not affect *ilvE* expression, whether growth-inhibitory (Fig. 2B) or noninhibitory concentrations of decoyinine were used (data not shown). Comparison of the absolute amounts of *ilvE* mRNA observed in the wild-type and

Δ codY strains clearly indicated that CodY acts as a repressor of *ilvE* expression (Fig. 2B). Unexpectedly, a small but significant induction of *ilvE* was observed in the *codY* mutant when it was grown in the presence of decoyinine. We also assessed the expression of *ilvE* in cells treated with mupirocin and found that *ilvE* is strongly induced by this analog of isoleucine (Fig. 2B). Because a similar pattern of induction was observed in both the parental and *codY* mutant strains, it appears that the effects of mupirocin, which increases (p)ppGpp pools and lowers GTP levels, are independent of functional CodY. We also assessed the expression patterns of *ilvB* in the wild-type and Δ codY strains treated with decoyinine or mupirocin. The results obtained for *ilvB* were nearly identical to the results obtained for *ilvE* (data not shown). Based on the results described above, it is tempting to speculate that (p)ppGpp positively regulates *ilv* gene expression. Of note, (p)ppGpp has been shown to exert a positive effect on transcription from promoters controlling amino acid biosynthetic operons in *E. coli* (33).

Collectively, the results presented above provided strong evidence that *S. mutans* CodY is not activated by GTP, consistent with the results of studies of *L. lactis* and *S. pneumoniae* CodY. Based on the consensus sequence of the putative GTP-binding motifs G1, G3, and G4 of CodY proteins (35), *L. lactis*, *S. pneumoniae*, and *S. mutans* each have multiple amino acid substitutions at normally conserved residues. Notably, a single amino acid change in G1 was shown to cause loss of CodY-mediated repression in *B. subtilis* (35, 40).

Effects of *codY* inactivation on biofilm formation and acid tolerance. The pathogenic potential of *S. mutans* is directly related to the organism's ability to adhere and form biofilms on the tooth surface and to survive large and rapid changes in environmental pH (18). To evaluate the physiological consequences of loss of CodY in *S. mutans* and to better understand the relationship of (p)ppGpp/GTP ratios with CodY activation in controlling critical virulence attributes of *S. mutans*, biofilm and stress susceptibility assays were carried out with the Δ codY

FIG. 3. Biofilm formation by *S. mutans* UA159 (wild type) and its derivatives. Cultures were grown in a microtiter plate containing BM supplemented with glucose (A) or sucrose (B) at 37°C for 24 h. The graph shows the averages and standard deviations (error bars) of the results of three independent experiments. Asterisks indicate results that differ significantly from the results for UA159 ($P \leq 0.05$; Student's *t* test).

(JLcodY), $\Delta relAPQ$ (JLT1), and $\Delta relAPQ \Delta codY$ (JLQ1) strains.

In *S. mutans*, there are two distinct mechanisms to colonize tooth surfaces: sucrose-independent adherence that is important for the initial attachment to the salivary constituents in enamel pellicle and a sucrose-dependent pathway involving the production of glucans, extracellular homopolymers of glucose which are formed through the action of glucosyltransferase enzymes and bound by glucan binding proteins (5, 32). Biofilm assays were conducted by using semidefined BM medium containing either glucose or sucrose as the supplemental carbohydrate sources. The ability of the $\Delta codY$ and $\Delta relAPQ \Delta codY$ strains to form biofilms in the presence of glucose or sucrose was significantly reduced in comparison to that of the parental strain (Fig. 3). The results of recent studies have demonstrated the involvement of CodY in biofilm maturation in other GPB. In *Bacillus cereus*, the disruption of *codY* resulted in a biofilm-defective phenotype (15). In an *S. aureus* clinical isolate, transposon mutagenesis identified CodY as important for proper biofilm formation (45). However, the results in a more-recent report suggested the opposite and showed that the production of the polysaccharide intercellular adhesin was enhanced in a *codY* mutant (23). Notably, the $\Delta relAPQ$ strain did not show altered biofilm accumulation in either glucose or sucrose. This latter finding was somewhat unexpected because a strain lacking RelA alone showed significant reductions in biofilm formation when grown in glucose (19). However, as noted above, the RelA enzyme is the only protein in *S. mutans* that contains the conserved (p)ppGpp hydrolase domain, and we have shown that the $\Delta relA$ strain accumulates (p)ppGpp in vitro (19). Thus, the biofilm-deficient phenotype of the $\Delta relA$ strain could be a result of unregulated accumulation of (p)ppGpp generated by the RelP and RelQ enzymes, which could affect the expression of adhesins or genes required for intercellular interactions.

To test the susceptibilities of the various strains to low pH, exponentially growing cultures were serially diluted and aliquots were spotted onto BHI agar plates adjusted to pH 7.0 or

5.0. The results obtained indicate that strains lacking a functional CodY, the $\Delta codY$ and $\Delta relAPQ \Delta codY$ strains, were more sensitive to low pH, whereas the $\Delta relAPQ$ strain grew as well as the wild type (Fig. 4). All strains grew equally well in plates adjusted to pH 7.0. In addition to sudden and abrupt changes in pH, organisms in the oral cavity also have to cope with other environmental stress, including oxidative and osmotic shock (18). Thus, we used a disk diffusion assay to test the susceptibility of the strains to H₂O₂ and NaCl. For this purpose, filter paper disks were saturated with either 0.5% H₂O₂ or 0.5 M NaCl and placed on the surface of agar plates that contained a uniform layer of an exponentially grown bacterial culture. Based on the diameters of the zones of inhibition, no differences in sensitivity to H₂O₂ or NaCl were observed among the wild type and all knockout strains tested (data not shown).

Global transcriptional profile of the $\Delta codY$ mutant. Given that CodY affects biofilm formation and acid tolerance, which are two critical virulence attributes of *S. mutans*, and that there is considerable support for the idea that the evolution of the role of CodY in this oral pathogen may be substantively different than in some other bacteria, we assessed the scope of the CodY regulon of *S. mutans* by transcription profiling. RNA

FIG. 4. Growth of *S. mutans* UA159 and its derivatives on BHI plates adjusted to pH 7.0 or 5.0. Strains were grown in BHI to an OD₆₀₀ of 0.3 and serially diluted in PBS, and 5- μ l aliquots were spotted onto plates that were incubated at 37°C for 72 h. Numbers correspond to the dilution factors for each individual culture, and images representative of the results of three individual experiments are shown.

FIG. 5. Number of genes, grouped in functional categories, that were differentially expressed in strain JLCodY ($\Delta codY$) relative to their levels of expression in UA159 (wild type). Gene annotations are based on information provided by the Los Alamos National Laboratory (www.oralgen.lanl.gov) or by published literature available at the same website.

was isolated from mid-exponential-phase ($OD_{600} = 0.3$) cultures of UA159 and the $\Delta codY$ strain grown in complete FMC medium, and microarray analysis was performed as previously described, with the modifications noted in Materials and Methods. A total of 79 genes were differentially transcribed at an assigned P value of ≤ 0.005 , with 63% ($n = 50$) upregulated and 37% ($n = 29$) downregulated (see Table S1 in the supplemental material). For simplicity, the 79 differentially expressed genes were grouped into eight functional categories (Fig. 5). The higher number of upregulated genes was consistent with the finding that CodY functions as a repressor. In contrast, the number of downregulated genes in the *S. mutans* $\Delta codY$ mutant was surprisingly high in comparison with the results of previous microarray analysis of $\Delta codY$ strains of other GPB (11, 12, 14, 28). The elevated number of downregulated genes may be a result of indirect effects of CodY inactivation but, as is discussed below, may also indicate that CodY can function both as a repressor and an activator. To validate the microarray data, real-time quantitative RT-PCR was performed on 11 genes found to be differentially expressed in the mutant, and all genes displayed the same trends observed in the microarrays (see Table S1 in the supplemental material).

(i) Upregulated genes. Consistent with the knowledge that CodY represses amino acid biosynthetic operons, the deletion of $codY$ resulted in the upregulation of at least four genes involved in amino acid biosynthesis and transport, including *ilvC*, which codes for a ketol acid reductoisomerase that participates in the BCAA's biosynthetic pathway. Moreover, *livK*, a gene involved in BCAA binding and transport, *gdhA*, coding for a putative NADP-specific glutamate dehydrogenase, and *hisE*, a phosphoribosyl-ATP pyrophosphatase involved in histidine biosynthesis, were also upregulated in the $\Delta codY$ strain. Notably, three putative transcriptional regulators (*smu112c*, *smu1287*, and *smu2027*) were upregulated in the mutant, indicating that other regulators potentially amplify the effect of CodY inactivation in the transcriptome. Of the 50 genes found to be upregulated in the $\Delta codY$ strain, 66% encoded hypothetical proteins of unknown function (Fig. 5). The fact that the

$codY$ mutant has impaired capacity to form biofilms and displays an acid-sensitive phenotype suggests that some of these hypothetical proteins may contribute to the pathogenic potential of this organism, making those genes attractive targets for future studies.

(ii) Downregulated genes. In comparison to results for other GPB, a large number of genes were found to be downregulated in the *S. mutans* $\Delta codY$ mutant. A recent study of *S. pneumoniae* and two independent studies of *L. lactis* revealed a very limited number of genes that depend on CodY for optimal expression. For example, Guédon and colleagues identified 110 genes that were differentially expressed in an *L. lactis* $\Delta codY$ mutant in comparison to their levels of expression in the parental strain, but only 6 genes were found to be downregulated (12). In *S. pneumoniae*, 43 of 47 genes that were differentially expressed in the $\Delta codY$ mutant were upregulated (14). Among the 29 genes found to be downregulated in the *S. mutans* $\Delta codY$ mutant, almost half of the genes ($n = 13$) encode products involved in transport and binding processes. Among those genes, 11 encode subunits of ABC transporters predicted to participate in amino acid uptake. In addition to ABC transporters, a gene encoding a putative ferrous ion transport protein (*feoA*) and a putative sodium/amino acid symporter (*smu1175*) were repressed in the $\Delta codY$ mutant. This finding was unexpected because CodY has been shown to repress genes involved in amino acid transport in *B. subtilis* and *L. lactis* (11, 41). Among the genes found to be downregulated, many appear to be cotranscribed. For example, an entire five-gene operon (*smu932-smu936*) predicted to encode proteins for an ABC transporter and a six-gene cluster encoding hypothetical proteins of unknown function (*smu277-smu285*) were found to be downregulated in the $\Delta codY$ mutant. Moreover, many genes that appear to be cotranscribed with genes identified at a P value of ≤ 0.005 were often found to show the same trend at a P value of ≤ 0.01 (data not shown). Altogether, the 29 genes found to be downregulated in the $\Delta codY$ strain are organized in 16 transcriptional units.

Identification of CodY boxes upstream of genes affected by the $\Delta codY$ mutation. In *L. lactis*, a conserved 15-bp palindromic sequence (AATTTTCWGAAAATT) was shown to serve as a high-affinity binding site for CodY (11, 12). This "so-called" CodY box was found predominantly upstream of genes involved in amino acid transport and metabolism. Interestingly, this motif is also found in the upstream region of $codY$, suggesting that CodY could be autoregulated. The CodY box is also present in other GPB, including *S. mutans*, and probably serves as an operator site for CodY proteins in general (12). In an attempt to distinguish direct and indirect effects of the loss of CodY on gene expression in our microarray data, we searched for the CodY box in the upstream regions of the 79 differentially expressed genes. The criteria for assigning CodY-binding boxes included five or fewer mismatches with the consensus sequence and location within 100 bp of the start codon. Of the 50 genes that were upregulated in the $\Delta codY$ strain, 17 genes were shown to contain a putative CodY box in the region proximal to the start codon. Interestingly, our searches revealed the presence of putative CodY boxes in at least six genes that were downregulated in the $\Delta codY$ strain, emphasizing the possibility that CodY may also be able to function as a transcriptional activator in *S. mutans*. Previously, the gene en-

FIG. 6. ClustalW alignment of putative CodY-binding boxes in upstream regions of *S. mutans* genes. Conserved regions are in shaded boxes, and a consensus sequence is shown below the alignments. Symbols: *, gene was flagged on microarray experiments with Δ codY strain (JLcodY); **, gene was identified by Guédon et al. (12); #, gene was downregulated in JLcodY.

coding acetate kinase (*ack*) in *B. subtilis* was shown to be positively regulated by CodY and CcpA (37). Work is under way to evaluate whether CodY can specifically bind to the regulatory regions of the genes identified in the microarray experiments that contain a putative CodY box.

By searching for the CodY box in the genomes of GPB, Guédon and colleagues identified 13 genes in *S. mutans* that contain the CodY motif (12). Among those were genes involved in BCAA biosynthesis (*ilvB*, *ilvE*, and *leuA*), additional genes involved in amino acid metabolism (*akh*, *hom*, *hisC*, *serC*, and *thrC*), the oligopeptide ABC transporter operon (*opp*), and *codY* itself. Figure 6 displays an alignment of CodY motifs identified in this study and previously (12) which confirms the presence of consensus CodY-binding sequences in *S. mutans*.

Concluding remarks. In the oral cavity, the levels of free amino acids are relatively low (6). Thus, the capacity of *S. mutans* and other oral bacteria to obtain or synthesize amino acids required to make up proteins is probably essential for growth and competitive fitness. In addition, metabolic adaptation to nutritional fluctuations has frequently been linked to the expression of virulence genes in bacteria. In this report, we were able to provide the first evidence that basal levels of (p)ppGpp produced by RelP and RelQ play an important role in cellular homeostasis and that these effects are exerted, in part, through CodY. We also demonstrate that CodY globally affects gene expression and is intimately linked to the manifestation of virulence attributes in *S. mutans*. Molecular studies are under way to fully understand how CodY and (p)ppGpp pools affect the expression of virulence attributes by *S. mutans*.

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