Entercoccus faecalis is an opportunistic human pathogen commonly associated with nosocomial infections and persisting endodontic infections (8, 11, 13). The ability of E. faecalis to adhere and develop biofilms on host tissues and to survive under harsh conditions is thought to contribute to its pathogenesis during chronic infections (5, 7, 9). Therefore, a better understanding of the mechanisms underlying biofilm formation and survival by E. faecalis may facilitate the identification of pathways that could be targeted to control infection by this organism. One such pathway may involve the stringent response, a global bacterial response to nutritional stress that is mediated by the accumulation of the alarmones guanosine tetraphosphate and guanosine pentaphosphate, collectively known as (p)ppGpp (10). In E. faecalis, (p)ppGpp metabolism is controlled by two enzymes: the bifunctional (p)ppGpp-synthetase/hydrolase RelA (also known as Rsh) and the small alarmone synthetase RelQ. Previous studies revealed that RelA is responsible for the rapid accumulation of (p)ppGpp when cells are under stress, while RelQ appears responsible for expression of low levels of (p)ppGpp when cells are not under stress (1). Physiological characterization of Δrel strains revealed that a high basal level of (p)ppGpp, characteristic of ΔrelA, corresponded with enhanced tolerance toward antibiotics that target the cell wall but a reduced ability to grow under several stress conditions (1). A complete lack of (p)ppGpp, as seen in a ΔrelA ΔrelQ double mutant strain, restored most of the stress-sensitive phenotypes but led to decreased antibiotic tolerance and attenuated virulence in Caenorhabditis elegans (1).

Here, we used an in vitro biofilm model to demonstrate that (p)ppGpp is involved in biofilm development and survival in E. faecalis.

Biofilm formation by E. faecalis strains. E. faecalis OG1RF (4) and mutant strains JAL1 (ΔrelA), JAL2 (ΔrelQ), and JAL3 (ΔrelA ΔrelQ) (1) were used. To prepare cells for the biofilm assays, strains were grown in Todd-Hewitt broth (THB; Becton, Dickinson and Co., Sparks, MD) in a 5% CO2 atmosphere at 37°C to mid-exponential phase (optical density at 600 nm, 0.4 ± 0.01). Cells were harvested by centrifugation (3,000 × g, 5 min at 4°C), washed in 10 mM phosphate-buffered saline (PBS), and resuspended in 10 ml THB.

The biofilm chamber system µ-Slide VI for live cell analysis (Integrated BioDiagnostics) was used to create biofilms in static conditions as previously described (3). Each biofilm chamber was inoculated with 30 μl of washed cell suspension, followed by the addition of 100 μl of fresh THB for a final volume of 130 μl. Biofilm chambers were incubated in 5% CO2 at 37°C. Observations of biofilm development were made at 6, 12, 24, 48, and 72 h by using confocal scanning laser microscopy (CSLM). For long-term starvation assays, the culture fluids from chambers containing 72-h biofilms were replaced with 130 μl PBS.

After removing the culture fluid from the biofilm chamber and washing with PBS to remove nonadherent cells, cell viability of biofilms at 3, 7, 24, and 21 days was assessed by using the Live/Dead BacLight stain (Molecular Probes, Inc., Eugene, OR). In order to determine protease activity, other groups of biofilms were stained using fluorescein isothiocyanate (FITC)-labeled casein (QuantiCleave fluorescent protease assay kit; Pierce) as a substrate (14). Briefly, after washing the biofilms with PBS, 30-μl aliquots of the substrate (10 μg/ml in 25 mM Tris, 150 mM NaCl [pH 7.2]) were added and the biofilms were incubated for 1 h at 37°C. The fluorescence in both techniques, i.e., Live/Dead stain and FITC-labeled casein, was visualized by using an inverted confocal scanning laser microscope (Eclipse TE2000; Nikon, Tokyo, Japan). Cell viability and proteolytic activity were calculated as percentage values of number of cells. Experiments were performed in triplicate.

FIG 1 Plot graph showing biofilm biovolume accumulation over time (6 to 72 h) by E. faecalis OG1RF and mutant strains JAL1 (ΔrelA), JAL2 (ΔrelQ), and JAL3 (ΔrelA ΔrelQ).
cate using three independent biofilms per strain. The overall volume of the biofilm was calculated by the bioImage_L software as previously described (2). Differences between strains in biovolume (μm³), number of viable cells (%), and number of proteolytic active cells (%) were analyzed by one-way analysis of variance and post hoc Tukey-Kramer tests, with an alpha level of \( P < 0.01 \).

**Biofilm growth and detachment are influenced by (p)ppGpp.** In a previous study using a colorimetric assay, \( E. faecalis \) mutant strains lacking relA and/or relQ were shown to accumulate similar biomass compared to the parent strain OG1RF (1). However, samples were harvested only at one time point (24 h) and cell viability was not assessed. In the present study, we used a model to assess biofilm formation by \( E. faecalis \) Δrel strains during a 72-h period and to investigate the role of (p)ppGpp during prolonged starvation. The major advantages of the present system are that biofilm architecture and distribution as well as cell viability can be monitored in situ over time. Biofilm development measured by total biovolume accumulation of each strain in biofilm chambers at different times is presented in Fig. 1. The mean biofilm biovolume of \( E. faecalis \) OG1RF significantly increased with time of incubation over the initial 48-h incubation period \( (P < 0.01) \), with no difference between the 48-h and 72-h incubation periods. All mutant strains showed less biovolume accumulation compared to the wild-type strain OG1RF \( (P < 0.01) \). Mean biovolume of the ΔrelQ mutant was greater than that of the ΔrelA mutant at all incubation periods \( (P < 0.01). \) Biovolume of the ΔrelA ΔrelQ double mutant strain increased with time of incubation during the first 24 h but thereafter decreased at 48 h \( (P < 0.01) \) and 72 h \( (P < 0.01) \).

**FIG 2** Representative 3D reconstructions of biofilm sections stained with Live/Dead BacLight stain after different incubation periods.
Representative three-dimensional (3D) reconstructions of confocal micrographs of *E. faecalis* biofilms are shown in Fig. 2. The data reveal that the ΔrelA ΔrelQ mutant is able to develop biofilm architecture similar to that of the wild-type strain during the first 12 h and that the decrease in biofilm biovolume observed at 48 h and 72 h may be related to detachment of biofilm cells (Fig. 2).

**Increased proteolytic activity in *E. faecalis* ΔrelA ΔrelQ biofilms.** Physiological changes related to loss of the ability to maintain a stable biofilm in (p)ppGpp-deficient strains of *E. faecalis* were investigated by measuring protease activity in 48-h biofilms, the period at which detachment of biofilm cells was first observed in the ΔrelA ΔrelQ strain. There were low levels of proteolytic activity in strain OG1RF (8.2 ± 0.6%) and the ΔrelQ single deletion mutant strain (9.1 ± 0.3%) (Fig. 3). The ΔrelA strain showed significantly less proteolytic activity (<1%) than the other three strains (P < 0.01), which may indicate that high basal (p)ppGpp levels slow down proteolysis. In contrast, 78.3 ± 0.3% of the (p)ppGpp-null ΔrelA ΔrelQ cells were proteolytically active at the 48-h time point (P < 0.01). The increased proteolytic activity of the ΔrelA ΔrelQ strain is in line with the “dispersion phenotype” and correlates well with the inability of the (p)ppGpp-null strain to form stable biofilms. Additional experiments under planktonic conditions did not show an increase in proteolytic activity of the ΔrelA ΔrelQ strain (see Table S1 in the supplemental material), suggesting that these changes were only provoked in biofilm conditions. In stark contrast with the biofilm data, planktonic cultures of the ΔrelA ΔrelQ strain were less proteolytically active than the other strains at both 5-h and 48-h time points.

**Survival of cells during long-term starvation.** Several studies have shown that *E. faecalis* can withstand prolonged periods of starvation and remain viable in root-filled teeth for at least 12 months (6, 12). Because (p)ppGpp is known to play an important role during periods of nutrient starvation (10), we investigated the ability of the biofilms formed by the Δrel strains to survive under oligotrophic conditions. Biofilms grown for 72 h were subjected to a complete depletion of nutrients (by incubation in PBS), and cell viability was monitored over 21 days as described above. At the onset of starvation, all strains showed very high cell viability (>95%) (Fig. 4). While the wild type maintained >95% viability over the duration of the 21-day experiment, both ΔrelA and ΔrelQ strains maintained >95% viability for the first 3 days only, after which there was a progressive and significant (P < 0.01) reduction in viability (53% ± 2% and 44% ± 3%, respectively, by day 21). In agreement with the role of (p)ppGpp in nutrient stress responses, viability of the ΔrelA ΔrelQ biofilm was dramatically reduced compared to the other three strains. In particular, a significant loss of cell viability was much more rapid for the ΔrelA ΔrelQ strain (P < 0.01) (57% survival at 3 days for the ΔrelA ΔrelQ strain versus >95% viability for the other strains), with only 11% viable cells by day 21.

**Concluding remarks.** The data presented in this study reveal that changes in (p)ppGpp pools can have a profound effect on the ability of *E. faecalis* to form, develop, and maintain stable biofilms in vitro. Although the RelA-mediated stringent response is known to play a central role in cell survival by optimizing gene expression for growth and persistence during starvation, our results provide important insights into the relevance of the small (p)ppGpp-synthetase RelQ to biofilm homeostasis. Interestingly, recent microarray analysis of the wild-type and ΔrelA ΔrelQ strains grown under homeostatic conditions reveals striking differences between these two strains, with a large number of genes involved in energy metabolism upregulated in the (p)ppGpp-null mutant (Gaca and Lemos, unpublished data). Although it remains to be investigated, it is feasible that an uncontrolled consumption of energy resources, an
imbalance in NAD/NADH ratios, or even the accumulation of metabolic end products caused by a “relaxed” metabolism may account for the severe defects in biofilm formation, maturation, and viability that were observed in the ΔrelA ΔrelQ strain.

REFERENCES