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A Conserved Hydrolase Responsible for the Cleavage of Aminoacylphosphatidylglycerol in the Membrane of *Enterococcus faecium*

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Background: Aminoacylation of phosphatidylglycerol (PG) alters the charge of the bacterial membrane and confers resistance to antimicrobial peptides.
Results: AhyD catalyzes the hydrolysis of aminoacylated PG in *Enterococcus faecium* and increases tolerance to bacitracin.
Conclusion: AhyD participates in maintaining aminoacyl-PG homeostasis in the membrane.
Significance: Understanding how bacteria modulate their membranes is crucial to understanding how they adapt to environmental challenges.

Aminoacylphosphatidylglycerol synthases (aaPGSs) are enzymes that transfer amino acids from aminoacyl-tRNAs (aa-tRNAs) to phosphatidylglycerol (PG) to form aa-PG in the cytoplasmic membrane of bacteria. aa-PGs provide bacteria with resistance to a range of antimicrobial compounds and stress conditions. *Enterococcus faecium* encodes a triple-specific aaPGS (RakPGS) that utilizes arginine, alanine, and lysine as substrates. Here we identify a novel hydrolase (AhyD), encoded immediately adjacent to *rakPGS* in *E. faecium*, which is responsible for the hydrolysis of aa-PG. The genetic synteny of *aaPGS* and *ahyD* is conserved in >60 different bacterial species. Deletion of *ahyD* in *E. faecium* resulted in increased formation of Ala-PG and Lys-PG and increased sensitivity to bacitracin. Our results suggest that AhyD and RakPGS act together to maintain optimal levels of aa-PG in the bacterial membrane to confer resistance to certain antimicrobial compounds and stress conditions.

One strategy bacteria have developed to resist antimicrobial compounds and adapt to changes in their cellular environment is to alter the properties of their cellular envelope through aminoacylation of the membrane lipid phosphatidylglycerol (PG).[2] This process is carried out by aminoacylphosphatidylglycerol synthases (aaPGSs), which use aminoacyl-tRNAs (aa-tRNAs) as amino acid donors to modify PG in the bacterial membrane. Early studies revealed that the enzyme MprF (or multiple peptide resistance factor) is an aaPGS responsible for the lysylation of membrane PG in *Staphylococcus aureus* (1). Since that time, this lipid modification pathway has been shown (in *S. aureus* and other organisms) to result in increased resistance to a wide selection of antimicrobial molecules such as various antimicrobial peptides, β-lactams, and aminoglycosides and to certain lipopeptides and glycopeptides (*e.g.* daptomycin (2, 3) and vancomycin (4); for review, see Ref. 5). aaPGS was also determined to be an important virulence factor because it provides resistance to killing by human neutrophils and increases the virulence of several pathogens in various animal models (1, 6–9). Most aaPGSs identified to date are specific for attaching Lys to PG (LysPGS). However, a few organisms such as *Pseudomonas aeruginosa* (10) and *Clostridium perfringens* (11) harbor an AlaPGS, and enterococci such as *Enterococcus faecalis* and *Enterococcus faecium* harbor a triple-specific enzyme (RakPGS) responsible for the synthesis of Ala-PG, Lys-PG, and Arg-PG (12). In *E. faecalis*, *rakPGS* has been shown to be associated with increased resistance to several antimicrobial molecules (13).

Several studies demonstrated that the type of amino acid (aa) attached to PG affects the spectrum of antimicrobial resistances associated with lipid aminoacylation. For instance, it was demonstrated that modification by Lys, Ala, or a mixture of the two altered the antimicrobial resistance phenotypes of *P. aeruginosa*, suggesting that resistances are mediated by the positive charge conferred by the aa attached to PG and the nature of the aa lateral chain (14). The bacterial background where these modifications are found also determines the types of associated resistances. For instance, Lys-PG formation in *S. aureus* has been shown to be involved in daptomycin resistance (2, 3), whereas the presence of this lipid in *E. faecalis* does not contribute to resistance to this antibiotic (13). Another factor that modulates aa-PG-mediated resistance is the amount of aa-PG present in the membrane. It was shown that increased Lys-PG decreases the daptomycin susceptibility of *Bacillus subtilis* (15) and *S. aureus* (16). Although various culture conditions have been identified that trigger aa-PG synthesis, it is not known whether cellular mechanisms exist to limit the amount of aa-PG in the membrane.

Previous studies identified a gene (*atvA*) that resides in the same operon as *lysPGS* in *Rhizobium tropici* (17). AtvA belongs to the VirJ-like family of proteins and exhibits a characteristic LIPASE_SER motif (Prosite PS00120). *atvA*, along with *lysPGS*, was shown to be essential for acid tolerance in *R. tropici*. Although biochemical evidence is still lacking, Vinuesa and co-

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[2] The abbreviations used are: PG, phosphatidylglycerol; aa, amino acid; aa-, aminoacyl-; aaPGS, aminoacylphosphatidylglycerol synthase; MprF, multiple peptide resistance factor; PGS, phosphatidylglycerol synthase; THB, Todd-Hewitt broth; YPG, yeast extract, peptone, glucose.

workers proposed that AtvA might represent a novel family of lipolytic enzymes. More recently, it was suggested that AtvA (and other VirJ-related proteins) might exhibit a hydrolytic activity against Lys-PG to maintain a low level of modified lipids in the membranes of Gram-negative bacteria (18). On the other hand, *acvB*, which codes for an AtvA homolog downstream of *lysPGS* in *Agrobacterium tumefaciens* (12), was determined to be a periplasmic protein involved in the machinery for the transfer of tumorigenic DNA to plants. No functional connection between *acvB* and *lysPGS* was established in this organism (19–23).

During the course of this study, we performed a thorough analysis of the genomic context of *aaPGS* genes in bacteria to identify possible functional associations between aaPGSs and other proteins. Along with VirJ-like proteins, we identified two additional families of hydrolase-like proteins (α/β-hydrolases and esterases) that are encoded adjacent to *aaPGS* genes in many bacterial species. We studied the role of one gene, referred to here as *ahyD*, which belongs to the α/β-hydrolase family, and is located upstream and adjacent to *rakPGS* in *E. faecium*. We show that *ahyD* encodes an aa-PG hydrolase, which hydrolyzes Ala-PG and Lys-PG. These results suggest that AhyD may act in concert with RakPGS to maintain optimal levels of aa-PG in this organism. Deletion of *ahyD* or *rakPGS* resulted in increased susceptibility to bacitracin, indicating that both genes contribute to the adaptability of *E. faecium* to different environmental conditions.

EXPERIMENTAL PROCEDURES

Phylogenetic Distribution of Conserved Proteins Adjacent to aaPGS Genes—Our previous alignment of aaPGS sequences was expanded (12), and sequences from overrepresented species (*i.e.* those for which numerous genome sequences are available) were removed, bringing the total number of aaPGS sequences to 605, covering 149 representative bacterial genera. This alignment served as a set of aaPGS sequences for analyzing the genomic context of their corresponding genes among bacteria. A Perl script was written using the BioPerl module (24) to retrieve the annotated regions (40,000 bp) surrounding each *aaPGS* gene from the NCBI Reference Sequence Database. An automated BLAST search was scripted to compare sequences coded within the targeted regions to generate a graphic output for identifying conserved genes located directly upstream or downstream of *aaPGS* ORFs. This algorithm allowed for identification of 274 conserved sequences. Sequences were analyzed using the Pfam protein families database (25) and matched three distinct families of proteins (α/β-hydrolase PF12697, esterase PF00756, and VirJ PF06057) with an E-value $<4.2 \times 10^{-4}$. Prediction of secretory signal peptide sequences and transmembrane helices for each of the protein families was carried out using the SignalP 4.0 (26) and TopCons (27) algorithms, respectively.

Bacterial Strains and Culture Conditions—*E. faecium* cells were grown in Todd Hewitt broth (THB), or plated on THB supplemented with 0.25 M sucrose and 250 mg/liter gentamycin as needed. Cells for lipid analysis and growth curves were grown with vigorous shaking at 37 °C overnight in a low nutrient medium containing 70 mM Na_2HPO_4/KH_2PO_4 buffer (adjusted to pH 5.6 or 7.6), 15 mM NH_4Cl, 0.4% glucose, 1 mg/ml yeast extract, and 1 mg/ml peptone. For richer medium conditions, *E. faecium* cells were grown in YPG broth consisting of 5 mg/ml yeast extract, 5 mg/ml peptone, and 0.1% glucose. *Escherichia coli* cells were grown in Luria broth, or plated on Luria broth agar, supplemented with 30 mg/liter chloramphenicol, 50 mg/liter kanamycin, or 25 mg/liter gentamycin as needed.

Markerless Deletion of rakPGS and ahyD from E. faecium—Markerless deletion of the *E. faecium* genes, *rakPGS* and *ahyD*, was achieved using the methods of Nallapareddy *et al.* (28). *E. faecium* strains and the *E. coli-E. faecium* shuttle vector pTEX5500ts (thermosensitive for replication in enterococci) were kindly provided by the laboratory of Dr. Barbara Murray, University of Texas Medical School, Houston. Briefly, the upstream and downstream regions of the targeted genes (*aaPGS2* and *ahyD*) were amplified and stitched together by PCR using *E. faecium* TX1330 genomic DNA as template. PCR products were cloned into pTEX5500ts using the restriction enzymes NheI and HindIII. Plasmid constructs were transformed into *E. faecium* TX1330 by electroporation as described previously (29). Transformants were allowed to recover in THB supplemented with 0.25 M sucrose for 2 h at room temperature and plated on THB supplemented with sucrose (0.25 M) and gentamycin (250 mg/liter). Plates were incubated at 28 °C for 72 h. Isolated colonies were used to inoculate THB supplemented with gentamycin and grown overnight at the nonpermissive temperature (*i.e.* 42 °C) to allow for plasmid integration. Genomic DNA was isolated, and single crossover integration of the plasmid was verified by PCR. Positive clones were subjected to five serial passages in THB containing gentamycin to cure the cells of free plasmid. Plasmid excision by a second single crossover event was allowed during four additional passages in THB without antibiotics. Gentamycin-sensitive clones that had lost the plasmid were identified by replica plating on THB, and THB supplemented with gentamycin. Gentamycin-sensitive clones were subjected to PCR screening and sequence analysis to verify removal of the targeted genes.

Extraction of Total Lipids and TLC Analysis—Total unlabeled lipids were extracted as described by Roy and Ibba (30) using a modified protocol based on the Bligh-Dyer method (31). Harvested cells were resuspended in 1.8 ml of 0.1 M Tris-Cl, pH 8, with 2 mg/ml lysozyme (Amresco), and incubated at 37 °C for 15 min. Lipids were extracted by addition of 0.2 ml of 3 M sodium acetate, pH 4.5, and 7.5 ml of chloroform:methanol (1:2, v:v), and mixing vigorously for 50 min at room temperature. Subsequently, 2.5 ml of chloroform and 2.5 ml of 120 mM sodium acetate, pH 4.5, were added, and the organic phase was separated by centrifugation and dried under vacuum. Lipids were resuspended in 50 μl of chloroform:methanol (2:1, v:v). An amount of lipids corresponding to 10 OD of cells was spotted on 10-cm long, 250-μm HLF silica gel TLC plates (Analtech). Plates were developed with chloroform:methanol:water (14:6:1, v:v:v) for 20 min in a single dimension. For detection of phospholipids, the TLC plates were stained with molybdenum blue (Dittmer's reagent (32)). Detection of aminoacylated phospholipids was performed using ninhydrin spray (Acros). Radioactively labeled lipids were extracted and analyzed similarly, but at a smaller scale, as described previously (30) and below.

The level of Lys-PG (relative to other phospholipids) was determined by densitometry analysis of the Dittmer-stained TLC plates using the software ImageJ (33). All experiments were repeated in triplicate.

Expression of ahyD in E. coli and Preparation of Membrane Fractions—Two variants of the *ahyD* gene from *E. faecium* DO were PCR-amplified and cloned into vector pet33b (Novagen) using the restriction enzymes NcoI and BamHI. The first variant corresponds to full-length AhyD (GI:69249188), and the second variant (15-AhyD) lacks the first 14 amino acids at the N terminus of the protein. *E. coli* C41 cells harboring the variant plasmids were grown to an A_{600} of 0.6 in 250 ml of Luria broth containing 50 mg/liter kanamycin. Protein expression was induced by addition of 0.5 mM isopropyl 1-thio-β-D-galactopyranoside followed by 4 h of incubation at 37 °C. Membrane fractions were prepared as described previously (30) from cells expressing AhyD and 15-AhyD, and from cells harboring empty vector (*i.e.* without insert) to serve as a negative control for protein expression. Briefly, cells were washed with 50 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, and 3 mM β-mercaptoethanol; protease inhibitors were omitted because AhyD exhibits a motif containing putative catalytic Ser and His residues). Cells were disrupted with a Branson 450 Sonifier in 12 ml of lysis buffer. Extracts were brought up to 35 ml, and the cellular debris was sedimented by centrifugation for 15 min at 8000 × *g*. The cell membranes in the supernatant were recovered by ultracentrifugation at 150,000 × *g* for 45 min. Membrane pellets were washed with 5 ml of lysis buffer, and membrane extracts were prepared by dispersion of the pellets using low powered sonication in 2 ml of the same buffer. Membrane extracts were stored at −80 °C in small volume aliquots.

In Vitro Hydrolysis of aa-PG—Total lipids from *E. faecium* were labeled radioactively *in vivo* as described previously (11, 30) by growing cells overnight at 37 °C in 2 ml of medium containing 70 mM sodium acetate, pH 5.6, 15 mM NH_4Cl, 0.4% glucose, 5 mg/ml yeast extract, 5 mg/ml peptone, and 20 μCi of [^{32}P]PP_i (PerkinElmer Life Sciences). ^{32}P-Labeled lipids were extracted as described above with a few modifications. Briefly, harvested cells were resuspended in 200 μl of 120 mM sodium acetate, pH 4.5, and 750 μl of chloroform:methanol (2:1, v:v). Lysis was performed by addition of 100 μl of acid-washed zirconium beads (200 μm, VWR) and vortexing for 30 min at room temperature. Phospholipids were extracted by addition of 200 μl of chloroform and 200 μl of 120 mM sodium acetate, pH 4.5. Phospholipids in the organic phase were separated by centrifugation, dried, and resuspended in 100 μl of chloroform:methanol (2:1, v:v) and stored at −80 °C.

40 μl of radiolabeled *E. faecium* lipids (600 cpm/μl) and 87 μg of L-α-PG in chloroform (Avanti Polar Lipids) were dried under vacuum and resuspended in 65 μl of buffer containing 0.1 mM HEPES, 30 mM KCl, 15 mM $MgCl_2$, and 2 mM DTT. 15 μl of the lipid emulsion was combined with 5 μl of the AhyD-expressing membrane fractions (see above) and incubated at 37 °C. 5-μl aliquots were removed at various times up to 20 min and quenched with 400 μl of chloroform:methanol:120 mM acetic acid, pH 4.5 (4:8:3.2, v:v). Lipids were reextracted and separated by TLC with the mobile phase consisting of chloroform:methanol:H_2O (28:12:2, v:v). Radiolabeled phospholipids were detected by phosphorimaging, and the relative abundance of PG, cardiolipin, and Lys-PG was determined. Hydrolysis experiments were performed in triplicate.

TABLE 1

Phylogenetic distribution of conserved protein families encoded adjacent to *aaPGS* genes in bacteria

The genomic context of 605 *aaPGS* genes in 493 bacterial species was analyzed, and the percentage of occurrence of each family of conserved proteins (encoded adjacent to *aaPGS*) is shown for each bacterial group. For example, 18% of 306 total Gram-positives possess at least one α/β-hydrolase. The sum of percentages is >100 within a bacterial group when more than one protein family was found in synteny with a single *aaPGS* gene in one or more species.

Bacterial groups[a]	Protein families (Pfam)			No ORF[b]
	α/β-Hydrolase	Esterase	VirJ	
	%	%	%	%
Gram-positive (306)	18	32	0	51
Firmicutes (147)	25	0	0	75
Bacillales (61)	0	0	0	100
Clostridia (28)	43	0	0	57
Lactobacillales (59)	42	0	0	58
Enterococcus (28)	100	0	0	0
Lactobacillus (25)	0	0	0	100
Lactococcus (17)	53	0	0	47
Leuconostoc (6)	100	0	0	0
Actinobacteria (159)	12	61	0	30
Gram-negative (187)	5	2	53	42
Bacteroidetes (6)	0	0	67	33
Chloroflexi (3)	0	0	0	100
Cyanobacteria (6)	33	67	0	33
Fusobacteria (4)	0	0	0	100
Proteobacteria (158)	4	0	61	37
α (73)	1	0	45	53
β (27)	0	0	96	4
δ (11)	25	0	50	45
γ (47)	4	0	68	30

[a] The number of species included in each bacterial group is indicated in parentheses. Only the main groups of bacteria are shown.

[b] Represents the percentage of species within a group that do not exhibit any of the conserved protein genes adjacent to an *aaPGS* gene.

Growth Curves and Resistance Phenotypes—Growth curves of wild-type and mutant *E. faecium* strains were compared in 96-well plates containing 100 μl of limited nutrient medium, pH 5.6 or 7.6, or YPG broth supplemented with 80 mg/liter bacitracin or 3.3 mM DL-lactic acid. Starter cultures were grown in THB at 37 °C and washed with 100 mM NaCl. 20,000 cfu was used to inoculate each well (1 OD = 185,000 cfu/μl), and plates were incubated in a Synergy H1 Hybrid Microplate Reader (BioTek) over 30 h at 37 °C with constant, linear agitation. The A_{600} was measured at 6-min intervals. Each growth condition was tested in triplicate or quadruplicate. To quantify small changes in bacterial growth, maximal growth levels (A_{max}) and maximal growth rates (μ_{max}) were determined with the Grofit package using the model-free spline method (34, 35).

RESULTS

Phylogenetic Distribution of Three Conserved Families of Proteins in Operon with aaPGSs—To predict the possible functional association of *aaPGSs* with other genes, we analyzed the genomic context of 605 *aaPGS* ORFs from 493 species of bacteria. This analysis revealed 274 conserved proteins encoded immediately upstream or downstream of, and bearing the same orientation as, *aaPGS*, indicating that the genes may be a part of an operon. These proteins belong to three distinct groups of hydrolytic enzymes (*i.e.* α/β-hydrolases (PF12697), esterases (PF00756), and VirJ proteins (PF06057)) based on the Pfam protein families database (25). Table 1 summarizes the phylo-

genetic distribution of these proteins among bacteria. VirJ-like proteins are found exclusively in Gram-negative bacteria, whereas α/β-hydrolases and esterases are found predominantly in Gram-positive bacteria. Esterase-like proteins are mainly found adjacent to *aaPGS* genes in actinobacteria. Although hydrolase-like proteins were found to be encoded next to *aaPGS* in 52% of the species we analyzed, these proteins are totally absent from some bacterial groups. For instance, all Bacillales (*e.g.* species belonging to the *Staphylococcus*, *Bacillus*, and *Listeria* genera), and some Lactobacillales and fusobacteria, are deprived of hydrolase-like proteins encoded adjacent to *aaPGSs*.

Investigation of predicted transmembrane helices and secretory signal protein sequences revealed a high degree of variability across the three families of proteins. A single transmembrane helix and an N-terminal signal peptide were detected in 58% of the VirJ sequences analyzed. Analysis of the esterase family of proteins did not reveal any putative secretory signal peptides. However, a variable number (2–4) of transmembrane helices, located at the N termini, were predicted for members of this group of enzymes. Finally, no signal peptide sequences or transmembrane helices were identified in any of the proteins belonging to the α/β-hydrolase family. The gene *ahyD*, encoded upstream of *rakPGS* in *E. faecium*, belongs to the latter group of enzymes. The α/β-hydrolase-like proteins exhibit a characteristic fold common to a variety of hydrolytic enzymes of differing phylogenetic origin (*e.g.* lipases, proteases, epoxide hydrolases) and with different catalytic functions (36). A sequence alignment of AhyD with other members of this family revealed the presence of conserved Ser and His residues (data not shown), which constitute the catalytic residues found in the α/β-hydrolase family of proteins (36).

Aminoacyl-PG Levels in E. faecium Are Up-regulated in Acid Conditions—Previous studies have shown that aa-PG levels are variable across bacterial species and are modulated depending on environmental conditions. Specifically, acidification of the medium was shown to contribute to increased formation of aa-PG in a range of Gram-negative and Gram-positive bacterial species (*e.g. R. tropici* (18), *S. aureus* (37), *B. subtilis* (38), *Bacillus megaterium* (39), and *Streptococcus faecalis* (13)). To analyze the lipid content of *E. faecium* strain TX1330, and determine whether aa-PG content in this organism is dependent on pH, cells were grown in a minimal medium supplemented with low amounts of peptone and yeast extract and adjusted to either pH 5.6 or 7.6. Lipids were analyzed by one-dimensional TLC and subsequent staining with Dittmer dye (for visualization of phospholipids) and ninhydrin (for detection of aa-PG). Ninhydrin-reactive spots exhibiting migration patterns consistent with Ala-PG and Lys-PG were observed in *E. faecium* wild-type cells grown in either pH condition (Fig. 1, *fifth* and *sixth lanes*). This is in agreement with *in vitro* studies that showed that RakPGS efficiently utilizes Lys-tRNA^{Lys} and Ala-tRNA^{Ala} as aminoacyl group donors (12). Although RakPGS was also shown to use Arg-tRNA^{Arg} as substrate, this activity was found to be >6-fold less efficient than for the other two aa-tRNAs, and thus the Arg-PG content in *E. faecium* lipid extracts is likely to be too low to detect by ninhydrin straining. The other major phospholipids that were observed in the wild-type strain (by Dittmer staining) exhibited migration patterns consistent with cardiolipin and unmodified PG (Fig. 1, *first* and *second lanes*). Inspection of the predominant aa-PGs (*i.e.* Lys-PG and Ala-PG) revealed an increased proportion of these lipid components in the lower pH culture (Fig. 1, compare *fifth* and *sixth lanes*), with the Lys-PG fraction increasing by 2.1-fold (±0.19). These results suggest that in *E. faecium*, as in some other organisms (see above), aa-PG expression may be important for bacterial adaptability to acidic pH conditions.

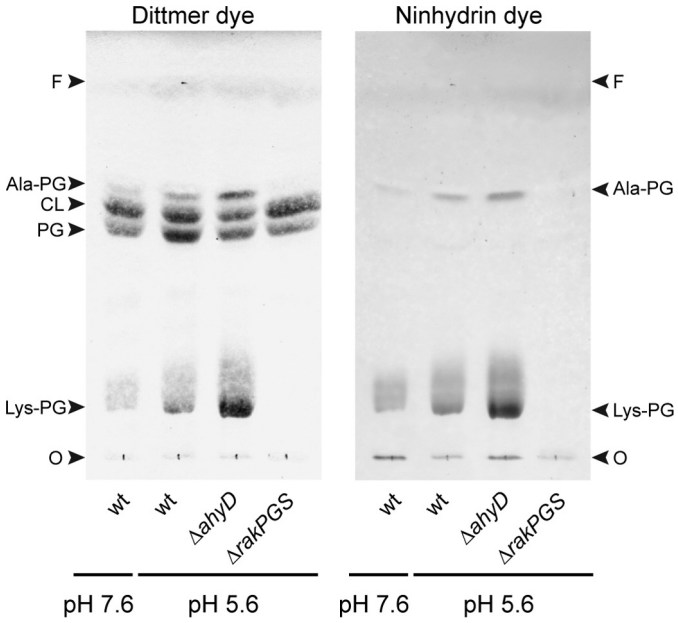

FIGURE 1. **Analysis of aa-PG content in *E. faecium* wild-type and mutant strains grown at pH 7.6 or 5.6.** Total lipids were isolated from wild-type (*wt*) and mutant strains (Δ*ahyD* and Δ*rakPGS*) grown at pH 7.6 or 5.6 and were analyzed by one-dimensional TLC. Phospholipids were visualized by staining with Dittmer dye, and aa-PGs were visualized by ninhydrin staining. *Lanes* shown are from a single TLC. The solvent front (*F*) and origin (*O*) are indicated for each *panel*, and predominant lipids are indicated. *CL*, cardiolipin.

Deletion of ahyD Results in Increased Levels of aa-PG—To investigate the role of *rakPGS* and *ahyD* we constructed markerless deletion strains of *E. faecium* TX1330 using a two-step integration-excision method with a replication-thermosensitive plasmid (pTEX5500ts). Determination of the lipid composition by TLC and ninhydrin staining revealed that deletion of the *rakPGS* gene is sufficient to completely disrupt formation of both Ala-PG and Lys-PG in *E. faecium* (Fig. 1, *eighth lane*). These results mirror recent findings in the related organism, *E. faecalis*, which showed that deletion of a homologous gene (*mprF2*) leads to complete loss of aa-PG formation in that organism (13). Deletion of the putative hydrolase, *ahyD*, resulted in an increase in Ala-PG and Lys-PG compared with the wild-type strain (Fig. 1, *sixth* and *seventh lanes*). These results suggest that AhyD may be responsible for the hydrolysis of aa-PG in *E. faecium*.

Two Putative Translational Start Sites of ahyD—Inspection of the sequences of the *ahyD* and *rakPGS* ORFs in *E. faecium* led to identification of a potential ambiguity in the location of the *ahyD* translational start site. Specifically, 14 amino acids downstream from the N-terminal Met-1 residue (as annotated in the NCBI RefSeq data bank) lies a Val residue preceded by a putative ribosome binding site, which we postulated might con-

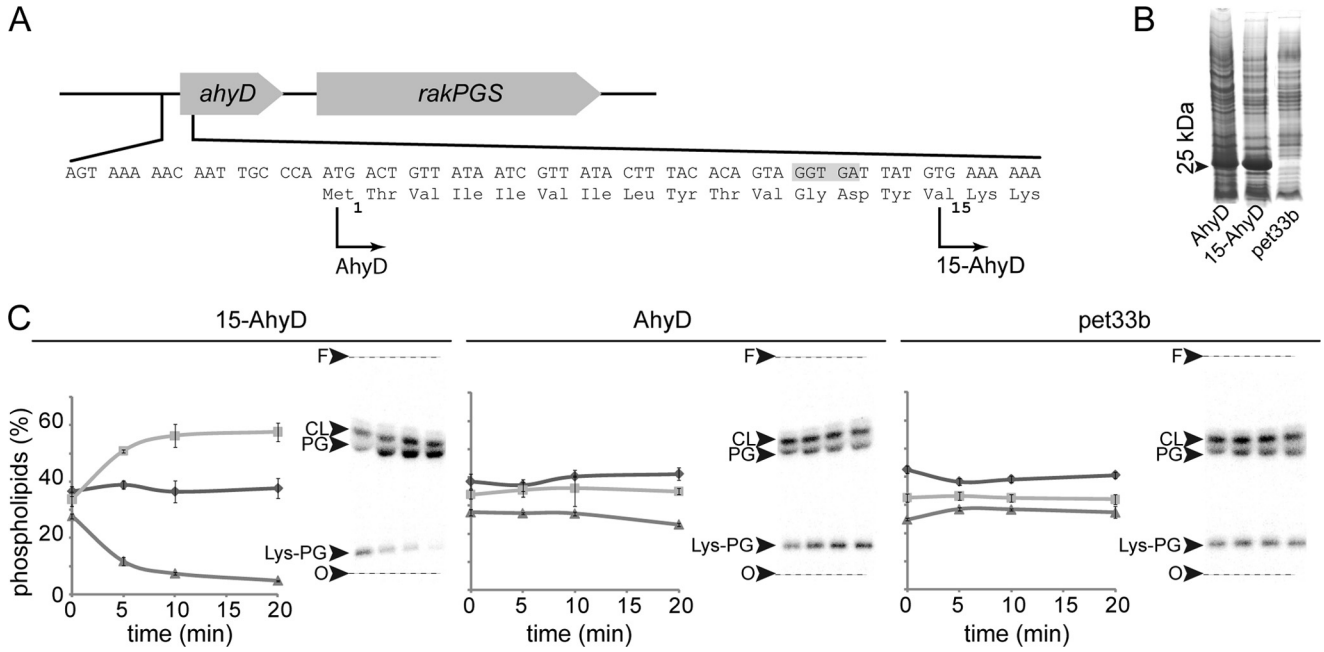

FIGURE 2. **Hydrolysis of Lys-PG is catalyzed by AhyD.** *A*, the *ahyD* gene from *E. faecium* TX1330 has two putative translational start sites (*arrows*). The first one initiates at Met-1 (yielding the protein, AhyD), and the second one initiates 14 amino acids downstream at Val-15 (for translation of 15-AhyD). A putative ribosome binding site is highlighted in *gray*. *B*, SDS-PAGE analysis of membrane fractions from *E. coli* expressing AhyD or 15-AhyD, or harboring empty expression plasmid (pet33b) is shown. *C*, ^{32}P-radiolabeled lipids isolated from *E. faecium* were incubated with membrane extracts from *E. coli* expressing either AhyD or 15-AhyD or harboring empty vector. Hydrolysis was monitored over time by reextraction of radiolabeled lipids and TLC analysis. *Lanes* on the TLC plates correspond to the lipid content at 0, 5, 10, and 20 min (from *left* to *right*). Percentages of lipid components were plotted *versus* time; *diamonds* indicate cardiolipin (*CL*), *squares* represent PG, and *triangles* represent Lys-PG. The solvent front (*F*) and origin (*O*) are indicated with *dashed lines* for each TLC plate.

stitute the actual translational start site (Fig. 2*A*). Two ORFs corresponding to the *E. faecium ahyD* gene, with or without the 14-amino acid extension at the N terminus (AhyD and 15-AhyD, respectively), were cloned into vector pet33b and expressed in *E. coli* C41. Membrane fractions were isolated, and proteins corresponding to ~25 kDa (*i.e.* the relative molecular mass of AhyD) were detected by SDS-PAGE in each of the membrane preparations (Fig. 2*B*, *first* and *second lanes*), but not in the soluble cellular fractions (data not shown). A separate membrane fraction isolated from cells harboring empty vector was included as a negative control (Fig. 2*B*, *third lane*).

AhyD Catalyzes the in Vitro Hydrolysis of Lys-PG—Deletion of *ahyD* in *E. faecium* resulted in increased levels of Lys-PG and Ala-PG *in vivo* (see Fig. 1). This led us to hypothesize that AhyD may be involved in the hydrolysis of aa-PG. To test this hypothesis directly, we measured the stability of ^{32}P-radiolabeled aa-PG (isolated from *E. faecium*) over time after treatment with membrane fractions isolated from *E. coli* cells expressing AhyD from *E. faecium*. Because there was some uncertainty about the correct translational start site for AhyD (see previous section), we tested membrane preparations containing either AhyD or 15-AhyD. When lipids were incubated with membrane extracts from *E. coli* expressing vector alone, or vector containing *ahyD*, the amount of Lys-PG remained steady at approximately 25% of the total lipid content for the duration of the 20-min time course (Fig. 2*C*). The cardiolipin and PG content also remained steady at approximately 40 and 30%, respectively. However, when radiolabeled lipids were incubated with membrane extracts isolated from *E. coli* cells expressing 15-AhyD, the Lys-PG content decreased by 2-fold after 5 min of incubation and by 4-fold after 20 min. Concomitantly, the relative amount of PG increased from approximately 30% to 55% over 20 min, as would be expected from the hydrolysis of the ester bond linking the amino acid to PG. These results demonstrate that AhyD from *E. faecium* catalyzes the hydrolysis of aa-PG and that the start codon of AhyD is likely to correspond to the Val-15 residue. In addition, these data show that *E. coli* membrane extract alone does not exhibit any specific hydrolytic activity against Lys-PG.

rakPGS Is Involved in Resistance to Low pH—Disruption of LysPGS and AlaPGS activity has been shown to result in decreased tolerance to acid, cationic antimicrobial peptides, and other antimicrobial compounds in a variety of bacterial species (for review, see Ref. 5). This prompted us to test the growth phenotypes of our mutant strains in several stress conditions to determine the importance of *rakPGS* and *ahyD*. First, we tested the growth of *E. faecium* strains in variable pH conditions to see whether the aa-PG pathway contributes to acid tolerance in this organism. Mutant strains were grown in low nutrient medium adjusted to pH 5.6 or 7.6 and compared with the wild-type strain. Deletion of *ahyD* or *rakPGS* did not result in major inhibition of overall growth at either pH (Fig. 3). However, a moderate effect on the maximal growth rate (μ_{max}) was observed with the Δ*rakPGS* mutant grown at pH 5.6 (relative to growth at pH 7.6), demonstrating that RakPGS plays a role in acid tolerance in *E. faecium*. The Δ*ahyD* mutant exhibited a moderate growth defect at both pH values, indicating that this gene may be important for maintaining optimal growth in neutral or acidic conditions.

Deletion of rakPGS or ahyD Decreases Tolerance to Lactic Acid and Bacitracin—Bacitracin is an antimicrobial peptide that inhibits dephosphorylation of undecaprenyl diphosphate,

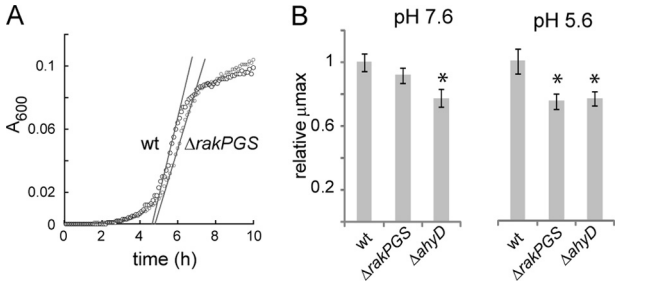

FIGURE 3. ***rakPGS* is involved in acid tolerance in *E. faecium*.** Wild-type and mutant *E. faecium* strains were grown at 37 °C in low nutrient medium adjusted to either pH 5.6 or 7.6. *A*, representative growth of wild-type and Δ*rakPGS E. faecium* (at pH 5.6) determined by measuring the optical density (*OD*) at 600 nm for 10 h at 6-min intervals. The maximal growth rate (μ_{max}) was determined using the Grofit package software (34). *B*, comparison of the μ_{max} values for each strain (normalized to the wild-type strain) at pH 7.6 and 5.6. *Asterisks* indicate significant differences relative to the wild-type strain as determined by Student's *t* test ($n = 3$, $p < 0.05$).

a lipid carrier that is essential for the synthesis of peptidoglycan during cell wall synthesis (40). This antibiotic has been shown to be effective in treating vancomycin-resistant *E. faecium* in the intestinal tracts of human patients (41). To investigate the role of AhyD and RakPGS in *E. faecium* resistance to this antibiotic, wild-type and mutant strains were grown in YPG broth supplemented with 80 mg/liter bacitracin. Wild-type cells grew well in these conditions with a maximal growth rate (μ_{max}) and maximal growth level (A_{max}) that was similar to that obtained with cells grown in the absence of bacitracin (Fig. 4*B*). However, there was a significant lag in growth, with the cells requiring nearly twice as long to enter the exponential phase and reach stationary phase compared with cells grown in the absence of the antibiotic (compare Fig. 4, *B* and *A*). No growth was observed with the Δ*rakPGS* cells after 30 h of incubation, indicating that aa-PG is essential for *E. faecium* resistance to bacitracin. An effect was also observed with the Δ*ahyD* mutant strain, albeit less dramatic. A significant lag in growth was detected, as well as a decrease in the μ_{max} and A_{max} values. These results suggest that the absence of AhyD has a negative impact on *E. faecium* growth in the presence of bacitracin. Results obtained with a double knock-out strain, containing deletions of both genes (Δ*rakPGS* and Δ*ahyD*), mimicked those obtained with the single deletion strain, Δ*rakPGS*; no growth was observed after 30 h.

Wild-type and mutant strains were also tested for tolerance to lactic acid, a naturally occurring compound produced by *E. faecium* and other lactic acid bacteria during fermentation, which can act as a proton carrier that diffuses across the cell membrane and leads to growth inhibition. Previously, it was shown that Ala-PG in the membrane of *P. aeruginosa* is important for resistance to lactic acid (10). Wild-type and mutant *E. faecium* strains were grown at 37° C for 20 h in YPG broth alone or supplemented with DL-lactic acid. In these conditions, growth of all the strains (including wild-type) were significantly inhibited compared with cells grown in the absence of lactic acid (Fig. 4*C*). However, an additional effect was observed with the Δ*rakPGS* mutant, suggesting that aa-PG production is important for tolerance to lactic acid in *E. faecium*. The Δ*ahyD* strain, on the other hand, grew similarly to the wild-type strain, indicating that the absence of AhyD has no detrimental effect

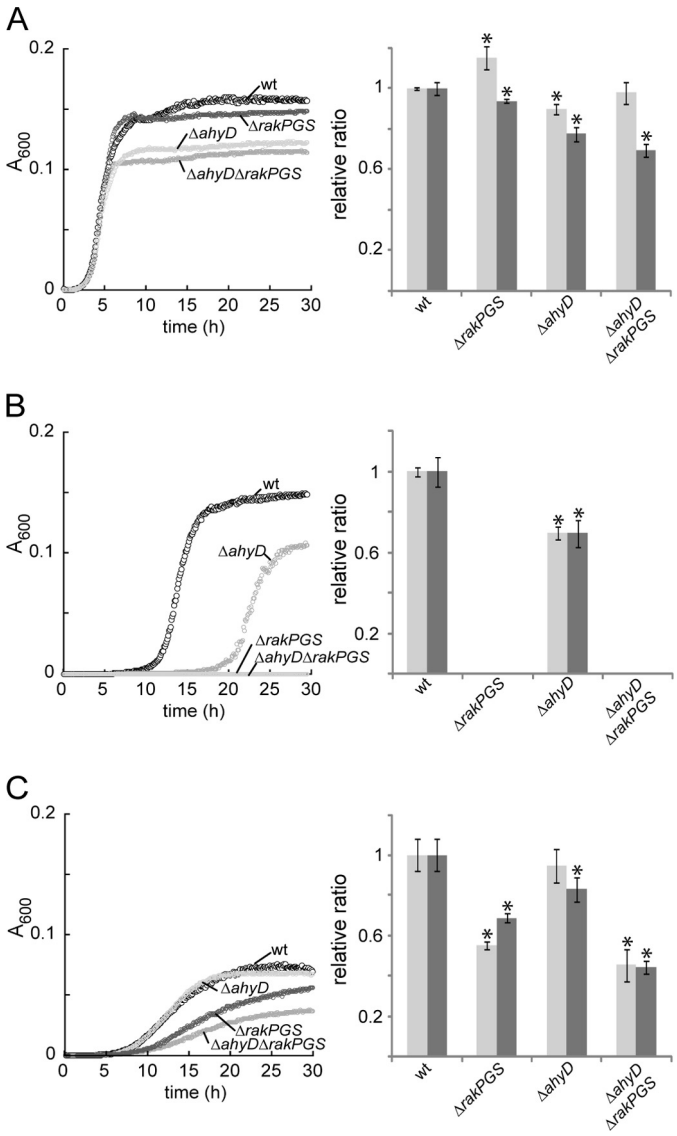

FIGURE 4. **Role of *rakPGS* and *ahyD* in *E. faecium* resistance to lactic acid and bacitracin.** *E. faecium* strains were grown at 37 °C in YPG (*A*), or YPG supplemented with 80 mg/liter bacitracin (*B*), or 3 mM DL-lactic acid (*C*). The optical density (*OD*) at 600 nm was monitored over 30 h at 6-min intervals, and the μ_{max} (*light bars*) and A_{max} values (*dark bars*) were determined using Grofit package software (34). Values were plotted after normalization to the wild-type strain, and significant differences in growth parameters between wild-type and mutants strains (indicated by *asterisks*) were determined by Student's *t* test ($n = 3$, $p < 0.05$).

on growth. Interestingly, a further decrease in the growth parameters was observed when *ahyD* and *rakPGS* were deleted simultaneously. Taken together, these findings suggest that AhyD may have a role outside of aa-PG hydrolysis that is necessary for optimal growth of *E. faecium* in these conditions.

DISCUSSION

Modification of phosphatidylglycerol by aminoacylation is one of the mechanisms utilized by bacteria to adapt their cellular envelope to stressors in their environment and to resist a wide array of antimicrobial molecules (for review, see Ref. 5). In addition to increased tolerance to antimicrobials, aa-PG has been shown to contribute to the virulence of several medically relevant human pathogens (*e.g. S. aureus*, *Mycobacterium*

tuberculosis, and *Listeria monocytogenes*) (7, 9, 42). In this work we reveal the function of a new hydrolytic enzyme (AhyD), which is responsible for the hydrolysis of aa-PG in the membrane of *E. faecium*. *ahyD* is located upstream and adjacent to *rakPGS*, which encodes a unique, relaxed-specificity aaPGS (RakPGS) that can utilize Ala, Lys, or Arg for modification of PG (12). Our findings suggest that AhyD acts together with RakPGS to maintain aa-PG homeostasis in *E. faecium* by limiting the amount of aminoacylated PG in the membrane to specific levels. The maintenance of aa-PG levels by AhyD has little effect on growth of cells exposed to certain conditions (*e.g.* acidic pH or elevated levels of lactic acid); however, it confers a significant advantage to *E. faecium* cultured in the presence of the antimicrobial compound bacitracin. This latter finding points to AhyD as a potential drug target, whose inactivation might enhance bacterial sensitivity to select antimicrobial molecules.

Our analysis of bacterial genomes revealed three families of hydrolase-like proteins (α/β-hydrolases, esterases, and VirJ-like proteins), which are encoded adjacent to *aaPGS* genes. Several studies suggested a role for the VirJ-like proteins in lipid metabolism in proteobacteria, but no biochemical evidence has been presented for this family of proteins (17, 18). The precise roles of VirJ proteins, which are located in the periplasm of certain proteobacteria (19–23), and the esterase-like proteins that are found exclusively in actinobacteria, have yet to be determined. AhyD, which belongs to the α/β-hydrolase family of proteins, may have evolved as a mechanism to maintain the aa-PG content in organisms in which too much aa-PG might be detrimental, due to genetic context and/or environmental habitat. Interestingly, some organisms that appear to lack an AhyD homolog, or any another putative hydrolytic enzymes (*i.e.* VirJ or esterase-like proteins), are able to produce a very high level of aa-PG. For instance, in *S. aureus* and *B. megaterium* the Lys-PG content was shown to constitute >80% of the total lipid content in certain conditions (39, 43). In these organisms, down-regulation of aa-PG by a mechanism similar to *ahyD* may not be essential, or there may be other mechanisms in place for fine-tuning individual lipid components.

Our results show that aa-PG levels in *E. faecium* increase in response to acidic conditions, a behavior that mirrors observations made with other species. Due to the increased expression of aa-PG in acidic conditions, we postulated that aa-PG might contribute to the acid tolerance of *E. faecium*. Indeed, deletion of the *rakPGS* gene resulted in a lower maximal growth rate of *E. faecium* cells cultured at pH 5.6 (relative to pH 7.6); however, the effect was somewhat modest (only approximately 15%). The direct role of aa-PG in adaptation of *E. faecium* to acidic conditions is unclear. It is possible that the increase in aa-PG content at low pH may not only provide slightly increased resistance to elevated proton concentrations, but may also confer resistance to other molecular species that are normally present in acidic conditions. For example, formation of aa-PG at low pH might be one mechanism utilized by *E. faecium* to cope with increased levels of lactic acid, which are produced during fermentation of glucose. Lactic acid is an osmolyte that is naturally produced by *E. faecium* and other Lactobacillales. This compound acts as a proton carrier leading to accumulation of protons inside the cell and cellular death (44). aa-PGS has been identified as a resistance factor against this osmolyte in *P. aeruginosa* (10). In *E. faecium*, deletion of *rakPGS* resulted in a ~50% decrease in the maximal growth rate of cells grown in the presence of lactic acid. These results support the notion that increased expression of aa-PG at low pH may enable *E. faecium* to cope with other acid-related stressors, such as increased levels of lactic acid.

In addition, our data suggest that AhyD may have an additional function outside of aa-PG hydrolysis in *E. faecium.* Deletion of *rakPGS* did not dramatically affect the growth of cells cultured in YPG alone (see Fig. 4*A*), indicating that aa-PG formation is not required in these conditions. Deletion of *ahyD*, however, had a negative impact on the growth phenotype, suggesting that increased levels of aa-PG may be disadvantageous. Deletion of both genes (*ahyD* and *rakPGS*) simultaneously, also had a detrimental effect. This result is surprising because there is presumably no aa-PG formation in this mutant strain; we would expect that the results would mimic those observed when *rakPGS* is deleted alone. The fact that we observed decreased growth with the double-mutant strain indicates that *ahyD* may be involved in a mechanism besides aa-PG hydrolysis. This idea is reinforced with the results from the lactic acid experiments. Deletion of *ahyD* did not have a negative impact on *E. faecium* growth in the presence of lactic acid (Fig. 4*C*). The double knock-out, Δ*ahyD*Δ*rakPGS*, on the other hand, exhibited lower growth than the Δ*rakPGS* mutant alone, pointing to an additional role for *ahyD* in the cell.

Many questions remain unanswered about the mechanism of action of AhyD in the cell. For instance, it is not clear whether AhyD is localized to the inner leaflet or the outer leaflet of the bacterial membrane. AhyD activity on the inner leaflet might be necessary to hydrolyze aa-PG that has not translocated to the outer leaflet where aa-PG exerts its role in resistance to antimicrobials (45). Alternatively, AhyD activity may be targeted primarily to the outer leaflet, where it directly modulates the total aa-PG content. Also, it remains unclear whether AhyD activity is directed toward a single aa-PG or whether the enzyme is able to hydrolyze, with similar efficiency, lipid components bearing different amino acids. This aspect is especially interesting in organisms expressing more than one type of aa-PG, such as *E. faecium* and *C. perfringens*. Experiments to address these and other questions regarding AhyD-directed hydrolysis of aa-PG will provide insight into the mechanisms employed by bacteria to modulate the overall lipid content in the cellular envelope.

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A Conserved Hydrolase Responsible for the Cleavage of Aminoacylphosphatidylglycerol in the Membrane of *Enterococcus faecium*

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