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The Molecular Alarmone (p)ppGpp Mediates Stress Responses, Vancomycin Tolerance, and Virulence in *Enterococcus faecalis*▽

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The stringent response is a global bacterial response to stress that is mediated by accumulation of the alarmone (p)ppGpp. In this study, treatment with mupirocin was shown to induce high levels of (p)ppGpp production in *Enterococcus faecalis*, indicating that this nosocomial pathogen can mount a classic stringent response. In addition, (p)ppGpp was found to accumulate in cells subjected to heat shock, alkaline shock, and inhibitory concentrations of vancomycin. Sequence analysis of the *E. faecalis* genome indicated that (p)ppGpp synthesis is catalyzed by the bifunctional synthetase/hydrolase RelA and the RelQ small synthase. The (p)ppGpp profiles of Δ*relA*, Δ*relQ*, and Δ*relAQ* strains revealed that RelA is the major enzyme responsible for the accumulation of (p)ppGpp during antibiotic or physical stresses, while RelQ appears to be responsible for maintaining basal levels of alarmone during homeostatic growth. Compared to its parent, the Δ*relA* strain was more susceptible to several stress conditions, whereas complete elimination of (p)ppGpp in a Δ*relAQ* double mutant restored many of the stress-sensitive phenotypes of Δ*relA*. Interestingly, growth curves and time-kill studies indicated that tolerance to vancomycin is enhanced in the Δ*relA* strain but diminished in the Δ*relQ* and Δ*relAQ* strains. Finally, virulence of the Δ*relAQ* strain but not of the Δ*relA* or Δ*relQ* strain was significantly attenuated in the *Caenorhabditis elegans* model. Taken together, these results indicate that (p)ppGpp pools modulate environmental stress responses, vancomycin tolerance, and virulence in this important nosocomial pathogen.

Enterococcus faecalis is a natural inhabitant of the human and animal intestinal flora. Although normally harmless to healthy individuals, enterococcal infections rank among the top three most common causes of nosocomial infections (43), with *E. faecalis* representing nearly 90% of the total cases of enterococcal infections (44). The strong association of this organism with infections in hospital settings has been attributed to its inherent capacity to withstand environmental stresses and to its innate and acquired resistance to many antibiotics (15, 17, 33). In particular, the appearance of vancomycin-resistant enterococcus (VRE) strains in the late 1980s gave these organisms a tremendous selective advantage, increasing the likelihood of their propagation and persistence in hospitals (33). From a clinical standpoint, *E. faecalis* is an opportunistic pathogen that causes a myriad of diseases in humans, infecting the bloodstream, abdomen, endocardium, urinary tract, biliary tract, burn wounds, and indwelling foreign devices (17, 32, 43, 44). Moreover, *E. faecalis* has also been involved in the dissemination of antibiotic resistance to other medically relevant bacterial species (51).

The stringent response is a global bacterial response to nutritional stress that is mediated by accumulation of the alarmones guanosine tetraphosphate and guanosine pentaphosphate, collectively known as (p)ppGpp (8, 42). These nucleotides are synthesized by enzymatic phosphorylation of GDP and GTP using ATP as a phosphate donor and are produced at low basal levels during favorable growth conditions. Accumulation of (p)ppGpp results in strong downregulation of genes for rRNAs and anabolic processes and upregulation of genes involved in amino acid biosynthesis and stress survival (5, 8, 35, 36, 48, 49). Thus, the (p)ppGpp alarmones function as chemical messengers that allow bacteria to switch their metabolism from a "growth mode" to a "survival mode."

In *Escherichia coli* and its close relatives, two homologous proteins are involved in (p)ppGpp metabolism, the ribosome-associated (p)ppGpp-synthetase RelA and the bifunctional synthetase/hydrolase SpoT (8, 41). In gram-positive bacteria, a bifunctional Rel/Spo ortholog, herein designated RelA, harbors both degradation and synthesis activities (30, 52). Recently two small proteins, designated RelP and RelQ, with weak (p)ppGpp-synthase activities were found in the oral pathogen *Streptococcus mutans* (26). It was demonstrated that while *relA* encoded a strong bifunctional synthetase/hydrolase enzyme responsible for the rapid accumulation of (p)ppGpp upon amino acid starvation, the RelP and RelQ proteins were responsible for constitutive expression of (p)ppGpp in non-stressed cells (26, 35). Subsequent work with *Bacillus subtilis* and *Streptococcus pneumoniae* further confirmed the functionality of these enzymes as (p)ppGpp synthases (3, 34). Homologues of at least one of the two small (p)ppGpp synthases are found in the genomes of gram-positive bacteria but absent in the genomes of proteobacteria (26, 34).

In the past few years, the role of (p)ppGpp in bacterial virulence has become an area of extensive research. Studies have correlated changes in (p)ppGpp levels to the expression

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TABLE 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant characteristics/application	Reference or source
E. faecalis		
OG1RF	Laboratory strain; Rif^r Fus^r	Laboratory stock
V583	Clinical isolate; Van^r	Laboratory stock
551-05	Clinical isolate; Van^s	CDC[a]
555-05	Clinical isolate; Van^s	CDC[a]
94-06	Clinical isolate; Van^r	CDC[a]
CK111	OG1Sp *upp4*::P_{23}*repA4*	22
JAL1	*relA* deletion mutant of OG1RF, Δ*relA*	This study
JAL2	*relA* deletion mutant of OG1RF, Δ*relQ*	This study
JAL3	*relA relQ* double-deletion mutant of OG1RF, Δ*relAQ*	This study
E. coli		
DH10B	Cloning host	Laboratory stock
EC1000	Host for cloning RepA-dependent plasmids	24
Plasmids		
pGEM5	Cloning vector	Promega
pCJK47	Donor plasmid, carries $oriT_{pCF10}$, P-*pheS**, pORI280 derivative; Erm^r	22

[a] CDC *Streptococcus* Laboratory.

of virulence traits, including stress survival (12, 20, 25, 54), biofilm formation (2, 25, 46), antibiotic resistance (14, 19, 20, 40), and persistence (5, 14, 20, 21, 35, 42). In some cases, animal models have been used to provide unequivocal evidence of the role of (p)ppGpp in virulence (6, 9, 10, 16, 39, 46). Despite its strong association with bacterial virulence, the stringent response has not been studied for *E. faecalis*. In this article, we characterize the stringent response of *E. faecalis* and demonstrate that (p)ppGpp pools play a fundamental role in growth under adverse conditions and may be a key factor regulating tolerance and growth in the presence of antibiotics, in particular vancomycin. Finally, we show that virulence of a (p)ppGpp^0 strain was highly attenuated in a *Caenorhabditis elegans* killing assay.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* strains were routinely grown in brain heart infusion (BHI) medium at 37°C. *E. coli* strains were grown in Luria broth and used for plasmid construction and propagation. When required for selective growth of strains, erythromycin (10 μg ml^{-1} for *E. faecalis*, 300 μg ml^{-1} for *E. coli*), spectinomycin (1,000 μg ml^{-1}), fusidic acid (25 μg ml^{-1}), or rifampin (rifampicin) (200 μg ml^{-1}) was added to the growth medium. To evaluate the capacity of *E. faecalis* strains to grow under different stress conditions, mid-exponential-phase cultures were diluted 1:100 into fresh medium and growth was monitored using a Bioscreen C growth monitor (Oy Growth Curves AB Ltd., Helsinki, Finland). Disc inhibition assays were used to assess the sensitivity of the strains to H_2O_2, HCl, or sodium hypochlorite. Briefly, a uniform layer of exponentially grown cells was spread onto BHI agar plates using a sterile swab, and paper filter discs (1 mm in diameter) saturated with 5% H_2O_2, 3 N HCl, or 10% sodium hypochlorite were placed onto the agar. All plates were incubated at 37°C for 48 h before the inhibition halo was recorded.

Construction of mutant strains. A markerless genetic exchange system (22) was used to construct *relA*, *relQ*, and *relAQ* mutants in *E. faecalis* OG1RF. Briefly, two PCR products flanking the *relA* or *relQ* gene were obtained with the primers listed in Table 2. The PCR amplicons were approximately 1 kb in size and included the first 10 to 15 residues and the last 10 to 15 residues of the *relA* or *relQ* gene, which were retained in the mutants to avoid unanticipated effects on the expression of adjacent genes. After digestion with the appropriate restriction enzymes, the two PCR products flanking the gene of interest were simultaneously cloned onto pGEM5 (Promega, Madison, WI) to give the plasmids pGrelA and pGrelQ. The 2-kb fragments containing the *relA* (pGrelA) and *relQ* (pGrelQ) up-down fragments were subcloned into pCJK47 (22) using *E. coli* EC1000 (24) as the host strain. The resulting plasmids, pCJK-relA and pCJK-relQ, were electroporated into competent *E. faecalis* CK111 (donor strain) (23). *E. faecalis* CK111 containing the pCJK-relA or pCJK-relQ plasmids was conjugated with wild-type *E. faecalis* OG1RF, and single-crossover insertions were selected on BHI agar medium containing rifampin and erythromycin. Single colonies were subjected to the PheS* negative counterselection system to isolate double-crossover deletions as described elsewhere (22). The *relA* and *relQ* gene deletions were confirmed by PCR sequencing of the insertion site and flanking sequences. Subsequently, a double Δ*relA* Δ*relQ* strain was constructed by conjugation of the donor strain harboring pCJK-relQ with the Δ*relA* mutant.

Detection of (p)ppGpp accumulation patterns. For (p)ppGpp measurements in *E. faecalis*, cells were grown in the chemically defined FMC medium (47) containing a lower phosphate concentration (8.6 mM) in order to improve ^{32}P labeling (26). Overnight cultures grown in FMC were diluted 1:100 in fresh low-phosphate FMC, grown to an optical density at 600 nm of ≈0.2, and prelabeled with 50 μCi of carrier-free [^{32}P]orthophosphate (Amersham Biosciences, Piscataway, NJ) for one generation. At this point, experimental cells were treated with mupirocin (50 μg ml^{-1}), vancomycin (10 μg ml^{-1}), or ampicillin (32 μg

TABLE 2. Primers used for gene deletion

Primer	Sequence[a]	Application
FW relA UP	CGATTATCTTGGCATGCCCAGAGGAAG	*relA* deletion
RV relA UP	CCGCTGGGCCGATATCTATTTCTTCTTTTG	*relA* deletion
FW relA DOWN	GAACCAATGGGGATATCTTAAAAGTGAG	*relA* deletion
RV relA DOWN	GTGGTATCGCGCGGCCGCGAGATATTAC	*relA* deletion
FW relQ UP	CCGTCGTTTAATCTAGATAATCGGAATATG	*relQ* deletion
RV relQ UP	GATCCCTCAATAAGCTTGAATTTCATTC	*relQ* deletion
FW relQ DOWN	ATAAGCAAATTAAGCTTGAAACACCAG	*relQ* deletion
RV relQ DOWN	CGTCATTTTAACGGATCCGCCAGTTTGTA	*relQ* deletion

[a] The underlined bases correspond to restriction sites included to aid in the subsequent cloning of the PCR products.

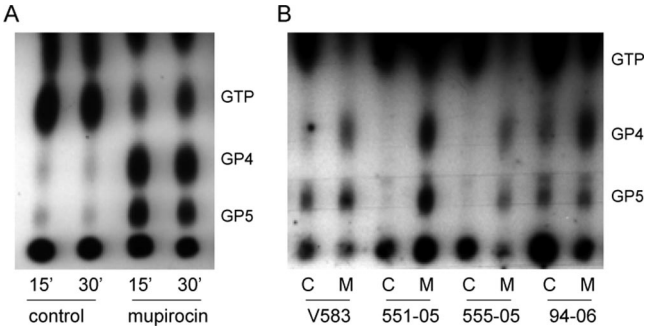

FIG. 1. Accumulation of (p)ppGpp in *E. faecalis* after mupirocin treatment. (A) *E. faecalis* OG1RF treated with mupirocin for 15 or 30 min. (B) *E. faecalis* clinical isolates treated with mupirocin for 30 min. Exponentially grown cells were labeled with [^{32}P]orthophosphate in FMC and treated with 50 μg ml^{-1} mupirocin for the times (minutes) indicated. Nucleotide acid extracts were spotted onto PEI-cellulose plates for TLC in 1.5 M KH_2PO_4. The identities of the radioactive spots were confirmed by comigration with GTP, GP5, and GP4 standards.

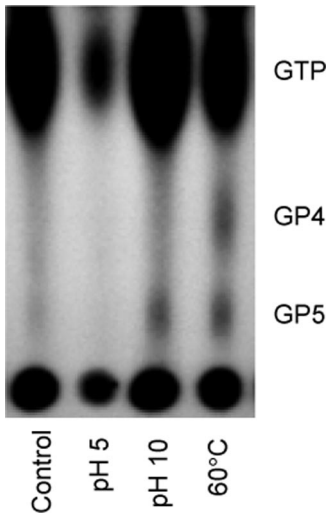

FIG. 2. (p)ppGpp profile of *E. faecalis* OG1RF under selected stress conditions. Exponentially grown cells were labeled with [^{32}P]orthophosphate in FMC and subjected to the stress condition indicated for 60 min. Acid extracts were spotted onto PEI-cellulose plates for TLC in 1.5 M KH_2PO_4.

ml^{-1}) or subjected to acid shock (pH 5, using 3 N HCl), alkaline shock (pH 10, using 2 N NaOH), and heat shock (60°C). Control cells consisted of aliquots that were labeled for the duration of the experimental cultures. Nucleotide pools were extracted by adding an equal volume of 13 M formic acid, followed by two freeze-thaw cycles. Acid extracts were centrifuged briefly, and the supernatant fluids were spotted onto polyethyleneimine (PEI)-cellulose plates (Selecto Scientific, Inc., Suwanee, GA) for separation by thin-layer chromatography (TLC) in 1.5 M KH_2PO_4 (pH 3.4).

Biofilm assay. Biofilm formation on polystyrene microtiter plates was quantified essentially as described previously (1). Biofilms were grown in a semidefined biofilm medium (28) containing glucose as the carbohydrate source for 24 h at 37°C before adherent bacteria were stained with 0.1% crystal violet. The bound dye was extracted from the stained cells with 33% acetic acid solution in water, and the biofilms were quantified by measuring the optical density of the solution at 575 nm.

Determination of MIC. The MIC for ampicillin, mupirocin, or vancomycin was determined in BHI using twofold serial dilutions prepared in the wells of microtiter plates. Bacteria from mid-log-phase cultures were inoculated into each well at ≈10^5 CFU ml^{-1}. Microtiter plates were incubated at 37°C for 24 h before the optical density at 600 nm of each well was measured using the Benchmark Plus microplate spectrophotometer (Bio-Rad, Hercules, CA). The lowest concentration of antibiotic that prevented cell growth was assigned as the MIC.

Time-kill kinetics. Cultures were grown in BHI to exponential phase and then diluted 1:10 in fresh BHI to obtain a starting inoculum of 5×10^6 to 1×10^7 CFU ml^{-1}. Time-kill studies were initiated by adding five times the MIC of vancomycin for the wild-type strain. Viable counts were determined at time zero and then every 24 h for up to 6 days.

***C. elegans* killing assay.** The ability of *E. faecalis* OG1RF and its derivatives to kill *C. elegans* was investigated as described previously (11). Briefly, a single colony of OG1RF was inoculated in BHI broth and grown at 37°C overnight, and 10 μl of culture was spread on a 35-mm BHI plate containing 50 μg ml^{-1} gentamicin. The plates were allowed to incubate overnight at 37°C and were brought to room temperature before 60 to 90 L4-stage, wild-type (N2) worms (obtained from the Caenorhabditis Genetics Center) were set on the bacterial lawns. The Kaplan-Meier method was used to determine survival over time, and curves were compared using the log-rank test to generate *P* values testing the null hypothesis that a given pair of survival curves was identical. *P* values of 0.05 or less were considered statistically significant. All data were analyzed with the GraphPadPrism 5.0 software program.

RESULTS

***E. faecalis* strains can mount a classic stringent response.** To determine whether *E. faecalis* can mount a stringent response, the accumulation of (p)ppGpp during mupirocin treatment was examined. Mupirocin is an inhibitor of isoleucyl-tRNA synthetase that has been shown to elicit a potent stringent response in streptococci (26, 31, 53). In *S. mutans*, we have previously determined that (p)ppGpp accumulation is best observed when cells are treated with 5 to 10 times the MIC for mupirocin (26). Thus, we determined the MIC for mupirocin in *E. faecalis* OG1RF and V583 and found that *E. faecalis* was intrinsically more resistant to mupirocin than *S. mutans* (MIC of 10 μg ml^{-1} for the *E. faecalis* strains versus MIC of 50 ng ml^{-1} for *S. mutans*). Time-course experiments using 50 μg ml^{-1} mupirocin showed dramatic increases in (p)ppGpp pools in *E. faecalis* OG1RF (Fig. 1A), with (p)ppGpp levels remaining elevated for up to 90 min (data not shown). Consistent with increases in (p)ppGpp pools was a fast drop in GTP levels, the acceptor nucleotide necessary for pppGpp synthesis (8). Similar results, e.g., rapid accumulation of (p)ppGpp at the expense of GTP upon mupirocin treatment, were obtained when using clinical isolates of *E. faecalis* (Fig. 1B), suggesting that this response is conserved among *E. faecalis* strains. Notably, the amounts of (p)ppGpp accumulated by the different isolates showed significant differences. Since all strains displayed the same MIC for mupirocin (data not shown), it appears that the differences in (p)ppGpp levels are due to inherent heterogeneity among different isolates. Based on the fact that all isolates can mount a classic stringent response, the experiments described below were conducted using strain OG1RF and its isogenic mutants. The advantages of using strain OG1RF is that it is readily transformable, does not carry plasmids, and is not resistant to antibiotics commonly used for genetic studies (4).

(p)ppGpp accumulates under selected environmental stress conditions. Enterococci are well known for their capacity to survive in harsh environments. Here we assessed (p)ppGpp levels in OG1RF cells subjected to acid, alkaline, or heat shock. Under the conditions tested, (p)ppGpp was found to accumulate in cells subjected to heat (60°C) or alkaline (pH 10) shock but not in cells subjected to acid shock (pH 5)

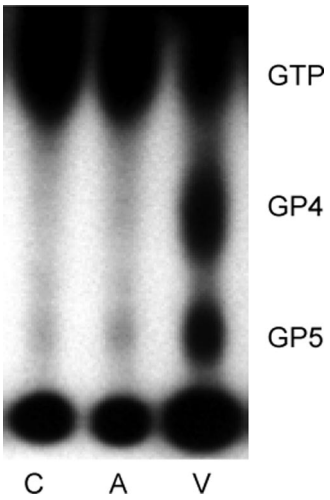

FIG. 3. Accumulation of (p)ppGpp in *E. faecalis* OG1RF after vancomycin treatment. Exponentially grown cells were labeled with [^{32}P]orthophosphate in FMC and treated with 32 μg ml^{-1} ampicillin (A) or 10 μg ml^{-1} vancomycin (V) for 60 min. Control cells (C) were kept untreated and labeled for the duration of the experiment. Acid extracts were spotted onto PEI-cellulose plates for TLC in 1.5 M KH_2PO_4.

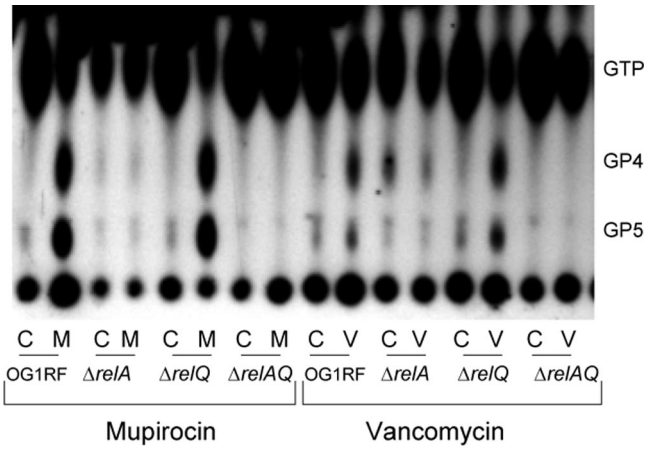

FIG. 4. Accumulation of (p)ppGpp in *E. faecalis* OG1RF, Δ*relA*, Δ*relQ*, and Δ*relAPQ* strains after mupirocin or vancomycin treatment. Exponentially grown cells were labeled with [^{32}P]orthophosphate in FMC and treated with 50 μg ml^{-1} mupirocin for 15 min or 10 μg ml^{-1} vancomycin for 60 min. C, untreated control cells; M, mupirocin-treated cells; V, Vancomycin-treated cells. Acid extracts were spotted onto PEI-cellulose plates for TLC in 1.5 M KH_2PO_4.

(Fig. 2). Time course experiments confirmed the transient accumulation of (p)ppGpp pools in cells subjected to heat or alkaline shock (data not shown).

Vancomycin treatment triggers (p)ppGpp accumulation. The spread of VRE strains constitutes a major clinical problem in hospital settings. Vancomycin is a glycopeptide that acts on gram-positive organisms by inhibiting cell wall biosynthesis. This molecule attaches to the lipid II precursor at its D-Ala-D-Ala peptide side chain, thereby inhibiting transglycosylation of cell wall subunits. Cell envelope stress responses have been linked to general stress responses (7) and to the overall homeostasis of gram-positive bacteria (18). Moreover, it has been reported that the intracellular concentration of (p)ppGpp is linked to the intrinsic resistance to antimicrobial agents in bacteria (14, 19, 20, 40). TLC analysis of nucleotide extracts clearly indicated that (p)ppGpp accumulates to significantly high levels in OG1RF cells treated with inhibitory concentrations of vancomycin (Fig. 3). Interestingly, treatment with inhibitory concentrations of ampicillin, a β-lactam antibiotic that also inhibits cell wall biosynthesis, did not result in (p)ppGpp accumulation (Fig. 3). The kinetics of (p)ppGpp accumulation, peaking after 60 min, were considerably slower than the kinetics observed in mupirocin-treated cells.

Identification of genes encoding (p)ppGpp synthetases in *E. faecalis*. Previously we have shown that production of (p)ppGpp by *S. mutans* is catalyzed by three enzymes: RelA, RelP, and RelQ (26). While RelA and RelQ appear to be ubiquitously distributed in the genome of low-GC gram-positive bacteria, BLAST searches indicated that RelP homologues are not ubiquitously distributed in gram-positive cocci, including *E. faecalis* V583 (37). The *E. faecalis* RelA (EF1974) and RelQ (EF2671) putative proteins share high levels of identity with their counterparts in *S. mutans* (78% and 80% similarities, respectively). Recently the complete genome of *E. faecalis* OG1RF became available (4). BLAST searches of the OG1RF genome and searches in the preliminary sequences of strains TX0104 and H22 available at the Baylor College of Medicine Human Genome Sequencing Center (http://www.hgsc.bcm.tmc.edu) identified putative RelA and RelQ homologues but not RelP. Thus, it appears that (p)ppGpp synthesis is catalyzed by only two enzymes in *E. faecalis*: RelA and RelQ.

RelA is the major synthetase responsible for (p)ppGpp accumulation. A markerless genetic exchange system was used to construct in-frame deletions in the *relA* and *relQ* genes of *E. faecalis* OG1RF. In order to rule out the possibility that polar effects on genes downstream of *relA* and *relQ* (ef1974 and ef2671, respectively) may have contributed to phenotypes of the Δ*relA* and Δ*relQ* strains, real-time PCR was used to confirm that expression of the genes apparently cotranscribed with *relA* and *relQ*, ef1973 and ef2670, respectively, was not affected in the mutant strains (data not shown). The (p)ppGpp profile of Δ*relA*, Δ*relQ*, and Δ*relAQ* indicates that RelA is the major enzyme responsible for the transient accumulation of (p)ppGpp during mupirocin or vancomycin treatment (Fig. 4). Although it does not appear to be involved in the transient accumulation of (p)ppGpp, RelQ appears to be responsible for maintaining basal levels of alarmone during homeostatic growth, since only the *relAQ* double mutant displayed an apparent (p)ppGpp^0 phenotype (Fig. 4).

Growth characteristics, stress tolerance, and biofilm formation by the Δ*relA*, Δ*relQ*, and Δ*relAQ* strains. The results described above prompted us to investigate the role of RelA and RelQ in the physiology of *E. faecalis*. Analysis of growth rates of the mutants in BHI indicated that the Δ*relQ* and Δ*relAQ* strains grew as well as the parent strain whereas the Δ*relA* strain grew slightly slower (Fig. 5). The ability of the Δ*relA*, Δ*relQ*, and Δ*relAQ* strains to grow under a variety of stress conditions was examined (Fig. 6). No differences in growth between the mutants and parent strain were observed when cells were grown at pH 9 or in the presence of detergents (0.003% sodium dodecyl sulfate or 0.02% deoxycholate) (data

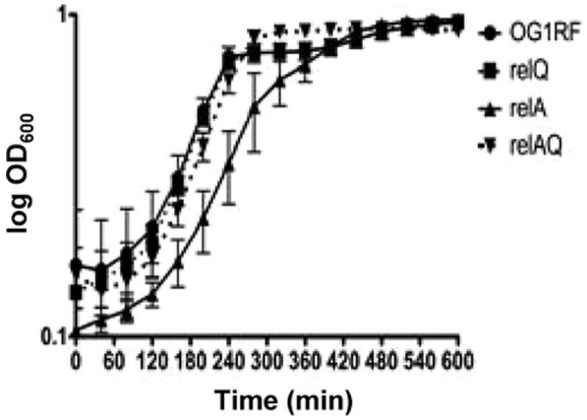

FIG. 5. Growth curves of *E. faecalis* OG1RF, Δ*relA*, Δ*relQ*, and Δ*relAQ* strains in BHI, determined using the Bioscreen growth reader monitor. The results represent the means ± standard deviations of three independent experiments.

TABLE 3. Phenotypes of the Δ*relA*, Δ*relQ*, and Δ*relAQ* strains in relation to that of OG1RF (wild-type strain) in disc diffusion and plate titration assays

Genotype	Result of plate test with indicated condition[a]				
	Disc diffusion assay			Plate titration assay	
	3 N HCl	5% H_2O_2	10% NaClO	45°C	50°C
Δ*relA*	S	S	ND	ND	ND
Δ*relQ*	ND	ND	ND	ND	ND
Δ*relAQ*	ND	ND	ND	ND	S

[a] S, sensitive in comparison to OG1RF; ND, no differences in comparison to OG1RF.

not shown). In comparison to its parent, the Δ*relA* strain grew poorly or considerably slower at 48°C, at pH 5, or in the presence of 5% NaCl or 2 mM H_2O_2 (Fig. 6). Inactivation of *relQ* alone did not affect cell growth under any of the conditions tested (Fig. 6). Complete elimination of (p)ppGpp in the Δ*relAQ* strain restored many of the growth defects of the Δ*relA* strain, with the exception of growth at 48°C or in the presence of H_2O_2. Compared to the parent strain, the Δ*relAQ* strain grew slowly and had low growth yields at 48°C. Surprisingly, the Δ*relAQ* strain grew faster and to a higher growth yield than its parent in the presence of H_2O_2 (Fig. 6).

We also tested the susceptibilities of the mutant strains to HCl, H_2O_2, and sodium hypochlorite using disk diffusion assays. Based on the diameters of the zone of inhibition for each compound tested, the Δ*relA* strain was more sensitive to HCl and H_2O_2, but no differences were observed in the inhibition zone caused by sodium hypochlorite (Table 3). No significant differences in sensitivity to any of the tested conditions were observed among wild-type, Δ*relQ*, and Δ*relAQ* strains. To test the abilities of the strains to grow on BHI plates at high temperatures, exponentially growing cells were serially diluted and aliquots were spotted onto BHI plates that were incubated at 45° and 50°C. No differences were observed when strains

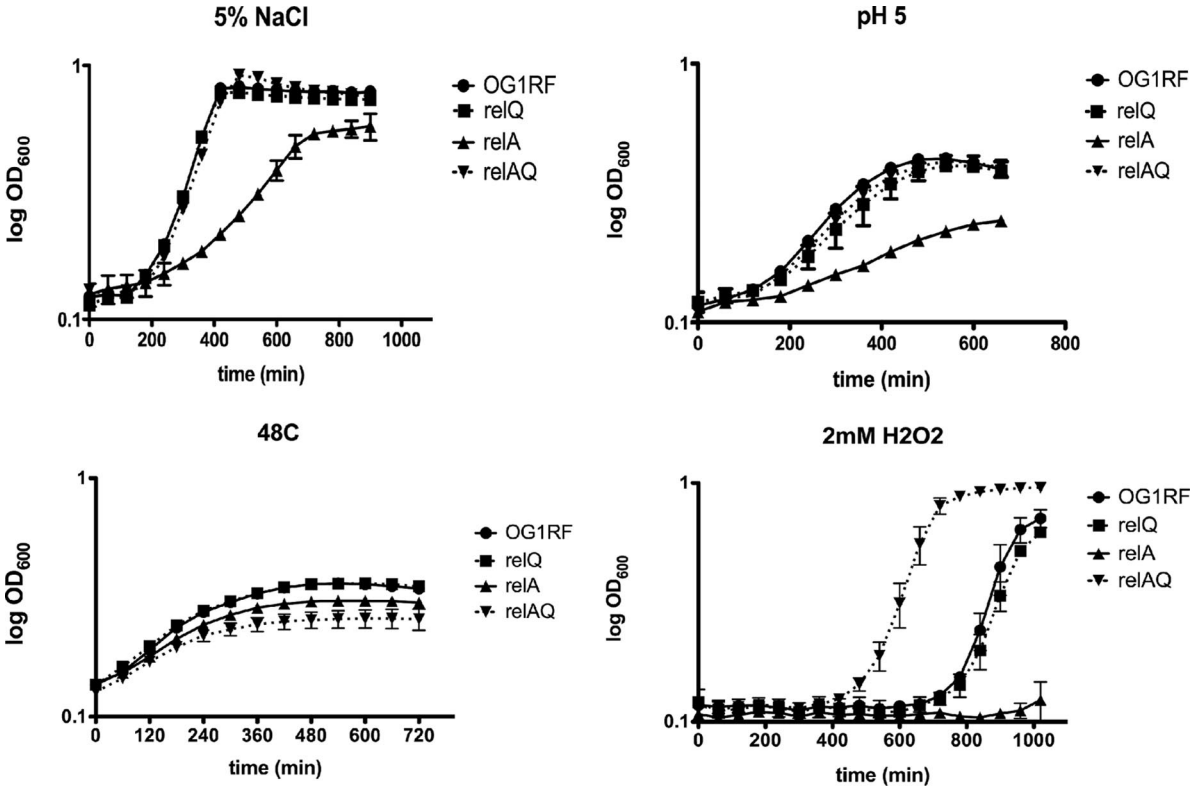

FIG. 6. Growth of *E. faecalis* OG1RF, Δ*relA*, Δ*relQ*, and Δ*relAQ* strains under stress conditions, determined using the Bioscreen growth reader monitor. The results represent the means ± standard deviations of three independent experiments.

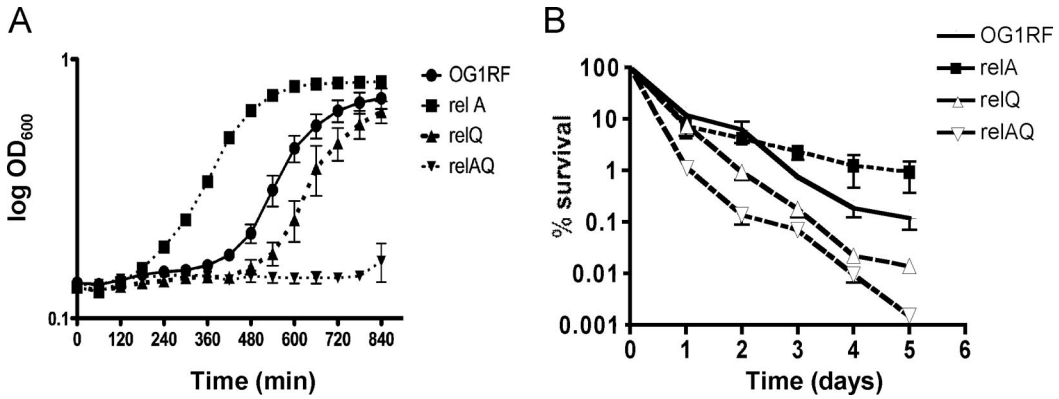

FIG. 7. Growth and time-kill curves of *E. faecalis* OG1RF, Δ*relA*, Δ*relQ*, and Δ*relAQ* strains in the presence of vancomycin. (A) Growth curves in BHI containing 2.5 μg ml^{-1} vancomycin. (B) Time-kill curves of logarithmic-phase cultures treated with 40 μg ml^{-1} vancomycin. Viable counts were determined by plating known dilutions of the samples on BHI plates every 24 h.

were grown at 45°C. However, consistent with the slow-growth phenotype at 48°C in broth, the Δ*relAQ* strain was more sensitive to growth at 50°C by 1 log (Table 3).

The capacity of the mutants to form biofilms on polystyrene microtiter plates was also assessed. Quantitative analysis of biofilms formed by the Δ*relA*, Δ*relQ*, and Δ*relAQ* mutants did not differ from that of biofilms formed by the parent OG1RF strain (data not shown). However, it is important to acknowledge the limitations of the microtiter plate assay. This method is based on batch cultures grown under static conditions that fail to reproduce the physicochemical environment that is encountered in vivo. Therefore, these data must be interpreted with caution, and it is possible that under certain conditions, changes in (p)ppGpp pools may affect biofilm formation by *E. faecalis*.

Tolerance to vancomycin is enhanced in the Δ*relA* strain but diminished in the Δ*relQ* and Δ*relAQ* strains. The accumulation of (p)ppGpp in cells treated with vancomycin suggests that these nucleotides could be involved in the intrinsic tolerance of the organism to this antimicrobial drug. Compared to the wild-type strain (MIC = 8 μg ml^{-1}), the Δ*relQ* and Δ*relAQ* strains exhibited lower MICs for vancomycin (4 μg ml^{-1}). No differences in the MICs were observed between the parent and Δ*relA* strains. Next, we tested the ability of *E. faecalis* OG1RF and its Δ*rel* derivatives to grow in the presence of subinhibitory concentrations of vancomycin. Compared to the growth of the wild-type strain in the presence of 2.5 μg ml^{-1} vancomycin, the Δ*relQ* strain grew slower whereas growth of the Δ*relAQ* strain was severely impaired (Fig. 7A). Surprisingly, the Δ*relA* strain grew considerably faster than the wild-type strain (Fig. 7A). Time-kill kinetic studies were conducted using a concentration equal to five times the MIC of vancomycin for OG1RF (40 μg ml^{-1}). In comparison to the parent strain, OG1RF, the Δ*relQ* and Δ*relAQ* strains were more rapidly killed whereas the Δ*relA* strain was more resistant to vancomycin killing (Fig. 7B).

Virulence of the Δ*relAQ* mutant was attenuated in the *C. elegans* model. To determine whether disruption of one or both (p)ppGpp synthetases affected *E. faecalis* virulence, the Δ*relA*, Δ*relQ*, and Δ*relAQ* mutants were tested for their ability to kill *C. elegans* (Fig. 8). Compared with the wild-type strain, OG1RF, virulence of the Δ*relAQ* strain was highly attenuated ($P < 0.0001$). There were no significant differences in killing among the Δ*relA* and Δ*relQ* strains.

DISCUSSION

In this report, (p)ppGpp profiles of cells subjected to stress and mutational analysis of genes involved in alarmone metabolism provided unequivocal evidence that production of (p)ppGpp is an integral part of the core stress responses of *E. faecalis*. More specifically, it was demonstrated that *E. faecalis* accumulates the alarmone (p)ppGpp in response to amino acid starvation, and more importantly, it reveals that (p)ppGpp modulates environmental stress responses, vancomycin tolerance, and virulence in this important nosocomial pathogen.

Until recently, bifunctional RelA was considered the sole enzyme responsible for controlling (p)ppGpp metabolism in gram-positive bacteria. However, two related small enzymes, designated RelP and RelQ, were recently identified and shown to function as true alarmone synthetases in *S. mutans*, *S. pneumoniae*, and *B. subtilis* (3, 26, 34). While RelA and RelQ appear to be ubiquitous in the genome of many gram-positive

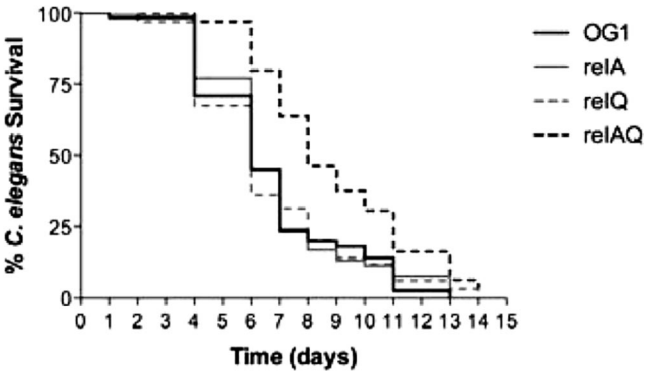

FIG. 8. Killing of *C. elegans* by *E. faecalis* OG1RF and its derivatives. Exposure of *C. elegans* to the *relA* and *relQ* mutants did not cause a significant difference in killing from that with the wild-type strain, OG1RF ($P = 0.8089$ and 0.8895, respectively). However, the Δ*relAQ* mutant was significantly attenuated ($P < 0.0001$). This experiment was repeated three times with similar results.

organisms, RelP was found to be absent in several organisms, including *E. faecalis* (4, 37). The contributions of each enzyme to (p)ppGpp metabolism have been partially assigned in *S. mutans* and *B. subtilis*. It was demonstrated that RelP and RelQ play an important role in cell homeostasis by producing low levels of alarmone during nonstressful conditions, whereas RelA appears to have retained the function of Rel/Spo-like proteins and is the major enzyme responsible for the stringent response, i.e., the rapid accumulation of (p)ppGpp in response to amino acid starvation (26, 35). Notably, RelA is the only enzyme also acting as a (p)ppGpp phosphohydrolase, suggesting that in addition to being responsible for the rapid and transient accumulation of (p)ppGpp during starvation, RelA plays a key role by controlling the intracellular (p)ppGpp/GTP/GDP ratios during homeostatic growth.

The (p)ppGpp measurements using the *E. faecalis* Δ*relA*, Δ*relQ*, and Δ*relAQ* mutants reveal a pattern similar to that described for *S. mutans* and *B. subtilis* (26, 34), with the caveat that RelP is not found in *E. faecalis* (4, 37). Moreover, the growth characteristics of the *E. faecalis* Δ*relA*, Δ*relQ*, and Δ*relAQ* mutant strains mirrored the growth behavior of the Δ*relA*, Δ*relQ*, and Δ*relAPQ* strains of *S. mutans* and *B. subtilis* (26, 34). In both *S. mutans* and *B. subtilis*, the slow-growth phenotype of Δ*relA* strains could be restored when the RelP and RelQ synthases were simultaneously inactivated, resulting in a (p)ppGpp^0 phenotype (26, 34). Similarly, the *E. faecalis* Δ*relAQ* strain grew as well as the parental strain; thus, the slow growth of the *E. faecalis* Δ*relA* mutant is most likely a result of the inability of the strain to hydrolyze (p)ppGpp synthesized by RelQ.

The association of (p)ppGpp levels with resistance to antibiotics has been observed in *E. coli* (14, 19, 38, 40, 50). In most cases, antibiotic resistance was linked to low growth rates due to the stringent response and to reductions in autolytic activity (19, 50). Moreover, it has been demonstrated that artificially raising (p)ppGpp levels increased β-lactam tolerance in *E. coli* (19), and mutant cells lacking RelA were more susceptible to β-lactams (38). More recently, a direct correlation between intracellular (p)ppGpp levels and resistance to microcin J25, a plasmid-encoded antibacterial peptide, was established (40). In this latter case, resistance to microcin J25 was shown to involve (p)ppGpp-dependent induction of an efflux pump responsible for lowering the intracellular levels of the peptide (40). Despite its association with antibiotic resistance in *E. coli*, the role of (p)ppGpp in multitolerant bacteria, such as VRE, has not been evaluated. Here, we demonstrated that tolerance to vancomycin is intimately associated with (p)ppGpp pools. Although RelA is the enzyme responsible for rapid accumulation of (p)ppGpp, when exposed to inhibitory concentrations of vancomycin, the Δ*relA* mutant had the same MIC as the wild-type strain, grew faster in the presence of subinhibitory concentrations of the drug, and survived better in time-kill studies. On the other hand, the Δ*relQ* and Δ*relAQ* strains had a lower MIC and slow or impaired growth in the presence of subinhibitory concentrations and were killed more rapidly by vancomycin, characteristics that were exacerbated in the Δ*relAQ* strain. Considering that the Δ*relA* strain, which is unable to break down (p)ppGpp, has high background levels of alarmone (synthesized by RelQ), that the Δ*relQ* mutant produces lower basal (p)ppGpp pools (through RelA), and that the Δ*relAQ* strain is completely unable to synthesize (p)ppGpp, one can speculate that higher basal levels of (p)ppGpp increase the expression of vancomycin tolerance/resistance determinants in *E. faecalis*. In many ways, because the Δ*relA* strain grew better in the presence of subinhibitory concentrations of vancomycin, this result is in stark contrast with earlier findings for *E. coli* that indicated that antibiotic tolerance was linked to slow growth and reduced autolysis that was dependent on a functional RelA (13, 50). However, it is important to note that recent studies revealed that bacteria have evolved different modes of (p)ppGpp regulation and that the effects of (p)ppGpp on cell physiology vary greatly among different organisms (26, 34, 41). Presently it is not clear whether (p)ppGpp has a direct role in the expression of genes that confer vancomycin tolerance to *E. faecalis* or if it initiates a regulatory cascade that leads to tolerance. Future efforts aiming to dissect the scope of the (p)ppGpp regulon will help us identify potential downstream genes and pathways responsible for these effects.

It was interesting to note that there were clear phenotypic differences between strains expressing different levels of (p)ppGpp. While it appears that basal levels of (p)ppGpp produced by RelQ play a fundamental role in vancomycin tolerance, a functional RelA protein seems to be of greater relevance when cells are under environmental stress. However, it is possible that the reduced ability of the Δ*relA* strain to grow under stress was due to toxic accumulation of (p)ppGpp through RelQ. If this is true, the key function of RelA during environmental stress may be to fine-tune basal levels of (p)ppGpp by virtue of its unique capacity to control (p)ppGpp/GTP ratios. This appears to be the case in *S. mutans*, since several experiments have suggested that one of the key roles of RelA is to limit the amount of (p)ppGpp that is allowed to accumulate in the cells from the action of RelP and RelQ (26, 35).

In the nematode *C. elegans* model, adult worms feeding on lawns of *E. faecalis* were killed over the course of several days in an infectious process that reproduces several aspects of human gram-positive pathogenesis (11). Furthermore, known virulence factors required for *E. faecalis* pathogenesis in mammalian model systems were also shown to be important for nematode killing and vice-versa (11, 29, 45). Here we showed that virulence of the Δ*relAQ* double mutant, which is completely unable to produce (p)ppGpp, was highly attenuated in the *C. elegans* model. Interestingly, this was not the case for the Δ*relA* and Δ*relQ* single mutants, which produce basal levels of (p)ppGpp, and in the case of Δ*relQ*, the mutant is capable of synthesizing large amounts of (p)ppGpp as part of a RelA-dependent stringent response. Therefore, it appears that basal levels of (p)ppGpp, rather than the stringent response, constitute the key factor controlling *E. faecalis* virulence in the *C. elegans* model.

In summary, this report reveals that (p)ppGpp plays an important role in stress tolerance and may be a key factor regulating tolerance and growth in the presence of vancomycin, two major factors that contribute to the emergence of *E. faecalis* as a pathogen. In addition, by using the *C. elegans* model of infection, we were able to demonstrate that (p)ppGpp is required for full virulence of *E. faecalis*. The discovery that the (p)ppGpp alarmone is associated with vancomycin tolerance in *E. faecalis* and possibly tolerance to other antibi-

otics is of particular importance. Although tolerant strains are not drug resistant, these strains retain the capacity to grow once the drug is discontinued and are thereby implicated in treatment failure and relapsing disease, especially when grown in biofilms (27). Moreover, bacterial tolerance facilitates the appearance of drug resistance, especially in organisms such as enterococci and *Staphylococcus aureus*, which can rapidly acquire resistance determinants from its surrounding environment. Although the (p)ppGpp-mediated stringent response is well known to protect the cells during starvation by shutting down global gene expression and by selectively activating transcription of genes involved in amino acid biosynthesis and stress survival, this report and others (26, 34) indicate that subtle changes in (p)ppGpp pools can dramatically affect cell homeostasis. Studies are under way to fully understand how and to what extent (p)ppGpp pools control virulence-related events in *E. faecalis*.

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