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Role of RelA of *Streptococcus mutans* in Global Control of Gene Expression▽†

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The production of (p)ppGpp by *Streptococcus mutans* UA159 is catalyzed by three gene products: RelA, RelP, and RelQ. Here, we investigate the role of the RelA (Rel) homologue of *S. mutans* in the stringent response and in the global control of gene expression. RelA of *S. mutans* was shown to synthesize pppGpp in vitro from GTP and ATP in the absence of added ribosomes, as well as in vivo in an *Escherichia coli relA-spoT* mutant. Mupirocin (MUP) was shown to induce high levels of (p)ppGpp production in *S. mutans* in a *relA*-dependent manner, with a concomitant reduction in GTP pools. Transcription profiling after MUP treatment of *S. mutans* revealed that 104 genes were upregulated and 130 were downregulated ($P \leq 0.001$); mainly, genes for macromolecular biosynthesis, translation, and energy metabolism were downregulated. When a derivative of UA159 carrying a complete deletion of the *relA* gene was treated with MUP, 72 genes were upregulated and 52 were downregulated ($P \leq 0.001$). The expression of 50 genes ($P \leq 0.001$) was commonly affected by MUP treatment in the two strains, suggesting that *S. mutans* can mount a *relA*-independent response to MUP. Consistent with the gene expression profiling, RelA was shown to play major roles in the regulation of phenotypic traits that are required for establishment, persistence, and virulence expression by this oral pathogen. Thus, RelA is the major (p)ppGpp synthase controlling the stringent response in *S. mutans*, and it coordinates the expression of genes and phenotypes that contribute to the pathogenic potential of the organism.

In the oral cavity, bacteria are continually exposed to dramatic changes in pH, oxygen tension, nutrient availability, and carbohydrate source. Fluctuations in pH, as well as in the source and availability of carbohydrates, are considered to have the most profound impact on supragingival plaque ecology and the development of dental caries (8). It has been shown that carbohydrate concentrations can almost instantly increase from around 10 μM during fasting periods to more than 10 mM during the intake of sweetened foodstuffs. This so-called "feast-or-famine" existence (12) demands that organisms in dental plaque adjust their metabolism and gene expression patterns to maximize the use of available substrates and to provide protection from stresses induced by fluctuations in nutrient pools; including rapid and considerable acidification of the environment when carbohydrates are abundant. *Streptococcus mutans* is a gram-positive bacterium considered to play a major role in the development of dental caries. This organism relies on a biofilm lifestyle to persist in the oral cavity and has developed sophisticated strategies to cope with the various stresses encountered in the oral environment (27). Because *S. mutans* is strongly aciduric and acidogenic, it takes advantage of periods of high concentrations of carbohydrates and low pH to gain a selective advantage over less-aciduric species. The organism must, however, achieve a genetic and physiologic balance that allows it to remain competitive under low-nutrient conditions while retaining the capacity to rapidly assimilate the spectrum of nutrients offered intermittently in the diet.

One key bacterial adaptation to changing environments is the stringent response, which allows for global readjustments in gene expression in response to nutrient limitation or other conditions that slow or arrest growth. The hallmark of the stringent response is the accumulation of the bacterial alarmones, guanosine tetraphosphate and guanosine pentaphosphate, (p)ppGpp. The levels of (p)ppGpp have been found to be inversely correlated with growth rates (14) and to affect the expression of traits important to the virulence of many different bacteria, including biofilm formation (5, 55), quorum sensing and competence development (23, 58), antibiotic synthesis (54), and bacteriocin production (25). It has been proposed that (p)ppGpp adjusts metabolism by favoring transcription of genes involved in amino acid biosynthesis and stress tolerance at the expense of those essential for growth (34).

In *Escherichia coli* and related gram-negative enteric bacteria, two enzymes are involved in the accumulation of (p)ppGpp: the (p)ppGpp synthetase RelA and the bifunctional (p)ppGpp synthetase/hydrolase SpoT (14). Inactivation of *relA* and *spoT* leads to a (p)ppGpp-null phenotype, (p)ppGpp^0. Studies of these "relaxed" mutants have contributed to the identification of pathways that are affected by (p)ppGpp levels (17, 36, 64). In gram-negative α-proteobacteria and gram-positive bacteria, a single bifunctional synthetase/hydrolase enzyme, known as Rel or RelA, produces (p)ppGpp. In a previous study (28), measurements of (p)ppGpp pools in a RelA-deficient strain of *S. mutans* treated with serine hydroxamate (SHx) revealed that

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the mutant retained the capacity to synthesize and accumulate (p)ppGpp, and also that RelA plays a critical role in the capacity of *S. mutans* to form stable biofilms and tolerate acid stress. More recently, we identified two novel (p)ppGpp synthetases in *S. mutans*, designated RelP and RelQ (29). A combination of mutants lacking *relA*, *relP*, and *relQ* were constructed and used to demonstrate the contributions of each synthetase to (p)ppGpp metabolism by *S. mutans*. RelP appears to be responsible for the bulk of (p)ppGpp produced in cells growing exponentially or treated with SHx (29), but inactivation of RelA alone had the greatest impact on biofilm formation and stress tolerance. Notably, RelA is the only (p)ppGpp enzyme harboring both a synthetase and hydrolase domain, so some of the effects of loss of RelA may be due to increases in the pools of (p)ppGpp. Importantly, the discovery of these new synthases and the apparent ability of *S. mutans* to utilize different synthases under normal or stress conditions raises the question of whether the RelA enzyme of this organism has retained the functions generally ascribed to the Rel/SpoT-like enzymes of gram-positive bacteria.

The effects of a variety of translational inhibitors that are effective at stimulating (p)ppGpp synthesis and accumulation in cells have been described (15, 21, 24, 45, 59). However, SHx is only poorly effective at inducing alarmone production in *S. mutans* (28), and increased production of (p)ppGpp by SHx occurs in the absence of the RelA enzyme. Mupirocin (MUP), also known as pseudomonic acid, is a potent inhibitor of isoleucyl-tRNA synthetase (20) and has been used to elicit a stringent response in several bacteria, including streptococci (15, 38, 45, 63). In the present study, the synthetic capacity of the RelA enzyme was demonstrated, and MUP was found to elicit vigorous accumulation of (p)ppGpp by *S. mutans*. Microarrays revealed major changes in the transcriptomes of *S. mutans* UA159 and a RelA-deficient derivative of UA159 after treatment with MUP. The effects of loss of RelA on particular aspects of cellular physiology related to virulence were investigated. Our results indicate that even though *S. mutans* has evolved alternative pathways to modulate cellular levels of (p)ppGpp, an intact RelA protein is required for the full expression of a classical stringent response, and RelA strongly influences the expression of traits that are essential for virulence expression by *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. mutans* UA159 and a strain carrying a replacement of the *relA* gene with an antibiotic resistance cassette (JLrelA) (28) were maintained in brain heart infusion (BHI) medium in a 5% CO_2, aerobic atmosphere at 37°C. *E. coli* strains CF1648 (wild type) and CF1693 (Δ*relA* Δ*spoT*) were kindly provided by Mike Cashel (National Institutes of Health, Bethesda, MD) and were grown in Luria broth or M9 minimal medium supplemented with 0.2% glucose as a carbohydrate source (46) at 37°C. For microarray analysis, cells were grown in the chemically defined medium FMC (56) to an optical density at 600 nm (OD_{600}) of 0.3, and the cultures were divided into two aliquots. To one aliquot, 500 ng ml^{-1} of MUP was added, and the cells were incubated at 37°C for 20 min (MUP-treated cells), while the other aliquot was collected by centrifugation and immediately frozen (MUP-control cells). Since MUP treatment resulted in rapid growth arrest, the final OD_{600} values of experimental and control samples were essentially identical.

To assess the ability of cells to grow in selected carbohydrates and to measure sugar transport by the phosphoenolpyruvate-sugar phosphotransferase system (PTS), mid-exponential-phase cultures growing in BHI were diluted 1:100 into tryptone-vitamin (TV) base medium (11) supplemented with the desired carbohydrates. Growth was monitored spectrophotometrically or by using a Bioscreen C growth monitor (Oy Growth Curves AB, Ltd.).

In vitro synthesis of (p)ppGpp by *S. mutans* RelA. The *relA* gene was amplified from *S. mutans* UA159 with primers containing restriction sites (5′-TTAGAGA GGCGGATCCATGGCGAAAG-3′ and 5′-CAATTTTCATTCTAGATTACC CGTTAG-3′) to facilitate cloning into the plasmid expression vector pMAL-c2x (New England Biolabs, Beverly, MS). The *relA* gene of *S. mutans* was cloned downstream from *malE*, which encodes a maltose-binding protein (MBP). The resulting plasmid was isolated in *E. coli* DH10B and produced a soluble MBP fusion protein. Expression of the fusion protein was induced by addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h, and the recombinant protein was purified by one-step affinity chromatography specific for MBP according to the supplier's instructions. The resulting plasmid was also used for the complementation studies in *E. coli* strains. For detection of (p)ppGpp synthetic activity, different concentrations of purified *S. mutans* RelA were mixed with 8 mM ATP, 6 mM [α-^{32}P]GTP, 25 mM Bis-Tris propane (pH 9.0), 150 mM NaCl, and 15 mM $MgCl_2$ and then incubated at 37°C for 1 h as detailed elsewhere (39). The reaction was terminated by the addition of an equal volume of 13 M formic acid, and 5-μl samples were resolved by polyethyleneimine thin-layer chromatography (PEI-TLC) in 1.5 M KH_2PO_4.

Detection of (p)ppGpp accumulation patterns. To measure the accumulation of (p)ppGpp in *S. mutans*, cells were grown in modified FMC broth lacking isoleucine and containing a reduced amount of phosphate (8.6 mM). Briefly, cultures were grown to an OD_{600} of 0.15 and uniformly labeled for one generation with carrier-free [^{32}P]orthophosphate, at which point 500 ng of MUP ml^{-1} was added to the experimental aliquots. Control samples consisted of aliquots that were not treated with MUP but were labeled with ^{32}P for the duration of the experiment. Measurements of (p)ppGpp in *E. coli* were performed using cells grown in low-phosphate morpholinepropanesulfonic acid (MOPS) labeling medium treated with 1 mg of SHx and 0.5 mg of L-valine ml^{-1} for 20 min, as described elsewhere (13). Nucleotides were extracted by adding an equal volume of 13 M formic acid, followed by two freeze-thaw cycles in a dry-ice–ethanol bath. Extracts were spotted on a PEI-cellulose plate (Selecto Scientific, Inc., Suwanee, GA) for separation of the phosphorylated nucleotides by PEI-TLC in 1.5 M KH_2PO_4 (pH 3.4), using [^{32}P]GTP (Amersham Biosciences, Piscataway, NJ) and (p)ppGpp and ppGpp (gifts from M. Cashel) as standards.

RNA methods. To isolate RNA from *S. mutans*, cells were harvested by centrifugation at 4°C and then treated with the RNA protect reagent (Qiagen, Inc., Chatsworth, CA). Total RNA was isolated by the hot acid-phenol method as described previously (1). After extraction, RNA was precipitated using a 1/10 volume of 3 M sodium acetate (pH 5.0) and an equal volume of isopropanol after incubation at −20°C for 1 h. RNA was resuspended in diethyl pyrocarbonate-treated water, digested with DNase I (Ambion, Austin, TX), and purified with the RNeasy minikit (Qiagen). RNA concentration was determined spectrophotometrically in triplicate, and 1 μg of RNA was run in a formaldehyde gel to verify RNA quality.

Microarray experiments. Transcriptome analysis was performed using the *S. mutans* UA159 microarrays provided by The Institute for Genomic Research (TIGR). 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. Additional details regarding the arrays are available at http://pfgrc.tigr.org/descriptions/S_mutans.shtml. A reference RNA prepared from a large-scale culture of *S. mutans* UA159 cells that had been grown in BHI broth to an OD_{600} of 0.5 was used in every experiment (1). A description of the advantages of using a reference RNA in microarray studies is presented elsewhere (32, 48). Total RNAs were purified as described above and used to generate cDNA according to the protocol provided by TIGR, with minor modifications as detailed elsewhere (1). The reaction mixture, consisting of 10 μg of total RNA, Superscript III reverse transcriptase (Invitrogen, Gaithersburg, MD), random hexamer primers, and deoxynucleoside triphosphate mix (Sigma Chemical Co.), was incubated at 42°C for 17 h. Purified cDNAs from *S. mutans* UA159 and JLrelA were labeled with Cy3-dUTP, and the reference cDNAs were labeled with Cy5-dUTP (Amersham Pharmacia Biotech). Four individual Cy3-labeled cDNA samples originating from four different cultures of UA159 and JLrelA strains grown under control (FMC) and experimental (FMC containing MUP) conditions were hybridized to the arrays, along with Cy5-labeled reference cDNA, generating a total of 16 slides. The ratios of the signals of the test RNAs with that of the uniform reference RNA were analyzed within and between slides. Hybridized slides were washed and scanned by using a GenePix Scanner (Axon Instruments, Inc., Union City, CA) at 532 nm (Cy3 channel) and 635 nm (Cy5 channel). The 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 carried out by

TABLE 1. Real-time PCR primers

Gene identification no.	Gene name	Orientation[a]	Sequence
SMU.37	*purH*	F	CCTGCTGACTACGCTGAGATTC
		R	TTGCTGCCAGACGCTTACG
SMU.1672	*clpP*	F	AGCGGTGCCAAAGGAAAACG
		R	GCCATATCAGACTGCTGTGTGC
SMU.80	*hrcA*	F	TGGAATTTGCTGGGCTTAACAC
		R	CATCTTCAGGTAGGCTTTGACG
SMU.81	*grpE*	F	AGAGCGACAAAGTTTGCAGAGG
		R	CAACAGCAAGAGCACGTTCAAG
SMU.105	*scrR*	F	TCGCATGGCATCCTTGAATCAG
		R	TCAGCAGCTAATTGACCTCCAC
SMU.128	*adhB*	F	CAGGCTCAGGTATCGGCTCAG
		R	GGCGCAACCACCTTGATTCC
SMU.179	*HP*[b]	F	ATGATTGTGGGCTGTTCG
		R	CGTGTTCAGAGTGACCTAG
SMU.182	*sloA*	F	F:CAATGGTGCGGGCAAGTC
		R	CGCATTCCTTGACTTTAATGGG
SMU.233	*ilvC*	F	TTCTGCGCGTGTAGGTCTCC
		R	AACCACCCATAAGAACCGCTTG
SMU.255	*oppA*	F	AAATTCGTGCCGTTCAAACAGC
		R	TCGCCGCCCCAAAGAGAC
SMU.872	*fruI*	F	GGGACTTGGGAAGTACGAGAAG
		R	AAACAAGAGCTGCTGCACCG
SMU.1745	*HP*	F	GGCGCGTTGTTTATTTAACCC
		R	CATCCATTGCTTCTGTGATTCG
SMU.1924	*gcrR*	F	TGGACGGGTATGAAGTGATTCG
		R	CATCTGCTCCACGATCAAGACC
SMU.2020	*rl16*	F	AGGCTGCTCGTATCGCTATGAC
		R	ACGTACACCGATGGCCTTAGC
SMU.1517	*covR*	F	ACACGATTACAGCCTTTGATGG
		R	CTTCCTTAGCCACTTCAAGACC
SMU.78	*fruA*	F	GGGACTTGGGAAGTACGAGAAG
		R	AAACAAGAGCTGCTGCACCG
SMU.2057	*cadD*	F	TCCATTCAAGACAGACCACAAGC
		R	TTCCTAATTCACGCCCAACAGC
SMU.602	*HP*	F	GCAACAGCAGCAGGAGTC
		R	GGGAACAACAACAATTCGTAAAG
SMU.426	*copA*	F	AGTGTCCTTAGCAACAACAGC
		R	GAACCTTAATCTCCTCGCCATC
SMU.2005	*adk*	F	ACCTTAAGCTAGATGGCGTTATC
		R	GAGCAATGTTGACATCTAAGCG
SMU.942	*mvaA*	F	TGAGTGTTGACACCAAAGAGG
		R	GACTGAGGAAACGAAGATTAACC
SMU.1591	*ccpA*	F	ATTTATGATGTTGCCCGTGAAGC
		R	GATAACCACACCTACCGTAGTCG
SMU.1288	*rplS*	F	CCGTACAGACATTCCTAACTTCC
		R	ACACCAACACCGCTTGAAATC
SMU.562	*clpE*	F	TTGTTGGTGCTGGTTCTGC
		R	GGGCTGAATACCTTTAAGAATCG
SMU.256	*oppB*	F	ATCTGGTTGGGGCTCTTTTGC
		R	TGCGATAGGCGTGATGATTGG
SMU.382	*HP*	F	CCGCTGAGTATTATGGACATGG
		R	TGAGAGAGGAAATGGAAGTAGGG
SMU.646	*gph*	F	GGCTCTTGCGGTCACTTATAGG
		R	CATCACGCTTAACTTGCTCTTGG
SMU.1916	*comD*	F	TATGGTCTGCTGCCTGTTGC
		R	TGCTACTGCCCATTACAATTCC
SMU.2042	*dexT*	F	F-TTAAGGCTGGGATCATGAATCG
		R	AACAGGTTCACCATCAAGAGC
SMU.1877	*manL*	F	GCATCTGACACAGTTGCTAAGG
		R	CATTAGCTTTAACACCGCCAGG
SMU.765	*nox1*	F	GGGTTGTGGAATGGCACTTTGG
		R	CAATGGCTGTCACTGGCGATTC

[a] F, forward primer (5′–3′); R, reverse primer (3′–5′).
[b] *HP*, hypothetical protein gene.

using BRB ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) with a *P* value of 0.001. Microarray data has been deposited with the National Center for Biotechnology Information.

Real-time quantitative PCR. A subset of genes was selected to validate the microarray analysis. Gene-specific primers were designed by using Beacon Designer 2.0 software (Premier Biosoft International, Palo Alto, CA) and are listed in Table 1. To obtain cDNA, 1 μg of three independent RNA samples and the iScript kit containing random primers (Bio-Rad, Hercules, CA) were used. Reverse transcription and real-time reverse transcriptase PCR (RT-PCR) were carried out according to protocols described elsewhere (3). A Student *t* test was performed to verify the significance of the real-time RT-PCR quantifications.

PTS assays. To measure sugar transport through the PTS, cells were grown to mid-exponential phase in TV medium supplemented with 0.5% glucose, fructose, or mannose; collected by centrifugation and washed (2); permeabilized with acetone-toluene (9:1); and assayed for sugar-specific PTS activity by the method of LeBlanc et al. (26). PTS activity was expressed as nanomoles of NADH oxidized in a PEP-dependent manner per minute per milligram of protein. Protein concentration of permeabilized cells was determined by the bicinchoninic acid assay (Sigma) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

***S. mutans* RelA restores (p)ppGpp production in an *E. coli* (p)ppGpp^0 strain.** To confirm the functionality of the *S. mutans* RelA enzyme, *E. coli* CF1693 (64), a *relAspoT* mutant strain unable to produce (p)ppGpp, was transformed with a plasmid containing the *relA* gene from *S. mutans* fused to the coding sequence for MBP. *E. coli* CF1693 has been shown to grow well in rich medium but not on M9 minimal agar due to multiple amino acid auxotrophies arising from its (p)ppGpp deficiency (64). The ability of the *relA* gene of *S. mutans* to restore the growth of CF1693 on minimal medium in the presence of high concentrations of 3-amino-1,2,4-triazole (AT), which requires accumulation of adequate levels of (p)ppGpp, has been demonstrated (29). For (p)ppGpp measurements, strains were grown in low-phosphate MOPS, and starvation was induced by addition of SHx and L-valine as detailed elsewhere (13). Consistent with the restoration of growth in minimal medium by the *S. mutans* RelA enzyme, (p)ppGpp production was restored in CF1693 transformed with the *relA* plasmid, confirming that *relA* codes for a true (p)ppGpp synthetase (Fig. 1A).

The synthesis and degradation activities of the Rel/Spo homolog from *Streptococcus equisimilis*, called Rel$_{Seq}$, have been investigated (37–39). It was demonstrated that the enzyme

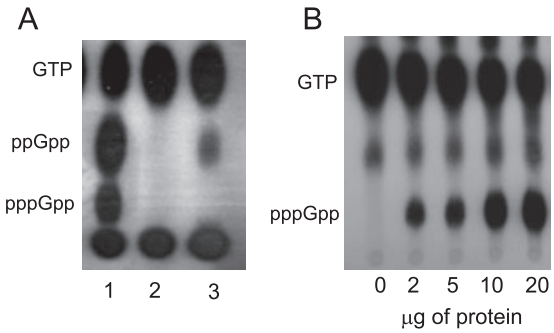

FIG. 1. Complementation of *E. coli* CF1693 (Δ*relA* Δ*spoT*) with an MBP fusion of the *S. mutans* RelA enzyme. (A) Accumulation of (p)ppGpp in *E. coli* strains after SHx-valine treatment. Strains were labeled with [^{32}P]orthophosphate in MOPS labeling medium. Nucleotides were extracted by adding an equal volume of 13 M formic acid, followed by freeze-thaw cycles. Acid extracts were spotted onto PEI-cellulose plates for TLC in 1.5 M KH_2PO_4. Lanes: 1, CF1648 (wild type); 2, CF1693 (Δ*relA* Δ*spoT*); 3, CF1693/pMAL-relA. (B) In vitro (p)ppGpp-synthetic activity of purified *S. mutans* RelA. Different concentrations (in micrograms as indicated) of purified RelA were incubated at 37°C for 1 h in a reaction mix containing 6 mM [α-^{32}P]GTP and 8 mM ATP as detailed in Materials and Methods. The reaction was terminated by addition of an equal volume of 13 M formic acid, and 5-μl samples were resolved by PEI-TLC in 1.5 M KH_2PO_4.

harbors a strong (p)ppGpp 3′ pyrophosphohydrolase and a weaker (p)ppGpp synthethase activity. More recently, work on the crystal structure of the enzyme demonstrated that the enzymatically active domain of Rel_{Seq} could acquire two mutually exclusive conformations, switching between synthetase-on/hydrolase-off and synthetase-off/hydrolase-on states that prevent futile cycles of (p)ppGpp synthesis and degradation (19). Although *S. mutans* RelA restored (p)ppGpp production in a $(p)ppGpp^0$ *E. coli*, the amounts of the penta- and tetraphosphorylated compounds in the complemented mutant strain were considerably lower than those accumulated in the wild-type *E. coli* parental strain. Similar to Rel_{Seq}, the expression of *S. mutans* RelA in wild-type *E. coli* lowered the levels of (p)ppGpp (data not shown). The *S. mutans* RelA enzyme is 93% similar to the *S. equisimilis* enzyme and possesses all of the synthetase and hydrolase residues mapped in Rel_{Seq}. Thus, it is likely that these enzymes function in a comparable way and that the *S. mutans* enzyme favors the degradation of (p)ppGpp under the conditions tested.

In vitro (p)ppGpp-synthetic activity of the *S. mutans* RelA. The *S. mutans* RelA protein (Smu-RelA) was expressed in *E. coli* as an MBP fusion and purified under soluble conditions. After purification, the Smu-RelA protein was released from the fusion protein by cleavage with Factor Xa. Purified preparations were used in an in vitro pppGpp synthesis assay with GTP and labeled ATP according to an established protocol (39). Under the tested conditions, pppGpp synthesis from purified Smu-RelA was demonstrated, confirming the results of the genetic complementation and providing evidence that the *S. mutans* RelA enzyme, like the *S. equisimilis* and *Mycobacterium tuberculosis* RelA enzymes (37, 39), does not require added ribosomes for pppGpp-synthetic activity in vitro (Fig. 1B). The fact that pppGpp was the only product detected in this assay is consistent with the knowledge that these enzymes produce the pentaphosphorylated compound when GTP is the acceptor and the tetraphosphorylated compound when GDP is the acceptor (14). However, it is not currently known whether the Smu-RelA enzyme has any preference for GTP or GDP as substrate.

RelA is the major (p)ppGpp-synthetase in MUP-treated cells. Previously, we demonstrated that *S. mutans* strains lacking RelA retained the ability to synthesize (p)ppGpp, and that the parental and RelA-deficient strains could accumulate comparable amounts of (p)ppGpp in response to treatment with SHx (28). Because the amounts of (p)ppGpp in SHx-treated cells were significantly less than what has been observed after similar treatment of *E. coli* and related organisms (40, 42, 51, 60), we assessed (p)ppGpp levels in *S. mutans* cells treated with MUP, an inhibitor of isoleucyl-tRNA synthetase that has been used to elicit a stringent response in other streptococci (15, 38, 45, 63). Treatment with MUP resulted in significant increases in (p)ppGpp pools in UA159 (an ~50-fold increase), but no increase in either compound was observed in JlrelA (see, for example, Fig. 2m in reference 29). Concurrent with the increase in (p)ppGpp levels, the amount of GTP was markedly diminished after MUP treatment (29). As observed previously in *S. equisimilis* (38) and *Streptococcus rattus* (63), the time course experiments of cells treated with MUP revealed that (p)ppGpp levels peaked after 20 min and remain elevated for up to 90 min in UA159, but no changes in the levels of (p)ppGpp were noted in response to MUP treatment in the Δ*relA* strain (data not shown). Thus, it was clear that RelA is the major enzyme responsible for (p)ppGpp production during MUP treatment. Also, consistent with our observations that there are additional sources of (p)ppGpp in *S. mutans* (28, 29), basal levels of (p)ppGpp were detected in the JLrelA strain after prolonged exposure of the X-ray films (data not shown).

Notably, increases in (p)ppGpp production were not related to increases in the transcription of *relA*. Previously, we demonstrated that there are no detectable differences in *relA* expression when cultures were subjected to SHx treatment or when comparing biofilm and planktonic cultures (28). We extended this analysis by testing the effects of other growth conditions using a gene fusion (*cat*) to the *relA* promoter (28). Specifically, cultures were grown to mid-exponential phase and subjected to treatment with MUP or SHx, low pH, excess glucose, or glucose starvation. None of the conditions tested elicited any changes in CAT activity (data not shown), suggesting that *relA* is not regulated at the transcriptional level, as has been observed in some organisms (55). Instead, (p)ppGpp levels are likely controlled by allosteric activation of RelA, presumably by mechanisms demonstrated for the Rel enzymes of other bacteria (62).

Gene expression profiling of UA159 treated with MUP. It is widely accepted that (p)ppGpp levels influence cellular physiology through transcriptional regulation of a large number of genes (16, 33, 45, 57). Here, microarrays were used to analyze gene expression in *S. mutans* UA159 treated with MUP, revealing that 234 genes were differentially transcribed ($P \leq 0.001$), with 42% ($n = 104$) upregulated and 58% ($n = 130$) downregulated (see Table S1 in the supplemental material). Consistent with the marked increase in (p)ppGpp levels in response to MUP exposure, many genes related to cell growth, including 30 involved in macromolecular biosynthesis, 15 encoding ribosomal proteins, and 10 related to energy metabolism, were downregulated in the presence of MUP (Fig. 2A). Among the upregulated genes, 12 participate in energy metabolism, 7 in amino acid biosynthesis, 2 encode components of the PTS, and 10 were predicted to have transcriptional or regulatory functions. Interestingly, a total of 79 genes encoding hypothetical proteins were affected by MUP (41 upregulated, 38 downregulated). Given the effects of loss of RelA on biofilm formation and acid tolerance (28), these hypothetical proteins could represent potential targets for subverting establishment or persistence of *S. mutans*. Real-time quantitative RT-PCR was performed on a subset of the genes (see tables in the supplemental material) to validate the microarray data, and all of the genes displayed the trends observed in the microarrays.

The upregulation of genes encoding biosynthetic enzymes for branched-chain amino acids (BCAAs) from the *leu* and *ilv* operons in *S. mutans*, such as *leuC* (SMU.1382), encoding an alpha-isopropylmalate isomerase large subunit, and *ilvE* (SMU.1203), encoding a BCAA aminotransferase, indicates that treatment of *S. mutans* with MUP does in fact lead to starvation for isoleucine, as has been verified in a genome profiling study of *E. coli* cells exposed to MUP (45). In *Bacillus subtilis*, expression of the *ilv* genes is negatively regulated by CodY (41, 49, 50), a global transcriptional repressor that controls many genes required for adaptation to nutrient limitation (23, 41, 50). DNA binding by CodY is activated by two differ-

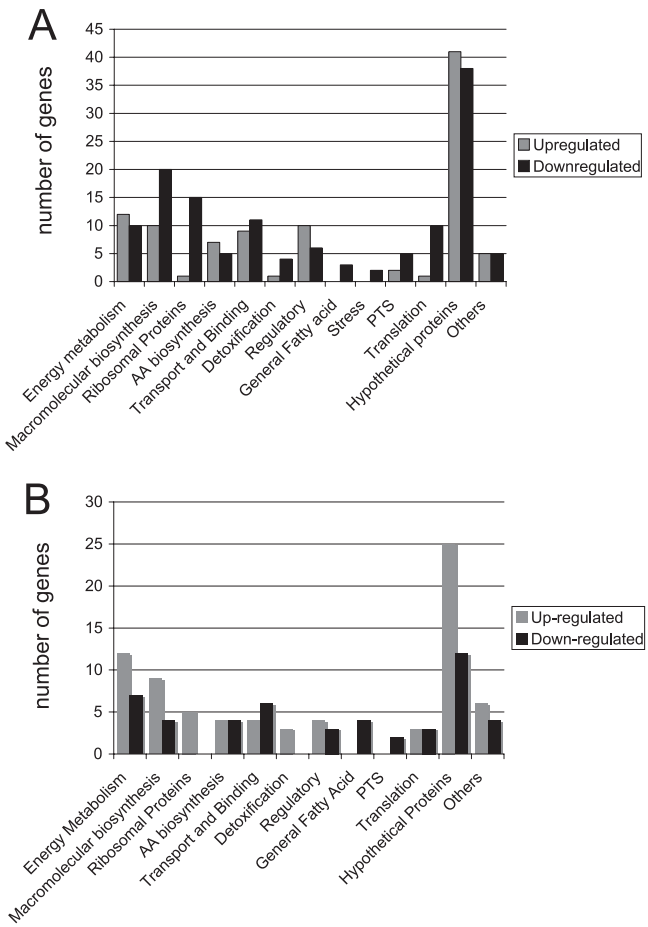

FIG. 2. Numbers of genes grouped by functional categories that were differently expressed after MUP treatment in comparison to control cells. (A) UA159; (B) JLrelA (Δ*relA*). Gene annotations are based on information provided by the Los Alamos National Laboratory (www.oralgen.lanl.gov) or by published literature available at the same website.

ent effectors, GTP and BCAAs (49). Given that high levels of (p)ppGpp are synthesized by RelA during the *S. mutans* MUP-response, which markedly reduces the intracellular levels of GTP (29) and potentially leads to derepression of CodY-regulated genes, our data provide further support for a link between RelA, CodY, and the stringent response (7, 35, 52). Consistent with our observation that the levels of GTP in MUP-treated cells may contribute to changes in gene expression patterns, we have revealed that an *S. mutans* strain lacking all (p)ppGpp synthase activity cannot grow in minimal medium lacking leucine or valine. However, growth was restored if a *codY* mutation was introduced into the strain lacking *relA*, *relP*, and *relQ*, indicating that CodY of *S. mutans* likely functions as has been demonstrated in certain other low G+C gram-positive bacteria (J. A. Lemos et al., unpublished data).

The relaxed phenotype of JLrelA. When the Δ*relA* mutant was treated with MUP, 124 genes ($P \leq 0.001$) exhibited differences in expression (see Table S2 in the supplemental material). Of those, 42% ($n = 52$) were downregulated, including 7 genes involved in energy metabolism and 4 related to amino acid biosynthesis. Fifty-eight percent ($n = 72$) of the differentially expressed genes were upregulated, including 25 hypotheticals, 12 that participate in energy metabolism, 9 associated with macromolecular biosynthesis, and 5 ribosomal protein genes (Fig. 2B). Interestingly, the majority of the genes associated with macromolecular biosynthesis and translation were induced by MUP in JLrelA, which is opposite to that observed in the wild-type strain. Similar to what was demonstrated in *E. coli* MUP-treated cells (45), the inversion of expression patterns of ribosomal protein genes in MUP-treated JLrelA supports that a lack of stringent control exists in the mutant strain.

Comparison of the UA159 and JLrelA transcriptomes in control and MUP-treated cells. To determine the effects of the absence of RelA on the MUP response, the transcriptomes of the wild-type and *relA* mutant strains of *S. mutans* were compared before and after addition of MUP. The transcriptome pattern comparison of the UA159 and JLrelA control cells grown in FMC containing glucose revealed that only six genes ($P \leq 0.001$) were differently expressed (data not shown), even though measurable differences in the growth rates of the strains exist (28). All of these genes were downregulated in the mutant compared to the parental strain, including four hypothetical genes and two genes involved in energy metabolism, *dexT* (SMU.2042) and *citZ* (SMU.671). Still, the results imply that, in nonstressed cells growing exponentially in broth culture, RelA has little impact on cellular homeostasis. This observation is consistent with our previous report that RelP, not RelA, is the major (p)ppGpp synthase functioning during exponential growth in the absence of stressors (29). In contrast, comparison of strains treated with MUP showed that 164 genes ($P \leq 0.001$) were expressed differently as a result of RelA deficiency, with 105 genes being upregulated and 59 being downregulated in the mutant compared to the parental strain (see Table S3 in the supplemental material), reinforcing the central role of RelA in the response of *S. mutans* to MUP. Of interest, a total of 20 genes dedicated to protein synthesis were upregulated in JLrelA compared to UA159 after MUP treatment, further confirming a relaxed phenotype of the mutant strain as a result of failure to accumulate (p)ppGpp.

RelA-independent response to MUP. The expression of 50 genes ($P \leq 0.001$) was commonly affected in both the parent and the mutant strains after the addition of MUP. Among these genes, *manL* (SMU.1877) and *manM* (SMU.1878), which encode components of the mannose PTS permease that are involved in global control of gene expression (1), especially energy metabolism, were repressed. A response regulator, *gcrR* (SMU.1924), of a two-component signal transduction system that has been shown to control the expression of the *S. mutans* glucan-binding protein *gbpC* (SMU.1396) and *gtfD* (SMU.910), encoding glucosyltransferase-S (22, 47), were also downregulated. In the presence of sucrose, the glucosyltransferase enzymes produce water-insoluble glucan polymers, which, in association with specific glucan-binding proteins, play key roles in adherence and biofilm formation by *S. mutans* (6, 65). Interestingly, the *gbpC* gene was also upregulated in the UA159 and JLrelA strains after MUP treatment. Moreover, *gtfD* and *gbpD* (SMU.772), which encode the water-soluble glucan synthase and glucan-binding protein D, respectively, were upregulated only in JLrelA MUP-treated cells, suggesting that regulation of these *S. mutans* virulence genes may be sensitive to (p)ppGpp levels. Notably, we previously demonstrated that the

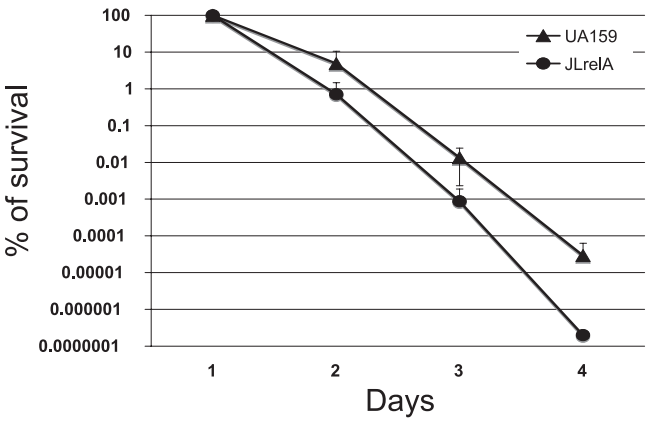

FIG. 3. Long-term survival of *S. mutans* UA159 (wild type) and JLrelA (Δ*relA*) in TY medium supplemented with 50 mM glucose. The results represent the means ± the standard deviations of three independent experiments.

S. mutans strain lacking RelA exhibited several important properties, including marked impairment in biofilm formation and enhanced acid tolerance when grown in biofilms (28). Of interest, *comD* (SMU.1916), which encodes the histidine kinase of a two-component signal that coordinates multiple environmental signals for the induction of *com* genes and development of competence (4, 30, 31), was induced by MUP in both strains. The competence regulon is important for the formation of biofilms and tolerance of environmental insults by *S. mutans* (30). Therefore, *S. mutans* appears capable of mounting a *relA*-independent, and possibly (p)ppGpp-independent, response to MUP that primarily involves regulation of genes essential for its pathogenicity, including those intimately involved in sugar metabolism, biofilm formation, competence development, and global control of gene expression.

Transcriptional analyses have been used to demonstrate a *relA*-independent amino acid starvation response in *Streptococcus pyogenes*, and this response was implicated in modulation of the expression of accessory and dedicated virulence genes (53). Using BLASTP searches, we identified potential homologues of the putative (p)ppGpp synthetases, RelP and RelQ, of *S. mutans* in the *S. pyogenes* genome and other bacteria (29). These findings suggest that *relA*-independent modulation of (p)ppGpp levels may be a general feature of bacteria harboring multiple synthetases, which may be particularly important for host-associated pathogens that encounter environments specific to the infection or transmission processes. Generally, pathogens that lack a free-living lifestyle do not encounter severely oligotrophic conditions, yet they do need to optimize growth and survival responses in relation to temporal or spatial variations during colonization and disease development in the host. Fine tuning of gene expression by modulation of (p)ppGpp levels through multiple enzymes, in response to multiple environmental or physiologic inputs, may be critical to persistence and pathogenesis.

Physiological characteristics of RelA-deficient *S. mutans*. Our microarray data support a central role for RelA in control of expression of genes that are intimately linked to the establishment, persistence, and pathogenesis of *S. mutans*. *E. coli* strains lacking both RelA and SpoT produce no detectable (p)ppGpp and exhibit a variety of phenotypes, including auxotrophy for several amino acids and poor long-term survival (14). Deletion of the *rel* gene was also found to adversely affect long-term persistence of *M. tuberculosis* (43). The long-term survival rates of the *S. mutans* RelA-deficient strain growing in BHI and TY containing 50 mM glucose were compared. In BHI, which has a glucose concentration of 11 mM, both parental and mutant strains were able to lower the pH to approximately 6.0 upon entry into stationary phase. Culture viability was assessed for up to 12 days, but no significant difference in the survival rates was observed between strains (data not shown). It has been demonstrated that *S. mutans* survival is reduced in media containing higher concentrations of carbohydrate, likely due to increased acidification of the environment through glycolysis (44). When we compared the survival of the cells in TY medium containing 50 mM glucose, in which a final pH of approximately 4.2 was attained on entry into stationary phase, the viability of the parental strain was substantially reduced compared to cells that were grown in BHI, with no viable cells detected after 3 days. Importantly, the viability of the *relA* mutant grown in TY with 50 mM glucose was significantly lower than that observed for the wild-type strain under the same conditions (Fig. 3). We have also observed that the loss of RelA enhances resistance to acid killing in biofilm populations of *S. mutans* (28), indicating that the role of RelA may be affected by growth domain, the growth of cells in densely packed populations, or quorum sensing.

We previously demonstrated that inactivation of *relA* of *S. mutans* results in a slower-growth phenotype and that this phenotype was more pronounced in FMC medium than in nutritionally rich BHI medium (28). Here, we tested the capacity of the *relA* mutant to grow on a variety of carbohydrates using TV medium supplemented with different sugars (Table 2). In TV base medium containing glucose, lactose, or fructose, the mutant grew slightly slower than the wild-type strain, but the differences observed in growth rates were not significant. Growth in mannose was significantly slower in the *relA* mutant, which displayed a doubling time of around 257 min compared to 227 min for the parental strain. In TV containing inulin, a homopolymer of fructose, the mutant strain grew faster and displayed a shorter lag phase after transfer from BHI medium compared to the wild-type strain (doubling times of approximately 338 and 434 min, respectively). Thus, lack of RelA in *S. mutans* leads to impaired long-term survival and atypical growth characteristics in selected sugars, suggesting that, in *S. mutans*, RelA plays a role in selectively balancing growth and

TABLE 2. Doubling time of *S. mutans* UA159 and JLrelA strains in TV broth supplemented with 0.5% glucose, lactose, fructose, mannose, or inulin

Supplement	Mean doubling time (min) ± SD[a] in broth with strain:	
	UA159	JLrelA
Glucose	80 ± 7	91 ± 4
Fructose	73 ± 8	70 ± 5
Mannose	227 ± 8	257 ± 7*
Inulin	434 ± 6	338 ± 5*

[a] *, Statistically significant ($P \leq 0.01$).

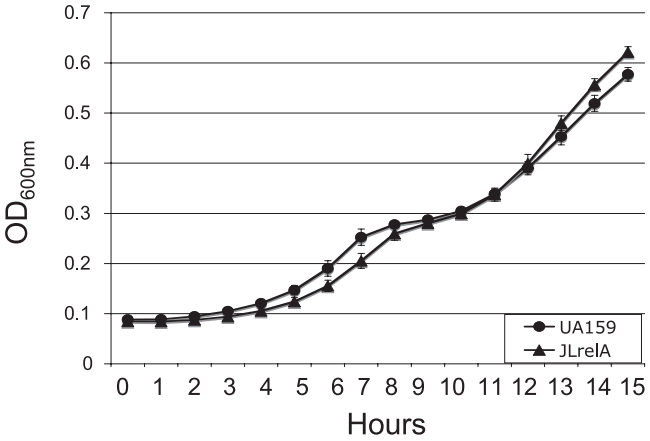

FIG. 4. Diauxic growth of *S. mutans* UA159 (wild-type) and JLrelA (Δ*relA*) strains. Mid-exponential-phase cultures grown in BHI were diluted 1:100 in TV medium supplemented with 0.05% glucose and 0.5% inulin, and cell growth was monitored every 1 h. The results represent the means ± the standard deviations of three independent experiments.

survival decisions and controlling the expression of key catabolic pathways. Since the RelA enzyme is the only protein in *S. mutans* in which a highly conserved (p)ppGpp hydrolase domain can be identified, we propose that a key role for RelA under the conditions tested may be to limit the amount of (p)ppGpp that is allowed to accumulate in the cells from the action of the RelP and RelQ enzymes.

Role of RelA in fructan metabolism. Taking into consideration that the Δ*relA* strain displayed slower growth on most carbohydrates, it was surprising that the mutant strain of *S. mutans* grew faster than the parental strain on inulin. Inulin is a fructose homopolymer that is degraded extracellularly to fructose by the *fruA* gene product (10), which is inducible by substrate, sensitive to carbohydrate catabolite repression (CCR) and contributes directly to the pathogenic potential of the organism. The fructosidase enzyme (FruA), an established virulence factor (10), is responsible for degradation of extracellular fructan polysaccharides into fructose, which is then directly consumed as an energy source. To better understand the role of RelA in inulin metabolism, diauxic growth of the strains cultured in TV containing 0.05% glucose and 0.5% inulin was monitored. Consistent with the shorter lag phase observed in the Δ*relA* mutant after transfer to inulin-containing medium, this mutant also displayed a shorter diauxie than did the wild-type strain (Fig. 4). Decreased diauxie is likely due to increased FruA expression in the Δ*relA* strain under the conditions tested. In fact, the levels of *fruA* mRNA from cells grown in TV-inulin were measured by real-time PCR and shown to be 2.3-fold higher in JLRelA than in UA159 ($P = 0.0032$). The most likely explanation for increased *fruA* expression is that CCR is partially alleviated in the absence of RelA. Of note, the expression of the *ccpA* gene (SMU.1591), which encodes the catabolite control protein CcpA (18), was decreased in the *relA* mutant compared to the wild-type strain, as revealed by microarray analysis and that CcpA can negatively regulate the *fruA* operon (J. Abranches et al., unpublished data). However, *fruA* regulation is complex (9) and involves the PTS (61, 66), so other changes induced by the loss of RelA could contribute to increased expression of *fruA*.

Another contributing factor to the enhanced growth phenotype of the Δ*relA* mutant on inulin could be that the mutant strain has a higher capacity to transport the fructose that is liberated from inulin by FruA through the PTS. At low sugar concentrations, the PTS is the major system for internalizing sugar, and we showed previously that glucose-PTS activity was higher in the *relA* mutant strain (28). Here, we found that fructose-PTS activity is also higher in the Δ*relA* strain (56% increase) compared to the wild-type strain (data not shown). Microarray analysis revealed that the expression of a variety of PTS genes, including *manL*, which encodes the EIIAB^{Man} of *S. mutans* was affected by MUP (see Tables S1 and S2 in the supplemental material). EIIAB^{Man} not only participates in sugar transport, including internalization of fructose, but also has key roles in global control of energy metabolism and CCR (1). Thus, our results support a major role for RelA in regulation of sugar catabolism, probably through modulation of (p)ppGpp pools during exponential growth.

Summary. *S. mutans* lives in multispecies oral biofilms where access to adequate nutrients is a key determinant for bacterial survival. To cause disease, the organism has to accomplish four major goals: attach, accumulate in significant numbers, generate acid, and tolerate environmental stress. The transcription profiling and physiologic and biochemical analyses presented here reveal an intimate role for RelA in all aspects of *S. mutans* pathogenesis. Both the endurance of periods of nutrient limitation, when saliva is the main source of nutrients, and the abrupt exposure to an excess amount of carbohydrate in the diet with associated environmental acidification, are major challenges for *S. mutans*. The RelA enzyme of *S. mutans* clearly plays a central role in optimizing gene expression for growth and persistence under these conditions by regulating sugar metabolism according to source and availability, modifying the cell envelope, and effecting changes in the expression of genes known to be essential for homeostasis, biofilm formation, and global regulation of gene expression. It is clear from our studies that *S. mutans* has evolved multiple routes to modulate (p)ppGpp production to adapt to unique niches in the oral cavity. We propose that (p)ppGpp is produced by the different enzymes in response to specific environmental conditions to fine-tune gene expression in a manner that allows the organisms to rapidly assimilate nutrients presented transiently in the diet while persisting mainly under carbohydrate limitation on salivary substrates during fasting periods. Efforts to dissect how RelA interacts with other (p)ppGpp synthases in control of homeostasis, growth, and virulence are ongoing, as are studies to determine how (p)ppGpp/GTP balances affect gene expression in *S. mutans*.

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