A Novel Phosphodiesterase of the GdpP Family Modulates Cyclic di-AMP Levels in Response to Cell Membrane Stress in Daptomycin-Resistant Enterococci

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ABSTRACT Substitutions in the LiaFSR membrane stress pathway are frequently associated with the emergence of antimicrobial peptide resistance in both *Enterococcus faecalis* and *Enterococcus faecium*. Cyclic di-AMP (c-di-AMP) is an important signal molecule that affects many aspects of bacterial physiology, including stress responses. We have previously identified a mutation in a gene (designated *yybT*) in *E. faecalis* that was associated with the development of daptomycin resistance, resulting in a change at position 440 (*yybT*^{440S}) in the predicted protein. Here, we show that intracellular c-di-AMP signaling is present in enterococci, and on the basis of *in vitro* physicochemical characterization, we show that *E. faecalis yybT* encodes a cyclic dinucleotide phosphodiesterase of the GdpP family that exhibits specific activity toward c-di-AMP by hydrolyzing it to 5'pApA. The *E. faecalis* GdpP^{440S} substitution reduces c-di-AMP phosphodiesterase activity more than 11-fold, leading to further increases in c-di-AMP levels. Additionally, deletions of *liaR* (encoding the response regulator of the LiaFSR system) that lead to daptomycin hypersusceptibility in both *E. faecalis* and *E. faecium* also resulted in increased c-di-AMP levels, suggesting that changes in the LiaFSR stress response pathway are linked to broader physiological changes. Taken together, our data show that modulation of c-di-AMP pools is strongly associated with antibiotic-induced cell membrane stress responses via changes in GdpP activity or signaling through the LiaFSR system.

KEYWORDS *Enterococcus faecalis*, *Enterococcus faecium*, GdpP, LiaFSR, membrane stress, c-di-AMP, cyclic dinucleotide, daptomycin, enterococci

Cyclic di-AMP (c-di-AMP) is a recently discovered second messenger molecule found in a wide range of bacteria and archaea (1), including *Bacillus subtilis* (2), *Listeria monocytogenes* (3), *Staphylococcus aureus* (4), *Streptococcus pyogenes* (5), and *Streptococcus pneumoniae* (6). *In vivo* levels of c-di-AMP are typically maintained by diadenyl cyclases that synthesize c-di-AMP from two molecules of ATP and by phosphodiesterases that degrade c-di-AMP to 5'pApA (7, 8). While c-di-AMP appears to be essential for bacteria that produce it, the accumulation of c-di-AMP can also be toxic or inhibit growth (9).

c-di-AMP plays important roles in the regulation of diverse cellular pathways, including potassium homeostasis, biofilm formation, and cell wall biosynthesis (4, 10, 11). Increased intracellular c-di-AMP levels in bacteria have been associated with increased tolerance to a wide range of environmental challenges, including cell wall damage, high temperatures, starvation, and acid stress, as well as some antibiotics,

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including lysostaphin, oxacillin, and penicillin G (4, 11, 12). While many genes regulated by c-di-AMP have been reported (10), the signal transduction pathways that convert environment stresses to c-di-AMP signals have not been identified, though it has been suggested that membrane-associated enzymes such as c-di-AMP synthase A (CdaA, also referred as YbbP) and its regulator CdaR (c-di-AMP synthase A regulator, also referred to as YybR) may sense damage directly (2, 7, 13). The cyclic dinucleotide signaling pathway and its potential role in stress response have not been characterized in enterococci.

Enterococci are facultative anaerobic Gram-positive bacteria and are the cause of multiple human infections, including endocarditis, bacteremia, and urinary tract infections (14). The acquisition of multiple resistance determinants has made vancomycin-resistant enterococci (VRE) a serious public health threat with strains that are resistant to almost all antibiotics clinically available (15). VRE infections usually affect critically ill and immunocompromised patients with increased prevalence in health care-associated infections (9,820 in 2000 to 20,000 in 2013 in the United States) (16). The cyclic lipopeptide daptomycin is frequently used as a drug of last resort against recalcitrant VRE infections (15, 17). Daptomycin is a bactericidal antibiotic that targets the cell membrane in a calcium- and phosphatidylglycerol-dependent manner leading to cell death after disrupting the cell membrane (18).

In a previous study, we used quantitative experimental evolution of a polymorphic population of *Enterococcus faecalis* S613 to identify common evolutionary trajectories leading to daptomycin resistance (19). All of the initial mutations associated with resistance appeared directly within the *liaFSR* pathway, including *liaF* and *liaR* (19, 20). LiaFSR is a three-component membrane stress response regulon (21) that includes a transmembrane histidine kinase (LiaS) that is predicted to activate its cognate response regulator (LiaR) by phosphorylation in response to cell envelope stress (22). Upon activation, LiaR regulates downstream genes by binding DNA in a sequence-specific manner, including the *liaFSR* and *liaXYZ* operons (19, 22, 23). Studies with *B. subtilis* have suggested that LiaF is an inhibitor of LiaS that attenuates the signal response (21, 22).

After an initial set of mutations within the LiaFSR pathway, a second group of genes showed clear indications of being important to adaptation to daptomycin and included *cls* (encoding a cardiolipin synthase) and a previously unidentified gene designated *yybT* (19). On the basis of primary sequence alignment, we reasoned that YybT might be a member of the GGDEF domain protein-containing phosphodiesterase family (*E. faecalis* *yybT* is referred to as *gdpP* here) (4). GdpP has been identified in several Gram-positive bacteria, including *B. subtilis* (24), *Lactococcus lactis* (12), *S. pyogenes* (5), and *S. aureus* (4) with roles in bacterial growth and virulence. In addition to *gdpP*, we were able to identify potential homologs of a c-di-AMP synthase A (CdaA) and another cyclic-dinucleotide phosphodiesterase, PgpH, in the *E. faecalis* genome ([ADDP01000030.1](#)) (2, 8). Thus, we postulated that if *yybT* is indeed a *gdpP* homolog, the c-di-AMP signaling pathway may be important in enterococci and may influence different processes, including the adaptive response to antimicrobial peptides like daptomycin. Interestingly, daptomycin-resistant strains containing *gdpP*^{I440S} were found in association with mutations in the LiaFSR system such as *liaR*^{D191N}, suggesting a potential epistatic link to the LiaFSR regulon (19). Although *gdpP*^{I440S} has not been observed in clinical isolates, the possibility that it is a phosphodiesterase provided the first indication that the LiaFSR pathway, cyclic dinucleotide signaling, and daptomycin resistance might be related.

In this report, we show that c-di-AMP is a second messenger in enterococci. Using *in vitro* enzymatic activity assays, we show that *E. faecalis* GdpP is a *bone fide* c-di-AMP-specific phosphodiesterase. The GdpP^{I440S} substitution identified during selection with daptomycin has reduced activity both *in vitro* and *in vivo* and leads to accumulation of c-di-AMP. Additionally, we provide data that link the LiaFSR system and changes in c-di-AMP levels. Taken together, our data suggest that the membrane stress response of enterococci via GdpP or LiaFSR signaling affects c-di-AMP levels. The changes in

TABLE 1 Enterococcal strains used in this study and measured c-di-AMP concentrations and daptomycin MICs

Strain	Genotype or description	Avg intracellular [c-di-AMP] (pM/OD ₆₀₀) ± SD ^a	Daptomycin MIC ^b (μg/ml)	Reference or source
<i>E. faecalis</i> strains				
S613	Wild-type clinical VRE ^c strain	113 ± 81	0.5	14, 41
OG1RF	Wild-type laboratory vancomycin-susceptible strain	112 ± 47	2	14, 41
R712	Clinical daptomycin-resistant strain derived from S613; <i>liaF</i> ^{Δ1177} <i>cls</i> ^{ΔK61} <i>gdpD</i> ^{Δ1170}	349 ± 104	12	14, 41
OG1RF_Δ <i>liaR</i>	Δ <i>liaR</i>	351 ± 94	0.094	41
OG1RF_Δ <i>liaR</i> :: <i>liaR</i>	OG1RF_Δ <i>liaR</i> with <i>liaR</i> complementation in <i>cis</i>	140 ± 29	2	41
OG1RF::pMSP3535::gdpP	pMSP3535::gdpP- <i>Efc</i>	115 ± 9	0.5	This study
OG1RF::pMSP3535::gdpP ^{Δ4405}	pMSP3535::gdpP ^{Δ4405} - <i>Efc</i>	152 ± 3	0.5	This study
OG1RF::pMSP3535	pMSP3535	158 ± 3	0.5	This study
<i>E. faecium</i> strains				
HOU503	Clinical daptomycin-tolerant VRE strain with <i>liaR</i> ^{W37C} and <i>liaS</i> ^{T120A}	766 ± 318	3	27
HOU503F	HOU503 fusidic acid-resistant derivative	542 ± 133	3	27
HOU503F_Δ <i>liaR</i>	Δ <i>liaR</i>	622 ± 104	0.064	27
HOU503F_Δ <i>liaR</i> :: <i>liaR</i>	HOU503F_Δ <i>liaR</i> with <i>liaR</i> complementation in <i>cis</i>	505 ± 54	3	27
R497F	Fusidic acid-resistant derivative of clinical daptomycin-resistant vancomycin-susceptible <i>E. faecium</i> strain R497 with <i>liaR</i> ^{W37C} , <i>liaS</i> ^{T120A} , and <i>cls</i> ^{ins110}	549 ± 150	24	27
R497F_Δ <i>liaR</i>	Δ <i>liaR</i>	736 ± 186	0.094	27
R497F_Δ <i>liaR</i> :: <i>liaR</i>	R497F_Δ <i>liaR</i> with <i>liaR</i> complementation in <i>cis</i>	525 ± 134	24	27

^aErrors correspond to standard deviations of three individual measurements.^bMICs were measured by Etest (bioMérieux) on Mueller-Hinton agar.^cVRE, vancomycin-resistant *Enterococcus*.

c-di-AMP may have important physiological implications for the adaptive response to antibiotics in enterococci.

RESULTS

The nucleotide signaling molecule c-di-AMP is present at physiologically relevant levels in enterococci. As c-di-AMP signaling has not been previously identified in enterococci, it was essential to test whether c-di-AMP was present at levels consistent with its being a second messenger *in vivo*. Thus, we measured c-di-AMP in *E. faecalis* S613 and its daptomycin-resistant derivative *E. faecalis* R712, a vancomycin-resistant strain pair isolated from the bloodstream of a patient before and after daptomycin treatment, respectively (14, 41). Additionally, we included a laboratory strain of *E. faecalis*, OG1RF (Table 1) (14, 25). Cells were collected at late exponential phase (optical density at 600 nm [OD₆₀₀], ~1.0), and the concentration of c-di-AMP was measured with a competitive enzyme-linked immunosorbent assay (ELISA) specific for c-di-AMP (26). As shown in Fig. 1A, *E. faecalis* strains S613 and OG1RF produced c-di-AMP. The c-di-AMP levels of *E. faecalis* S613 and OG1RF at late exponential phase were 113 ± 81 and 112 ± 47 pM/OD₆₀₀, respectively. In *E. faecalis* R712 (daptomycin-resistant derivative of *E. faecalis* S613), the c-di-AMP level was 349 ± 104 pM/OD₆₀₀, more than 3-fold higher than that of *E. faecalis* S613 (Table 1 and Fig. 1A).

To determine whether c-di-AMP signaling is present in other enterococcal strains, daptomycin-tolerant and vancomycin-resistant *Enterococcus faecium* clinical isolate HOU503 and its fusidic acid-resistant derivative *E. faecium* HOU503F were tested (Table 1) (27). The c-di-AMP levels of *E. faecium* HOU503 and *E. faecium* HOU503F were 766 ± 318 and 542 ± 133 pM/OD₆₀₀ at late exponential phase (OD₆₀₀, ~1.2) (Table 1 and Fig. 1B), both significantly higher than those of *E. faecalis*. Interestingly, it has been reported that the c-di-AMP level in *S. pneumoniae* is ~25 pM/OD₆₂₀ (6) and that c-di-AMP is present in cytoplasmic extracts from other Gram-positive bacteria. The use of alternative methods to measure c-di-AMP shows that *B. subtilis* has ~1.7 μM/1-liter culture (28) and 3.33 ± 0.44 ng/mg of bacterial dry weight in *S. aureus* (4). While c-di-AMP signaling

FIG 1 Enterococcal strains with mutations in the LiaFSR signaling pathway have increased c-di-AMP levels. Intracellular c-di-AMP concentrations were measured by an ELISA (26). c-di-AMP was detected in both *E. faecalis* (A) and *E. faecium* (B). (A) *E. faecalis* strains. The intracellular c-di-AMP level in *E. faecalis* OG1RF_Δ*liaR* that knocks out LiaFSR signaling increases to 351 ± 94 pM/OD₆₀₀ (26), and complementation of *liaR* in *cis* restores the c-di-AMP level. *, $P < 0.05$. (B) *E. faecium* strains. The nonpolar *liaR* deletion mutation causes a small but not statistically significant increase in *E. faecium* HOU503F_Δ*liaR* and R497F_Δ*liaR*. Each c-di-AMP value is from at least three independent biological measurements. See Table 1 for strain descriptions. WT, wild type.

molecules have been identified in a number of bacteria, the presence of c-di-AMP in *E. faecalis* and *E. faecium* had not been reported before, and our results demonstrate that c-di-AMP is present in enterococci at levels sufficient to be a *bone fide in vivo* signal.

***E. faecalis* GdpP has phosphodiesterase activity.** On the basis of primary sequence homology, we hypothesized that the gene product of unknown function previously annotated as *E. faecalis* YybT was a potential cyclic dinucleotide phosphodiesterase of the GdpP family (*E. faecalis* GdpP) (3, 4, 24). *E. faecalis* GdpP is a multidomain protein containing two putative N-terminal transmembrane helices, a Per-Arnt-Sim (PAS) domain, a GGDEF domain, and a DHH domain with a DHHA1 subdomain (Fig. 2A) (24). The PAS domain is a putative signal input module and is frequently involved in signal transduction in orthologous systems (see Discussion; also see Fig. S1 and Text S2 in the supplemental material) (29). The GGDEF domain often has diguanylate cyclase activity (discussed below) (30). Thus, on the basis of homology (see Fig. S2), we postulated that the putative DHH/DHHA1 domain of *E. faecalis* GdpP would have cyclic dinucleotide phosphodiesterase activity (3, 4, 24). To test this hypothesis, *E. faecalis* GdpP and the DHH/DHHA1 domain (residues 309 to 658, referred to as GdpP³⁰⁹⁻⁶⁵⁸) were characterized *in vitro*. Purified wild-type protein was incubated with c-di-AMP or c-di-GMP, and the products were analyzed by reverse-phase high-performance liquid chromatography (HPLC). As shown in Fig. 3A and B, incubation of GdpP with c-di-AMP resulted in a decrease of the cyclic dinucleotide peak and the formation of a new

FIG 2 Domain organization and predicted structure of *E. faecalis* GdpP. *E. faecalis* GdpP is a multidomain protein containing two putative N-terminal transmembrane helices (TM1 and TM2), a PAS domain, a GGDEF domain, and a DHH domain with a DHHA1 subdomain. (A) The DHH/DHHA1 domain contains the c-di-AMP phosphodiesterase active site, while the GGDEF domain has a low level of ATPase activity. The daptomycin adaptive mutation I440S is in the DHH domain. (B) Predicted structure model of the *E. faecalis* GdpP DHH/DHHA1 domain indicating the presumptive phosphodiesterase active site and proximal position of I440S. Two Mn^{2+} ions are shown as magenta spheres. Asp residues 419 and 499 are blue, and Ile 440 is orange. The structural model was built with the Phyre2 server (40). The Mn^{2+} positions were predicted by alignment with *M. tuberculosis* phosphodiesterase Rv2837c (PDB accession no. 5CET) (32).

product corresponding to linear dinucleotide 5'pApA without generating AMP or 3'pApA. This result indicates that *E. faecalis* GdpP hydrolyzes c-di-AMP solely into 5'pApA and is consistent with the activity of *B. subtilis* GdpP measured previously (24). We also determined the phosphodiesterase activity of GdpP toward the cleavage of c-di-GMP. On the basis of HPLC analysis, c-di-GMP is not the physiological substrate of *E. faecalis* GdpP, although it has a weak ability to hydrolyze c-di-GMP to 5'pGpG (Fig. 3C and D).

Once the specificity for c-di-AMP was established, we quantitated the phosphodiesterase activity of the DHH/DHHA1 domain alone (*E. faecalis* GdpP³⁰⁹⁻⁶⁵⁸) and the full-length protein with the coralyne activity assay fluorescence turn-on assay (31). The activity of the full-length protein was weak (see Fig. S3D), and saturating conditions of c-di-AMP were not practical to establish, while activity by *E. faecalis* GdpP³⁰⁹⁻⁶⁵⁸ was ~13 times as high, allowing us to reach saturating conditions and establish kinetic parameters. The kinetic data for GdpP³⁰⁹⁻⁶⁵⁸ were clearly multiphasic and could be fitted to two phases to produce two K_m s extending over the conditions ranging from 0 to 300 and from 300 to 1,750 μ M c-di-AMP. The Michaelis-Menten reaction rate constants calculated separately for the two phases produce very similar k_{cat} values but a 3.3-fold change in the K_m ($K_{m1} = 443.6 \pm 97.53 \mu$ M, $k_{cat1} = 0.42 \pm 0.16 s^{-1}$, $K_{m2} = 132.6 \pm 20.05 \mu$ M, $k_{cat2} = 0.49 \pm 0.01 s^{-1}$) (Fig. 3G; see Fig. S3). GdpP³⁰⁹⁻⁶⁵⁸ was difficult to purify, with a strong tendency to aggregate and adsorb nonspecifically. Upon purification, we found that the detergent fluorinated octyl maltoside at 1 mM could reduce aggregation and produced protein that was twice as active (see Fig. S3). Taken together, the poor behavior of the protein during purification and the impor-

FIG 3 *E. faecalis* GdpP has phosphodiesterase activity with specificity for c-di-AMP. (A to F) Reverse-phase HPLC analysis of products from incubation of wild-type *E. faecalis* (*Efc*) GdpP and variants with cyclic dinucleotides at 28°C for 2 h with 100 mM Tris (pH 8.3), 20 mM KCl, and 0.5 mM MnCl₂. Shown are wild-type *E. faecalis* GdpP and c-di-AMP before (A) and after (B) incubation, wild-type *E. faecalis* GdpP with c-di-GMP before (C) and after (D) incubation, *E. faecalis* GdpP^{D419A/D499A} after incubation with c-di-AMP (E), and *E. faecalis* GdpP^{I440S} after incubation with c-di-AMP (F). (G) Steady-state kinetic analysis of *E. faecalis* GdpP DHH/DHHA1 domain (*E. faecalis* GdpP³⁰⁹⁻⁶⁵⁸) phosphodiesterase activity at 37°C. The reaction mixture contained 2.5 μM *E. faecalis* GdpP³⁰⁹⁻⁶⁵⁸, 50 mM CHES (pH 9.2), 20 mM KCl, 0.5 mM MnCl₂, and 1 mM fluorinated octyl maltoside. mAU, milliabsorbance units; V_0 , reaction velocity.

tance of fluorinated octyl maltoside to increase activity suggest that the multiphasic kinetics we observed may reflect the tendency of the protein to adsorb/aggregate as a less active population and that increasing the substrate concentration to $>300 \mu\text{M}$ mitigated that effect to increase activity. Alternatively, a conformational change within the DHH/DHHA1 domain could occur at higher c-di-AMP concentrations and be a physiological feature of GdpP. Thus, when toxic levels of c-di-AMP are reached, GdpP phosphodiesterase activity may increase.

Aspartates 419 and 499 are essential to *E. faecalis* GdpP phosphodiesterase activity. On the basis of a comparison of the *E. faecalis* GdpP sequence with those of others phosphodiesterases, Asp residues 419 and 499 are predicted to be ligands for

FIG 4 Expression of the c-di-AMP phosphodiesterase *E. faecalis* GdpP in *trans* leads to reduced c-di-AMP levels *in vivo*. Intracellular concentrations of c-di-AMP in *E. faecalis* OG1RF during the expression of *E. faecalis* GdpP or *E. faecalis* GdpP^{I440S}. Expression of *E. faecalis* GdpP leads to a statistically significant decrease in the c-di-AMP level (~27%). Expression of *E. faecalis* GdpP^{I440S} does not lead to a significant change in c-di-AMP. Three independent biological measurements of each strain were made. *, $P < 0.05$; **, $P < 0.01$.

the metal ion binding site required for phosphodiesterase activity (Fig. 2B; see Fig. S2) (24). To test whether Asp 419 and Asp 499 are required for phosphodiesterase activity, we generated the double mutant *E. faecalis* GdpP^{D419A/D499A}. As expected, no activity was detected after the incubation of *E. faecalis* GdpP^{D419A/D499A} with c-di-AMP (Fig. 3E). These results indicate that alignment of the primary sequences of *E. faecalis* GdpP with those of other members of the DHH protein family of phosphodiesterases has correctly identified residues essential to activity and support our conclusion that *E. faecalis* GdpP is a *bona fide* cyclic dinucleotide phosphodiesterase.

Adaptive mutant protein GdpP^{I440S}, which correlates with daptomycin resistance in *E. faecalis* S613, strongly decreases phosphodiesterase activity. In *E. faecalis* GdpP, the adaptive mutation I440S is located in the putative DHH domain (Fig. 2A) and is proximal to the predicted substrate binding site (Fig. 2B) (32). To elucidate how the adaptive mutation may facilitate daptomycin resistance, we determined the phosphodiesterase activity of *E. faecalis* GdpP^{I440S} toward its potential physiological substrate c-di-AMP. As shown in Fig. 3F, incubation of c-di-AMP with GdpP^{I440S} showed an 11-fold reduction in the ability to hydrolyze c-di-AMP to 5'pApA. Thus, gdpP^{I440S} was identified in the daptomycin adaptive isolates (19) and may lead to c-di-AMP accumulation in these strains, suggesting that high intracellular c-di-AMP levels are important for *E. faecalis* physiology during daptomycin-induced membrane stress.

***E. faecalis* GdpP has phosphodiesterase activity *in vivo*.** To further test whether *E. faecalis* GdpP has phosphodiesterase activity *in vivo*, the gene coding wild-type *E. faecalis* GdpP was subcloned into nisin-inducible vector pMSP3535 (33) and transformed into *E. faecalis* OG1RF. As shown in Fig. 4 and Table 1, overexpression of *E. faecalis* GdpP decreased cellular c-di-AMP levels by about 27% compared with the empty plasmid. The decreased c-di-AMP levels in cells expressing *E. faecalis* GdpP from plasmid pMSP3535 were statistically significantly different from those of the control strain ($P = 0.008$). We expected that expression of the *E. faecalis* GdpP^{I440S} mutant protein from plasmid pMSP3535 would lead to restored levels of c-di-AMP. While there was a very modest decrease in c-di-AMP levels, it was not statistically significant ($P = 0.078$). There could be a number of reasons for the inability of *E. faecalis* GdpP^{I440S} to fully restore c-di-AMP levels. First, the mutation I440S decreases the phosphodiesterase activity by around 11-fold but does not completely abolish it. Second, since *E. faecalis* GdpP^{I440S} is being expressed along with the genomic copies of wild-type GdpP in addition to the c-di-AMP synthesis and regulatory genes, the net effect of *E. faecalis*

FIG 5 ppGpp is able to modestly inhibit *E. faecalis* GdpP phosphodiesterase activity. (A) Reverse-phase HPLC analysis of products from incubation of wild-type *E. faecalis* (*Efc*) GdpP and c-di-AMP with (red) and without (black) 1 mM ppGpp after incubation for 2 h at 28°C. (B) *E. faecalis* GdpP^{I440S} with c-di-AMP and ppGpp after 2 h of incubation at 28°C showing no significant 5'pApA production.

GdpP^{I440S} might be compensated for by the homeostatic machinery of the cell. We observed a slight c-di-AMP level increase in *E. faecalis* OG1RF::pMSP3535 compared with that of *E. faecalis* OG1RF (158 ± 3 versus 112 ± 47 pM/OD₆₀₀ [$P = 0.225$]; see Table 1 and Fig. 1A and 4 for details), which may be caused by the addition of nisin, since higher c-di-AMP levels were also detected when *E. faecalis* OG1RF::pMSP3535 was cultured with nisin versus in its absence (data not shown). Together, our data strongly suggest that *E. faecalis* GdpP has phosphodiesterase activity *in vivo* and can regulate intracellular c-di-AMP levels.

***E. faecalis* GdpP^{I440S} is more sensitive to inhibition by ppGpp *in vitro*.** Several studies on homologs to *E. faecalis* GdpP have reported that ppGpp can inhibit GdpP phosphodiesterase activity (24, 34). ppGpp is a stringent response signaling molecule and accumulates in some bacteria during nutrient starvation (35). In *B. subtilis*, ppGpp binds to *B. subtilis* GdpP and inhibits phosphodiesterase activity (referred to as YybT in reference 24). To determine whether ppGpp is a direct inhibitor of *E. faecalis* GdpP, 1 mM ppGpp was incubated with 10 μ M *E. faecalis* GdpP and 100 μ M c-di-AMP. Figure 5A shows that ppGpp inhibited *E. faecalis* GdpP phosphodiesterase activity very modestly and did not completely abolish it, even though the molar ratio of c-di-AMP to ppGpp was 1:10. Our data suggest that ppGpp is a very weak inhibitor of *E. faecalis* GdpP phosphodiesterase activity *in vitro*. When 1 mM ppGpp was added to the reaction system of 10 μ M *E. faecalis* GdpP^{I440S} and 100 μ M c-di-AMP, no c-di-AMP was hydrolyzed to 5'pApA after incubation for 2 h (Fig. 5B). Our data indicate that the residual phosphodiesterase activity of *E. faecalis* GdpP^{I440S} can be inhibited by ppGpp *in vitro*, which suggests that *E. faecalis* GdpP^{I440S} could be more sensitive to ppGpp than wild-type *E. faecalis* GdpP is *in vivo*.

***E. faecalis* GdpP has ATPase activity *in vitro*.** *E. faecalis* GdpP has a putative GGDEF domain, which is unusual, as the previously described GdpP family proteins typically lack the conserved GGD(E)EF motif (4, 5, 24, 29). The GGDEF domain in *B. subtilis* GdpP has only very weak ATPase activity (referred to as YybT in reference 24). Proteins containing GGDEF domains in Gram-negative bacteria often have diguanylate

FIG 6 *E. faecalis* GdpP and GdpP^{I440S} have comparable ATPase activities. Shown are results of reverse-phase HPLC analysis of ATP and wild-type *E. faecalis* (Efc) GdpP before incubation (A), ATP and wild-type *E. faecalis* GdpP after 2 h of incubation at 25°C (B), and ATP and *E. faecalis* GdpP^{I440S} after 2 h of incubation at 25°C (C). (D) ATPase activity measurement of 5 μ M *E. faecalis* GdpP at 28°C. (E) ATPase activity measurement of 5 μ M *E. faecalis* GdpP^{I440S} at 28°C. The reaction buffer contained 50 mM Tris (pH 7.5) and 1 mM MgCl₂. mAU, milliabsorbance units; V_0 , reaction velocity.

cyclase activity (30). Primary sequence alignment indicated that *E. faecalis* GdpP (similar to *B. subtilis* GdpP) contains a modified GGDEF domain with a highly divergent amino acid sequence (see Fig. S4). The GGDEF domain in *B. subtilis* GdpP can bind ATP and slowly convert it to ADP (24). To test whether *E. faecalis* GdpP exhibits ATPase activity, we incubated 100 μ M ATP with 10 μ M *E. faecalis* GdpP and analyzed the products by reverse-phase HPLC. The chromatographic analysis revealed that *E. faecalis* GdpP could hydrolyze ATP, leading to the formation of ADP after incubation (Fig. 6A and B). The ATPase activity of *E. faecalis* GdpP had a k_{cat} of $0.80 \pm 0.03 \text{ min}^{-1}$ and a K_m of $239.2 \pm 41.8 \mu\text{M}$ (Fig. 6D), while the *B. subtilis* GdpP homolog had a similar k_{cat} value but a markedly lower K_m ($k_{\text{cat}} = 0.59 \pm 0.03 \text{ min}^{-1}$, $K_m = 0.90 \pm 0.12 \text{ mM}$) (24). To assess whether the mutation of Ile 440 to Ser in the DHH domain could affect ATPase activity in the GGDEF domain, we measured the ATPase activity of *E. faecalis* GdpP^{I440S}. The ATPase activity of *E. faecalis* GdpP^{I440S} was comparable to that of wild-type GdpP (Fig. 6C), with a k_{cat} of $1.12 \pm 0.02 \text{ min}^{-1}$ and a K_m of $287.5 \pm 20.2 \mu\text{M}$ (Fig. 6E). Thus, it appears that the I440S mutation in the DHH domain does not affect the ATPase activity in the GGDEF domain ($P = 0.880$).

The weak ATPase activity of GdpP required us to use up to 2 mM ATP to provide sufficient data for our analysis. When performing the ATPase studies, we observed unexpected substrate inhibition of the ATPase activity. Interestingly, inhibition starts at about 2.5 mM ATP, which is in the range of its intracellular concentration (about 1 to 10 mM) (36). To confirm that the inhibition was not caused by the reagents used in the colorimetric ATPase assay, we incubated 10 μ M *E. faecalis* GdpP with 2 and 6 mM ATP at 28°C, respectively, and analyzed the products directly by reverse-phase HPLC (see Fig. S5). Our data suggest that *in vitro* substrate inhibition could be biologically relevant and led us to suspect that the local cellular ATP concentration could alter *E. faecalis* GdpP ATPase activity. Incubation of GdpP with ATP or GTP did not produce either c-di-AMP or c-di-GMP, and thus it appears that the GGDEF domain is not a c-di-AMP or c-di-GMP synthase *in vitro*.

Mutations in the LiaFSR signaling pathway can affect intracellular c-di-AMP levels. During adaptation to daptomycin, the first step toward resistance was a family of mutations that, while in different genes, upregulated the LiaFSR membrane stress response pathway (19, 37). In two of the evolutionary trajectories leading to daptomycin resistance, an Ile-to-Ser substitution at position 440, was observed in GdpP (19). Interestingly, GdpP^{I440S} was always found in association with a LiaR substitution (LiaR^{D191N}), suggesting an epistatic link between changes in LiaFSR signaling and GdpP (19). Since LiaR is the response regulator of the LiaFSR membrane stress response system (21), we investigated the possibility that the LiaFSR pathway regulates the levels of c-di-AMP.

To test whether LiaFSR leads to changes in intracellular c-di-AMP regulation, we initially used a strain in which we had generated a nonpolar deletion of *liaR* in *E. faecalis*. Cell extracts from such a mutant (*E. faecalis* OG1RF_Δ*liaR*, Table 1) were tested for c-di-AMP levels. Of note, the *liaR* deletion removes the response regulator of the LiaFSR pathway, effectively “shutting off” the LiaFSR system. Deletion of *liaR* (*E. faecalis* OG1RF_Δ*liaR*) upregulated the levels of c-di-AMP to 351 ± 94 pM/OD₆₀₀ (Fig. 1A and Table 1), a >3-fold increase over wild-type *E. faecalis* OG1RF ($P = 0.030$). When *liaR* expression was restored in *cis* in *E. faecalis* OG1RF_Δ*liaR* (*E. faecalis* OG1RF_Δ*liaR*::*liaR*, Table 1), cellular c-di-AMP levels returned to the levels observed in wild-type *E. faecalis* OG1RF (140 ± 29 pM/OD₆₀₀, $P = 0.436$, Fig. 1A and Table 1). These results suggest that decreases in LiaFSR signaling lead to increases in intracellular c-di-AMP levels.

To demonstrate that changes in LiaFSR signaling lead to changes in c-di-AMP more broadly, we measured the c-di-AMP levels in nonpolar *liaR* deletion mutants of *E. faecium* strains HOU503F and R497F (*E. faecium* HOU503F_Δ*liaR* and R497F_Δ*liaR*, Table 1) which are tolerant of and resistant to daptomycin (27). As observed with *E. faecalis*, deletion of *liaR* in *E. faecium* HOU503F_Δ*liaR* and R497F_Δ*liaR* showed modest increases in c-di-AMP levels compared to *E. faecium* HOU503F and R497F (622 ± 104 pM/OD₆₀₀ versus 542 ± 133 pM/OD₆₀₀ and 736 ± 186 pM/OD₆₀₀ versus 549 ± 150 pM/OD₆₀₀, Fig. 1B and Table 1) but the increases were not statistically significant ($P = 0.250$ and 0.461). It is interesting that the inactivation of the LiaFSR pathway in *E. faecium* does not increase c-di-AMP concentrations to the same extent as observed in *E. faecalis*. The basal level of c-di-AMP in *E. faecium* HOU503F and R497F was much greater than that in *E. faecalis* OG1RF or S613, and thus there are substantial differences in the basal c-di-AMP pools of these related organisms. *cis* complementation with *liaR* decreased the intracellular c-di-AMP levels of *E. faecium* HOU503F_Δ*liaR*::*liaR* and R497F_Δ*liaR*::*liaR* to 505 ± 54 and 525 ± 134 pM/OD₆₀₀, respectively, similar to the c-di-AMP levels in wild-type strains (Fig. 1B and Table 1).

DISCUSSION

Cyclic dinucleotide signaling in bacteria is widespread and is linked to a variety of regulatory responses, including DNA integrity reporting (28), cell wall biosynthesis (4, 9), potassium homeostasis (10), and biofilm formation (4). Experimental evolution of a polymorphic population of *E. faecalis* S613 to daptomycin resistance with a continuous-evolution bioreactor identified a point mutation (*yybT*^{I440S}) in a gene of unknown function initially named *yybT* (19). On the basis of primary sequence identity, we postulated that YybT might, in fact, be a cyclic dinucleotide phosphodiesterase, and on the basis of our studies, we have established phosphodiesterase activity consistent with its classification as a member of the GdpP family. Therefore, we have changed the name to GdpP. As c-di-AMP signaling in enterococci had not been established, we first tested if c-di-AMP was present at levels consistent with a role in *in vivo* signaling (from 44 to 1,133 pM/OD₆₀₀, Fig. 1). Having established that c-di-AMP was present *in vivo*, we went on to confirm the role of the novel GdpP in c-di-AMP homeostasis.

As shown in Fig. 2, GdpP is composed of three distinct domains. We purified and characterized full-length *E. faecalis* GdpP, as well as the DHH/DHHA1 domain (*E. faecalis* GdpP^{P309-658}). Our results showed that *E. faecalis* GdpP is indeed a c-di-AMP phosphodiesterase with a strong preference for c-di-AMP over c-di-GMP. *E. faecalis* GdpP^{I440S}

shows little phosphodiesterase activity, and as expected, strains encoding *E. faecalis* GdpP^{P1440S} have increased c-di-AMP levels *in vivo*. Our results showed that expression of *E. faecalis* GdpP from the inducible vector pMSP3535 in wild-type *E. faecalis* OG1RF decreased the cellular c-di-AMP level by 27% (Fig. 4 and Table 1). Although we could demonstrate weak ATPase activity in the GGDEF domain and c-di-AMP phosphodiesterase activity in the DHH/DHHA1 domain, we were unable to identify the *in vivo* function of either domain. The PAS domain could be a critical regulator of the activities of the GGDEF and DHH/DHHA1 domains *in vivo*. For example, the c-di-AMP phosphodiesterase activity of the DHH/DHHA1 domain alone (GdpP³⁰⁹⁻⁶⁵⁸) is ~13 times as high as that of the full-length protein, suggesting that under specific conditions or stimuli *in vivo*, the kinetic rates of the activities within the GdpP domains may be quite variable and well regulated. Undoubtedly, each of the GdpP domains has a relevant activity or role *in vivo*. A study of *S. aureus* GdpP showed that two mutations in its GGDEF domain could increase resistance to the antibiotics vancomycin and oxacillin and that complementation with an *S. aureus* GdpP N-terminal truncation with an inactive DHH domain could restore antibiotic susceptibility (38). Together, these observations suggest that the *E. faecalis* GdpP GGDEF domain has additional activities *in vivo* beyond weak ATP hydrolysis. Recently, the GGDEF domains from *Deltaproteobacteria* were also reported to have a hybrid dinucleotide cyclase activity and can synthesize c-di-AMP, c-di-GMP, and cyclic AMP-GMP (3',3'-cGAMP) (39). The functional role of the PAS and GGDEF domains is the subject of ongoing investigation.

On the basis of sequence homology, *E. faecalis* has only one gene encoding a potential c-di-AMP synthase A (CdaA, also referred as YbbP) (2). Our finding of a robust c-di-AMP pool in *E. faecalis* suggests that *E. faecalis* CdaA is active *in vivo*. *E. faecalis* has two potential genes encoding c-di-AMP phosphodiesterases, *gdpP* and *pgpH* (8). *L. monocytogenes* PgpH is a membrane protein that can hydrolyze c-di-AMP to 5'pApA via a His-Asp (HD) domain (8). A study of *L. monocytogenes* c-di-AMP phosphodiesterases showed that DHH/DHHA1 family phosphodiesterases are more important than HD family phosphodiesterases for bacterial virulence and intracellular growth (8), which may be the reason that we did not observe mutations in PgpH in the clinical or experimental evolution data for enterococci.

Since GdpP^{P1440S} was found to be associated with changes within the LiaFSR pathway, we tested the effect of the mutations in the LiaFSR pathway upon *in vivo* c-di-AMP levels. We found that the deletion of *liaR* (*E. faecalis* OG1RF_Δ*liaR*) led to increased c-di-AMP levels (Table 1 and Fig. 1). While it is tempting to link c-di-AMP levels to daptomycin resistance, the nature of this association is unclear. Nonetheless, we observed a significant increase in c-di-AMP levels in a nonpolar *liaR* deletion of *E. faecalis* OG1RF (Fig. 1A and Table 1). However, quantitative PCR of the potential target genes *cdaA*, *gdpP*, and *pgpH* did not show significant changes in transcription (see Fig. S6), suggesting that the effects of mutations in LiaFSR signaling on cellular c-di-AMP levels are indirect. It is possible that cellular c-di-AMP pools will also respond dynamically through several stress regulons, including LiaFSR. In addition, other as-yet-unidentified regulatory mechanisms may also be involved in the system. For example, there may be another unknown c-di-AMP synthase in enterococci that responds to LiaFSR activation. Additionally, an unknown cofactor or stress may switch the function of GdpP from c-di-AMP phosphodiesterase (DHH/DHHA1 domain activity) to c-di-AMP synthase (putative GGDEF activity) (39). While we were unable to demonstrate that the *E. faecalis* GdpP GGDEF domain has c-di-AMP synthase activity, GGDEF domains of other proteins do have demonstrated synthase activity (39). How the PAS domain of GdpP might regulate the known DHH/DHHA1 domain phosphodiesterase activity, as well as the potential cryptic activities of the GGDEF domain, remains unknown. Although the complete signaling mechanism by which LiaFSR regulates c-di-AMP level remains to be fully elucidated, it is evident that membrane damage is faithfully transduced and converted into altered levels of c-di-AMP.

In summary, we show that c-di-AMP is an important second messenger in enterococci and that a novel GdpP phosphodiesterase previously associated with daptomycin resistance is important for the modulation of c-di-AMP in response to cell membrane

damage caused by the antibiotic. Moreover, LiaFSR seems to influence c-di-AMP pools *in vivo*. Our findings suggest that c-di-AMP signaling constitutes a key aspect of the membrane damage response with important consequences for our understanding of how enterococci respond to daptomycin and other cationic antimicrobial peptides.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains used in this study are listed in Table 1. *E. faecalis* OG1RF harboring pMSP3535 and recombinant plasmids was grown in brain heart infusion (BHI) supplied with 10 μ g/ml erythromycin and nisin as described in reference 33. Other *E. faecalis* strains were grown in LBHI broth (80% lysogeny broth [LB] and 20% brain heart infusion [BHI]) as described in reference 19. *E. faecium* HOU503 was grown in LBHI broth. Other *E. faecium* strains were grown in BHI. When daptomycin was added, 50 mg/liter CaCl₂ was supplied to the medium (19). (see Text S1 for details).

Measurement of intracellular c-di-AMP by competitive ELISA. The c-di-AMP competitive enzyme-linked immunosorbent assay (ELISA) protocols of Underwood et al. (26) were optimized for the measurement of intracellular c-di-AMP concentrations in *E. faecalis* and *E. faecium* (see Text S1 for details).

Plasmid construction. *E. faecalis* *gdpP* and subdomains were amplified by PCR from *E. faecalis* S613 with specific primers (see Table S1) with PfuUltra II DNA polymerase. PCR products were digested with appropriate restriction endonucleases and cloned into pET-28a (Novagen) for expression in *E. coli* BL21 or into pMSP3535 (33) for expression in *E. faecalis* OG1RF. Plasmids for the expression of *E. faecalis* GdpP^{L440S} and GdpP^{D419A/D499A} were generated by site-directed mutagenesis (see Table S1).

Expression of *E. faecalis* GdpP and GdpP^{L440S} in *E. faecalis* OG1RF. *E. faecalis* OG1RF strains with recombinant plasmids were grown in BHI with 10 μ g/ml erythromycin. Overnight cultures of *E. faecalis* OG1RF with recombinant plasmids were diluted 1:100 to BHI with 10 μ g/ml erythromycin on the second day, and 20 ng/ml nisin was added when the OD₆₀₀ was ~0.5. Cultures were incubated at 37°C shaken at 225 rpm overnight. On the third day, the overnight cultures were rediluted in BHI with 10 μ g/ml erythromycin and 20 ng/ml nisin. Cells were harvested after the OD₆₀₀ reached 1.5 (late exponential phase of *E. faecalis* OG1RF strains with plasmid pMSP3535) for c-di-AMP measurements.

Expression and purification of *E. faecalis* GdpP. Overnight cultures of *E. coli* BL21 carrying *E. faecalis* GdpP expression vector pET28a-*E. faecalis*_GdpP was diluted 1:100 in 8 liters of 2 $\times$ YT medium supplemented with 50 mg/liter kanamycin and shaken at 37°C until the OD₆₀₀ reached 0.6 to 0.7. Isopropyl- β -D-thiogalactopyranoside (IPTG) at 0.5 mM was then added to induce protein expression. Cultures were shaken at 16°C for 20 h before being harvested by centrifugation. Cells were resuspended in Ni-nitrilotriacetic acid (NTA) buffer A (50 mM Tris [pH 7.4], 20 mM imidazole, 500 mM NaCl, 10 mM β -mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 5% glycerol [vol/vol]) and opened by sonication on ice with three cycles of 2 min on and 3 min off at output 6/50% duty cycle. Supernatants were collected by centrifugation at 24,000 rpm for 1 h at 4°C. The protein was purified initially with a gravity Ni-NTA column and step eluted with Ni-NTA buffer B (50 mM Tris [pH 7.4], 200 mM imidazole, 440 mM NaCl, 9 mM β -mercaptoethanol, 0.2 mM PMSF, 5% glycerol [vol/vol]). The fractions containing target protein were collected and dialyzed overnight at 4°C against gel filtration buffer (25 mM *N*-cyclohexyl-2-aminoethanesulfonic acid [CHES, pH 8.6], 200 mM NaCl). Protein was subsequently concentrated and further purified on a HiLoad 16/60 Superdex 200 gel filtration column. Fractions containing target protein were collected. Fresh protein should be used for the following experiments, as storage at -80°C reduces activity.

Product analysis of GdpP reactions by HPLC. For ATPase activity, 10 μ M protein and 100 μ M ATP were incubated at 28°C for 2 h in a reaction buffer containing 40 mM Tris (pH 7.5) and 10 mM MgCl₂. For phosphodiesterase activity, 10 μ M protein was incubated with 100 μ M c-di-AMP or c-di-GMP at 28°C for 2 h in a reaction buffer containing 100 mM Tris (pH 8.3), 20 mM KCl, and 0.5 mM MnCl₂. ppGpp at 1 mM was added to the reaction buffer in experiments to estimate ppGpp inhibition of GdpP phosphodiesterase activity. Reactions were stopped, and protein was precipitated by the addition of trifluoroacetic acid to a 1% (vol/vol) final concentration and incubated on ice for 15 min. Precipitants were removed by centrifugation. A total of 10 μ l of each nucleotide standard or sample was injected into a reverse-phase C₁₈ column (Hamilton catalog no. 79674) and eluted with a gradient of 0 to 100% (buffer A was 0.1 M KH₂PO₄ and 4 mM tetrabutylammonium bromide [pH 6.0], and buffer B was 60% buffer A and 40% methanol [pH 5.5]).

Kinetic measurement of phosphodiesterase activity. The protocols of the coralyne fluorescence turn-on assay of Zhou et al. (31) were optimized to determine the enzymatic activity of *E. faecalis* GdpP³⁰⁹⁻⁶⁵⁸ phosphodiesterase toward c-di-AMP (see Text S1 for details).

Kinetic measurement of ATPase activity. The steady-state kinetics of ATPase activity were determined with the EnzChek Phosphate Assay kit (Life Technologies) by measuring the release of inorganic phosphate hydrolyzed from ATP. The reactions were set up in a 96-well plate and monitored with a Bio-Rad plate reader.

Structural modeling. A structural model of the *E. faecalis* GdpP DHH-DHHA1 domain was built with the Phyre2 server (40). The Mn²⁺ ion positions were predicted by alignment with *Mycobacterium tuberculosis* phosphodiesterase Rv2837c (Protein Data Bank [PDB] accession no. 5CET) (32).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01422-16>.

TEXT S1, PDF file, 1.2 MB.

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X.W., M.D., C.A.A., and Y.S. designed the research, analyzed the data, and wrote the paper. X.W., J.R., and D.P. performed the experiments. C.A.A., M.D., and Y.S. edited the manuscript.

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