

Broad Range Amino Acid Specificity of RNA-dependent Lipid Remodeling by Multiple Peptide Resistance Factors*

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Aminoacylphosphatidylglycerol synthases (aaPGSs) are multiple peptide resistance factors that transfer amino acids from aminoacyl-tRNAs to phosphatidylglycerol (PG) in the cytoplasmic membrane. Aminoacylation of PG is used by bacteria to decrease the net negative charge of the cell envelope, diminishing affinity for charged molecules and allowing for adaptation to environmental changes. Lys-PGS, which transfers lysine to PG, is essential for the virulence of certain pathogens, providing resistance to both host cationic antimicrobial peptides and therapeutic antibiotics. Ala-PGS was also recently described, but little is known about the possible activities of other members of the highly diverse aaPGS family of proteins. Systematic deletion of the predicted membrane-inserted domains of several aaPGSs revealed that the carboxyl-terminal hydrophilic domain alone is sufficient for aminoacylphosphatidylglycerol transferase catalytic activity. In contrast to previously characterized aaPGSs, the *Enterococcus faecium* enzyme used an expanded repertoire of amino acids to modify PG with Ala, Arg, or Lys. Reexamination of previously characterized aaPGSs also revealed broader than anticipated substrate specificity, for example *Bacillus subtilis* Lys-PGS was shown to also catalyze Ala-PG synthesis. The relaxed substrate specificities of these aaPGSs allows for more elaborate remodeling of membrane lipids than previously thought, potentially providing bacteria that harbor these enzymes resistance to a broad spectrum of antibiotics and environmental stresses.

To adapt to changing environmental conditions, such as those encountered during host infection, bacteria must alter the properties of their cellular envelope by adjusting the composition and abundance of their membrane lipids. Bacteria choose from a variety of lipid components that vary in fatty acid chain length and saturation levels and that bear a repertoire of different polar head groups. Aminoacylphosphatidylglycerol synthases (aaPGSs)² are enzymes embedded within the bacterial membrane that are responsible for the transfer of amino acids from aminoacyl-tRNAs (aa-tRNAs) to the polar head groups of phosphatidylglycerol (PG). Addition of amino acids

to membrane PG is a critical mechanism evolved by bacteria to decrease the net negative charge of the cell membrane (1). This alteration of charge diminishes the membrane affinity for cationic antimicrobial peptides used by the host immune system (*i.e.* defensins), and for other bactericidal agents (2). During infection, aaPGSs have been shown to be essential for the virulence of several pathogenic microorganisms by facilitating the evasion of antibiotic activity. Their role in virulence and their broad distribution in bacterial species make aaPGSs attractive targets for new therapeutic strategies to combat pathogenic microbes.

Lys-PG in the membrane allows for the evasion of neutrophils and enhances the virulence of *Staphylococcus aureus* both in mice (3) and in endovascular infection of rabbits (4). Similar observations were made for *Listeria monocytogenes*, in which Lys-PG enhances infectivity of epithelial cells and macrophages in mice (5, 6). Moreover, Lys-PG provides *S. aureus* with resistance to other classes of cationic bactericidal agents such as vancomycin (glycopeptide) and daptomycin (lipopeptide) (7, 8), which are often used as a last resort to treat infections. A new aaPGS with altered specificity was recently discovered in *Clostridium perfringens* and *Pseudomonas aeruginosa* (9, 10). This enzyme, Ala-PGS, is responsible for the formation of Ala-PG in the membrane. Despite the net neutral charge of Ala-PG, this modification has been shown to enhance bacterial resistance to certain cationic antimicrobial peptides (10). Both Lys-PG and Ala-PG have also been shown to enhance the resistance of *S. aureus* and *P. aeruginosa* to several negatively charged β -lactams (*e.g.* oxacillin, methicillin, cefsulodin) (7, 10, 11). These latter findings suggest that aaPGs confer antibiotic resistance both by diminishing the net negative charge of the membrane and by modulating more general biophysical properties such as membrane fluidity and permeability (12). In addition, aaPGs not only decrease membrane permeability to cationic antimicrobial peptides, but also to protons and osmolytes (*e.g.* lactate), providing bacteria with resistance to challenging osmotic or acidic growth conditions such as those encountered during fermentative growth (10, 13, 14).

Despite recent progress, many questions remain unanswered concerning PG aminoacylation in bacteria. Since the identification of Lys-PGS and Ala-PGS and the discovery of resistances associated with these enzymes the amino acid specificities of only seven aaPGSs have been directly characterized (2, 5, 9, 10, 13, 15–18). Predicted aaPGS sequences from different organisms display a remarkable level of secondary structural diversity. To date, 348 putative aaPGS genes in 213 distinct species have been identified in 96 different genera of microorganisms,

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² The abbreviations used are: aaPGS, aminoacylphosphatidylglycerol synthase; aa-tRNA, aminoacyl-tRNA; Ala-RS, alanyl-tRNA synthetase; Arg-RS, arginyl-tRNA synthetase; Lys-RS, lysyl-tRNA synthetase; PG, phosphatidylglycerol.

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covering almost all known groups of bacteria. Also, 49 of the available genome sequences encode two or more aaPGS paralogs. This dual occurrence is most often encountered in Gram-positive bacteria, particularly in members of the Actinobacteria class (e.g. *Mycobacterium*, *Streptomyces*, etc.). Here, we describe structure/function-based assignment of aaPGS catalytic domains that subsequently allowed us to identify new amino acid specificities of PG aminoacylation. The coexistence of several such activities in the same organism expands the set of unique aaPGSs that are available for tuning of membrane properties and may provide these bacteria with resistance to a far broader spectrum of antimicrobial agents and environmental conditions than previously appreciated. In addition, our work shows that although some aaPGSs display strict substrate specificity (e.g. Lys-PGS from *C. perfringens*), some aaPGSs display relaxed substrate specificity *in vitro*. For example, *Enterococcus faecium* aaPGS exhibits a triple specificity for Ala-, Arg-, and Lys-tRNA, and *Bacillus subtilis* displays a dual specificity for Lys- and Ala-tRNA.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and General Methods—The open reading frames encoding the aaPGSs from *E. faecium* DO (gi: 69245409 and 69249189) and *Agrobacterium tumefaciens* (gi: 15889786) were amplified by PCR and cloned under the control of the T7 promoter at the NcoI/XhoI restriction sites of the vector pet33b (*kan*^R; Novagen). The same vector was previously used for the cloning of the aaPGSs from *B. subtilis* and *C. perfringens* (9, 15). Recombinant vectors were used to transform the *Escherichia coli* strain C41 DE3 containing the plasmid pRARE2 (*cam*^R; Novagen) expressing tRNAs for translation of rare codons. Protein production was performed according to procedures described before (15) in autoinduction medium incubated overnight at 30 °C under agitation. To remove the membrane domain of aaPGSs partially or totally, site-directed mutagenesis was performed with the QuikChange kit (Stratagene) according to the manufacturer's instructions. Affinity-tagged lysyl-tRNA synthetase (Lys-RS) and alanyl-tRNA synthetase (Ala-RS) from *E. coli* and arginyl-tRNA synthetase (Arg-RS) from jack bean were produced and purified according to procedures described previously (19–21).

Membrane Extracts and Lipid Analysis—Membrane suspensions from aaPGS-expressing strains were prepared by successive centrifugation steps. Briefly, the cells were disrupted by sonication in a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM diisopropylfluorophosphate, 1 mM phenylmethylsulfonyl fluoride, 3 mM 2-mercaptoethanol. Cell debris were removed by centrifugation for 15 min at 10,000 × *g*. Membranes were sedimented from the supernatant by ultracentrifugation for 45 min at 250,000 × *g*. Membrane suspension was obtained after gentle sonication of the pellet and was then stored at –80 °C in the lysis buffer containing 20% glycerol. The concentration of protein in the membrane suspension was 15–20 mg/ml, as measured by the method of Bradford (Bio-Rad). Total lipids were extracted from cells using the procedure of Bligh and Dyer (22) with 120 mM potassium acetate, pH 4.5, in the aqueous phase. Lipids were analyzed by TLC on Silica Gel HL plates (Analtech) developed in one dimension or two dimensions by using, suc-

cessively, the solvents chloroform:methanol:water (14:6:1, vol/vol/vol) in the first dimension and chloroform:methanol:acetic acid (13:5:2, vol/vol/vol) in the second dimension. Lipids were visualized by fluorescence after staining with primuline.

tRNA-dependent AaPG Synthesis—aaPGS activity within the membrane suspensions obtained from aaPGS-producing strains was assayed in a reaction medium containing 100 mM Hepes-NaOH, pH 7.2, 30 mM KCl, and 15 mM MgCl₂, 8 mM ATP, 2 mg/ml total tRNA from *E. coli* MRE 600 (Roche Applied Science), 20 μM ¹⁴C-labeled amino acids, 1 μM appropriate aminoacyl-tRNA synthetase, 3.25 mg/ml membrane extract, and a 2 mg/ml suspension of egg-PG (Avanti) prepared as described. After 30 min of incubation at 37 °C, lipids were extracted by the Bligh and Dyer procedure (22), dried, and resuspended in 20 μl of a solution of chloroform:methanol (2:1). 2 μl of the extract was then subjected to analysis by TLC (see above). Transcripts of tRNA^{Arg} from jack bean and tRNA^{Lys} UUU from *E. faecium* were synthesized as previously described, and used in the aaPG biosynthesis assay in the presence of *E. coli* Lys-RS or jack bean Arg-RS, respectively.

Alignment and Phylogenetic Analysis—Sequences were retrieved by Blast (23) using the Lys-PGS from *B. subtilis* as a template. 437 aaPGS sequences displaying an E value < 10E-6 were aligned using ClustalX 2.0 (24). The alignment was split according to the three different domains present in aaPGSs (membrane, hydrophilic, and Lys-RS domains), and the sequences were independently realigned. Phylogenetic analyses were conducted with the Phylip package 3.68 with a representative subset of 230 sequences displaying <95% identity. The tree was constructed from a bootstrap of 100 replicates of a degapped alignment of 218 residues using the neighbor-joining method. A consensus tree was edited with iTOL (25). Prediction of transmembrane helices in aaPGSs was performed using TMHMM (26) and TOPCONS (27).

RESULTS

***E. faecium* Possesses a Dual-specificity Arg/Lys-PGS**—Previous reports describe the presence of several amino acids associated with phosphatidylglycerol in the membrane of *E. faecium* (formerly *Streptococcus faecalis*) (28). These studies showed that Lys-PG is present, along with significant amounts of Arg-PG, in the membrane of this organism, suggesting the possible presence of two distinct aaPGSs responsible for the biosynthesis of these two aaPGs. The presence of two aaPGS paralogs responsible for the biosynthesis of two distinct aaPGs (Lys-PG and Ala-PG) has recently been reported in *C. perfringens* (9). Using *B. subtilis* Lys-PGS as a guide, BLAST search analyses of the publicly available genome sequences of *E. faecium* (*E. faecium* DO, TX1330, 1141733, 1231501, Com12, and Com15) revealed the presence of two aaPGS paralogs encoded in all six organisms. Both open reading frames (aaPGS1 and aaPGS2, accession numbers ZP_00603404 and ZP_00604896, respectively) from *E. faecium* DO were cloned in pet33b and expressed in *E. coli*. The aaPGS activities of membrane extracts from the producing strains were tested with Lys-tRNA^{Lys} and Arg-tRNA^{Arg} as aminoacyl group donors. This analysis failed to reconstitute the activity of aaPGS1 but showed unambiguously the dual aa-tRNA specificity (Lys/Arg) of aaPGS2 (Fig. 1).

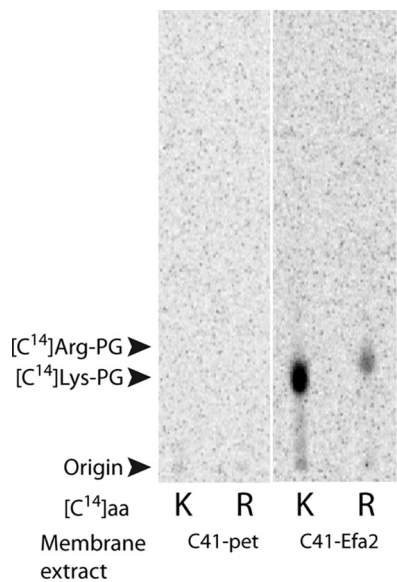


FIGURE 1. **Activity of aaPGS2 from *E. faecium*.** AaPGS activities in the membrane fraction of the *E. coli* strain C41 transformed with the vector pet33b empty (C41-pet) or producing aaPGS2 from *E. faecium* (C41-Efa2) were assayed as described under *Experimental Procedures* in the presence of [^{14}C]Lys (K) or [^{14}C]Arg (R).

These results suggest that the presence of Arg-PG and Lys-PG in the membrane of *E. faecium* may be due to a single aaPGS that bears two aa-tRNA specificities. Further *in vitro* characterization of these activities with tRNA transcripts (*i.e.* tRNA^{Lys} UUU from *E. faecium* and tRNA^{Arg} from jack bean) revealed that aaPGS2 displays a specific activity for lysylation of PG only 6.5-fold higher than that for arginylation. This result prompted us to reinvestigate the specificities of known aaPGSs using an expanded range of aa-tRNAs and to determine the specificity of a previously uncharacterized aaPGS.

AaPGSs Display Divergent Specificity for Aa-tRNAs *in Vitro*—The specificities of the Lys-PGS from *B. subtilis* and of the Ala-PGS from *C. perfringens* have been determined previously (9, 18) and were now reinvestigated using Lys-, Arg- and Ala-tRNA as substrates. An aaPGS homologous to Lys-PGS has also been reported in the Rhizobiales (*i.e.* *Sinorhizobium medicae*) for which it conferred resistance to lethal acidic conditions (14). However, the function of aaPGS in Rhizobiales remained unclear because no Lys-PG was detected (14). These previous results prompted us also to characterize the substrate specificity of a Rhizobiales aaPGS, in this case from the plant pathogen *A. tumefaciens*. All aaPGSs tested displayed activity *in vivo* (Fig. 2) and a moderate specificity toward their aa-tRNAs. For example, the *B. subtilis* enzyme, previously reported as a Lys-PGS, was also able to synthesize Ala-PG, but Arg-PG was not detected in this case (Fig. 2B). *A. tumefaciens* aaPGS synthesized Lys-PG, but no trace amounts of either Arg-PG or Ala-PG could be detected when this enzyme was assayed with Ala-tRNA^{Ala} and Arg-tRNA^{Arg} (Fig. 2C). Although the *B. subtilis* aaPGS displayed relaxed substrate specificity, Ala-PGS from *C. perfringens* exhibited a more stringent specificity for Ala-tRNA^{Ala} because neither Lys-PG nor Arg-PG was synthesized by this enzyme (Fig. 2D). Reexamination of *E. faecalis* aaPGS2 revealed that it was able to make Ala-PG in addition to Arg-PG

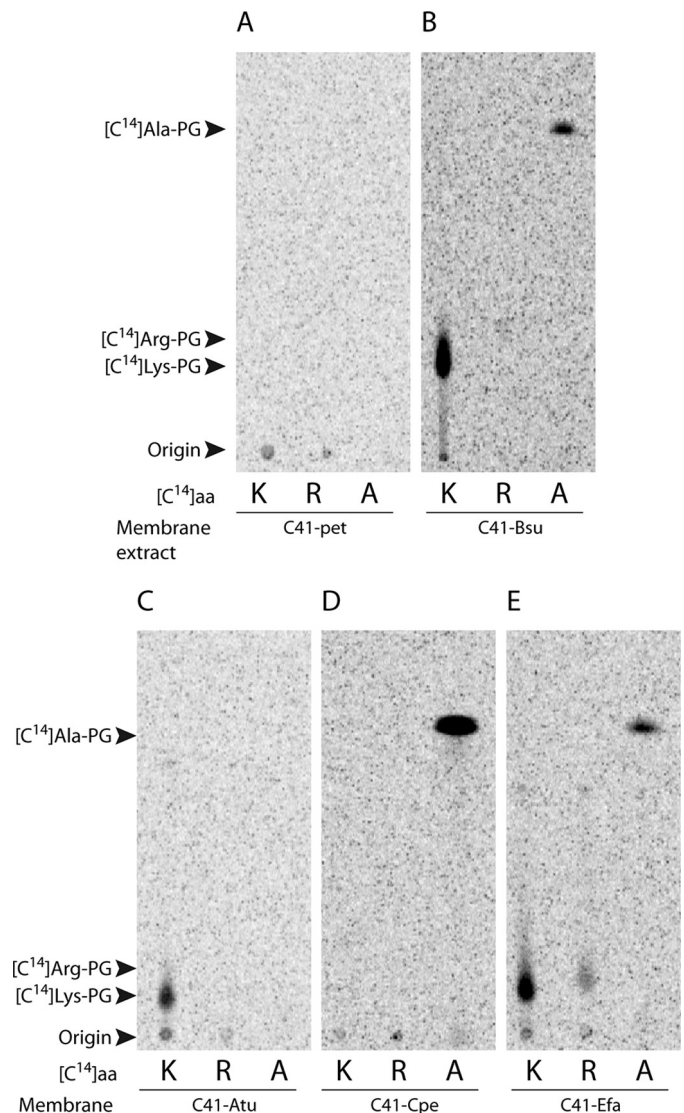


FIGURE 2. **Specificity of aaPGSs from various origins.** Membrane extracts from the *E. coli* strain C41 containing the empty vector pet33b (C41-pet; A) or producing the aaPGS from *B. subtilis* (C41-bsu; B), *A. tumefaciens* (C41-Atu; C), *C. perfringens* (C41-Cpe; D), or *E. faecium* (C41-Efa2; E). Activities were assayed as described under *Experimental Procedures* in the presence of [^{14}C]Lys (K), [^{14}C]Arg (R), or [^{14}C]Ala (A).

and Lys-PG (Fig. 2E). Our findings show that known aaPGSs fall into three categories according to their specificities: (i) aaPGSs that display a single, strict, specificity (*e.g.* Ala-PGS from *C. perfringens* and Lys-PGS from *A. tumefaciens*) and produce no detectable traces of other aaPGs; (ii) aaPGSs with a “primary” specificity (*i.e.* Lys-PGS from *B. subtilis*) that are also capable of producing several other aaPGs less efficiently; and (iii) aaPGS with relaxed specificity (Ala/Arg/Lys-PGS from *E. faecium*) able to synthesize several aaPGs at comparable levels.

AaPGS Structural Heterogeneity—To correlate the divergent specificity of aaPGSs with structural features, 230 representative sequences from 204 organisms were aligned and compared (Fig. 3). AaPGSs are predicted to be composed of a membrane-inserted amino terminus appended to a hydrophilic carboxyl terminus, but the precise role of each of these domains is unknown. The membrane domain varies in size and in

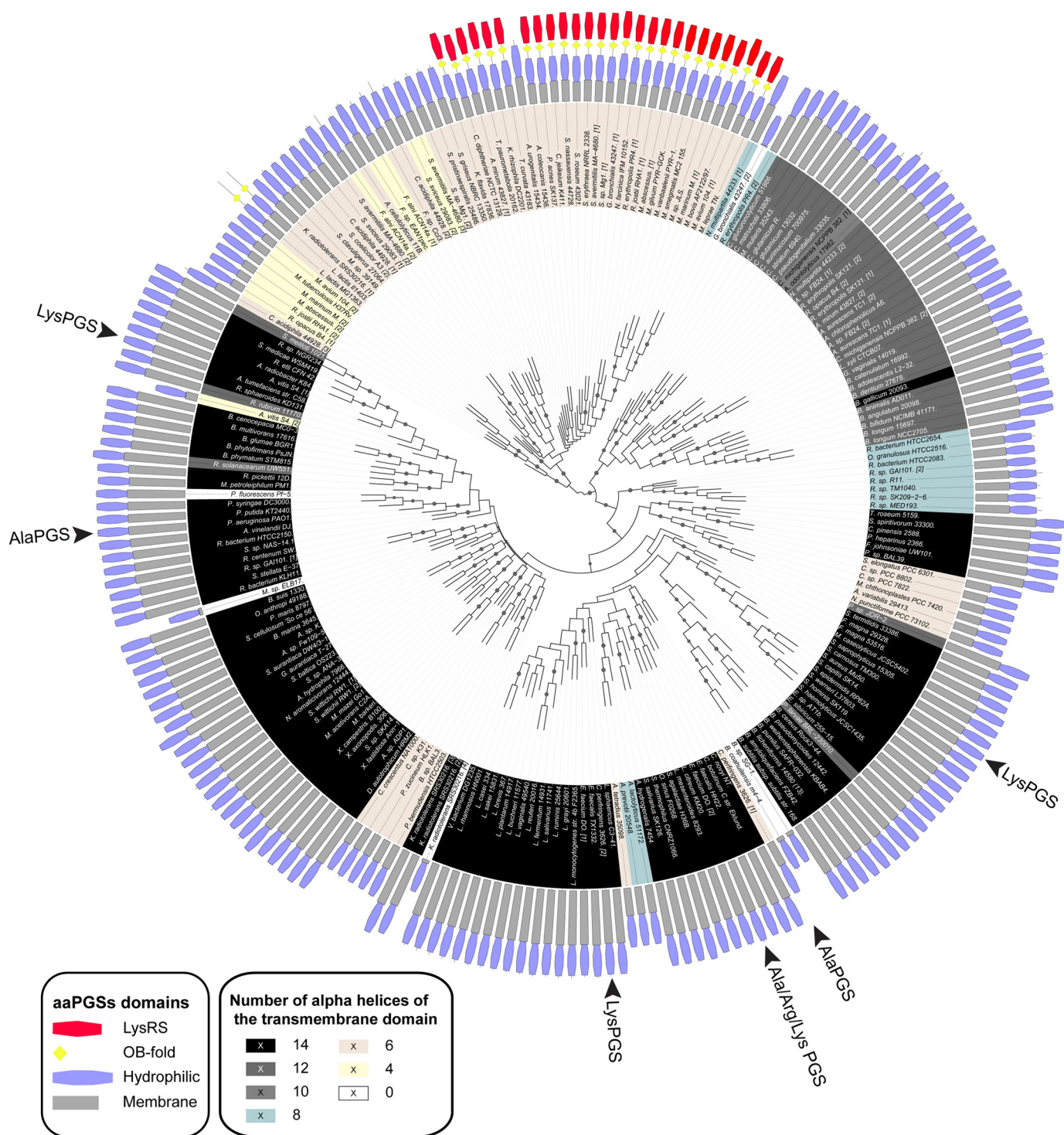


FIGURE 3. **Phylogeny and modular organization of aaPGSs.** Aa-tRNA specificity of the aaPGSs that have been characterized *in vitro* is indicated (black arrow). The modular organization and number of predicted helices within the integral membrane domain of each sequence, respectively, are reported beside each named organism and by a colored background. Branches displaying a bootstrap support >80% are indicated by gray circles. The number of aaPGS paralogs within a given organism is indicated in brackets.

sequence and is even absent from aaPGS homologs in a few cases (e.g. *Streptomyces*, *Burkholderia*). Based on the predicted secondary structure, the membrane-inserted domain is thought to consist of a variable number of α -helices (4 to 14) that orient the amino-terminal extremity and direct the hydrophilic carboxyl-terminal moiety toward the cytoplasm where aa-tRNAs, the substrate of aaPGSs, are synthesized. Biochemi-

cally characterized aaPGSs possess a membrane domain containing 14 predicted α -helices, except for the Ala-PGS from *C. perfringens* that contains only six helices. More distant aaPGS, such as the paralogs found in actinomycetes, are fused with a Lys-RS domain that displays 40% identity with *E. coli* Lys-RS. This domain exhibits all of the features associated with tRNA lysylation such as a tRNA anticodon binding domain (OB-fold)

along with all the conserved positions in Lys-RSs necessary for substrate binding (Lys, ATP, and tRNA) and enzyme dimerization (data not shown). The presence of this domain in aaPGS is probably involved in the channeling of the Lys-tRNA toward the membrane modification site. However, these enzymes possess a notably shorter membrane domain, containing six transmembrane α -helices. These secondary structural comparisons suggest that the aaPGS active site is likely located within the hydrophilic carboxyl-terminal moiety of the polypeptide.

AaPGSs Contain a Catalytic Carboxyl-terminal Domain—To determine whether the membrane-inserted domain of aaPGS plays a role in catalysis, the amino termini of the *C. perfringens* Ala-PGS and of the *B. subtilis* Lys-PGS were truncated to leave either one or none of the hydrophobic helices intact. It is worth noting that these predicted membrane domains are quite divergent: *B. subtilis* Lys-PGS contains 14 helices whereas *C. perfringens* Ala-PGS contains only 6. The activity of the different constructs was then investigated *in vivo* and *in vitro*. Although the presence of a single helix resulted in little or no activity *in vitro*, removal of the entire membrane domain led to variants with significant activity, indicating that aaPG synthesis is catalyzed by the hydrophilic amino terminus alone (Fig. 4). In contrast, only the full-length aaPGSs, but none of the truncated variants, displayed aaPG synthetic activity *in vivo* (Fig. 5).

DISCUSSION

Distinct Roles of the AaPGS Amino- and Carboxyl-terminal Domains—Recent studies of aaPGSs, particularly in the context of drug-resistant *mprF* alleles, provided preliminary indications of the possible roles of different domains of the protein. For example, loss of daptomycin susceptibility allowed the tentative assignment of lysyl-PG translocase (flippase) activity to the N-terminal eight transmembrane helices of *S. aureus* Lys-PGS (29, 30). It was also proposed that the synthase domain consisted of six transmembrane helices appended to the carboxyl terminus. Our data now show that the carboxyl terminus alone is sufficient for full synthase activity *in vitro*. The absence of activity *in vivo* for the carboxyl-terminal domain alone suggests that transmembrane helices position the catalytic domain in the cytosol during aaPG synthesis. The dual role of the membrane-inserted amino terminus, both as a translocase and for anchoring the catalytic moiety, is consistent with the high degree of heterogeneity observed in this region compared with the more conserved carboxyl terminus (Fig. 3). AaPGSs are encoded in many phylogenetically distant bacteria, and divergence in the membrane-inserted domain may reflect adaptation to a wide range of cell wall morphologies and lipid compositions. The carboxyl-terminal catalytic domain instead recognizes molecules that show relatively little phylogenetic variation, aa-tRNAs, consistent with the higher degree of conservation seen within this domain across different species.

Aa-tRNA Selection by AaPGS—Pioneering studies in *S. aureus* first identified lysyl-tRNA-dependent PG modification by Lys-PGS as a means to increase the net positive surface charge of bacteria (2, 31). More recently, tRNA-dependent Ala-PG synthesis was also demonstrated (9), thereby establishing that the aaPGS family does not exclusively catalyze lipid

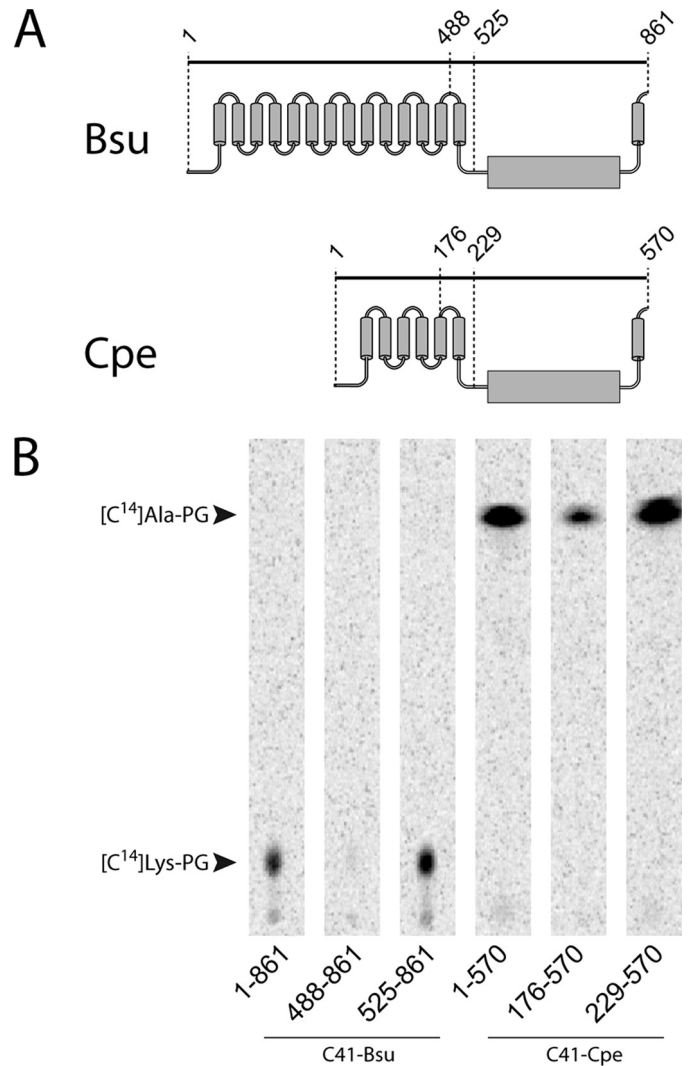


FIGURE 4. Activity of truncated aaPGSs. A, schematic representation of the *B. subtilis* (Bsu) and *C. perfringens* (Cpe) aaPGSs. Truncation within the amino-terminal membrane domain of both proteins is indicated. The membrane domains of *B. subtilis* and *C. perfringens* aaPGSs contain 14 and 6 α -helices, respectively (as predicted by TOPCONS). B, aaPGS activity of full-length and truncated proteins. Membrane extracts were tested as described under *Experimental Procedures* with the corresponding ^{14}C -labeled aa-tRNA in a medium containing ^{14}C -labeled amino acids, ATP, total tRNA from *E. coli*, and the cognate aa-RS.

lysylation. Both Arg and ornithine would be expected to have an impact on the charge of PG comparable with Lys, and both Arg-PG and ornithyl-PG have previously been identified in bacteria (for review, see 32). Although tRNA-dependent ornithyl-PG synthesis could not be detected,³ Arg-PG and Ala-PG synthase activities were readily observed for some Lys-PGS tested. The triple specific Ala/Arg/Lys-PGS from *E. faecium* displayed the broadest specificity because this enzyme was also able to synthesize Ala-PG at an efficiency comparable with Arg- or Lys-PG. These data suggest that aaPGSs capable of transferring bulkier amino acids such as Lys or Arg may display a moderate specificity compared with those that utilize smaller amino acids such as Ala. These observations imply that aaPGSs adapted to bind smaller amino acids (*i.e.* Ala) cannot accom-

³ H. Roy and M. Ibba, unpublished results.

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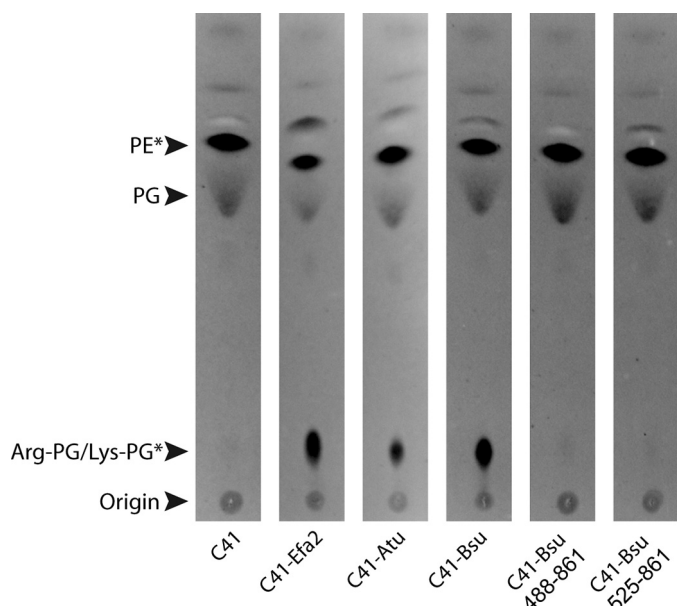


FIGURE 5. Biosynthesis of aaPG- in aaPGS-expressing strains. Full-length aaPGS2 from *E. faecium* (C41-Efa2), *A. tumefaciens* (C41-Atu), *B. subtilis* (C41-Bsu), and the truncated aaPGS from *B. subtilis* encompassing the residues 488–861 or 525–861 were expressed in the *E. coli* strain C41. Total lipids were extracted, separated on TLC, and revealed by primuline staining. Amino group-containing lipids were visualized by ninhydrine staining (*).

moderate bulkier substrates such as Lys or Arg. Conversely, those aaPGSs that preferentially utilize Lys or Arg as substrates can sometimes accommodate smaller amino acids such as Ala. A refined model for the molecular basis of amino acid recognition and discrimination by aaPGSs now requires detailed structural studies, but it is noteworthy that our initial findings are reminiscent of size-based amino acid discrimination strategies described for some aa-RS proteins (33, 34).

Functional Implications of Relaxed Substrate Specificity—It was shown recently that Ala-PG and Lys-PG impact cellular physiology in many similar ways, suggesting that the primary role of aaPGS is to neutralize the net negative charge of PG rather than place a particular aminoacyl group in the membrane (1, 10). In this respect, aaPGSs differ from other tRNA-dependent cell wall synthesis and remodeling enzymes that supply specific amino acids for processes such as peptidoglycan biosynthesis (35, 36). This functional difference between aaPGSs and related enzymes is consistent with the expanded range of aaPG synthesis described here. The relaxed specificity of aaPGSs allows cells to modulate their net charge using a wider range of substrates than previously anticipated. This, in turn, provides a means by which a single pathway can be used to adapt to environmental conditions under which different amino acids may become limiting at various points in the life cycle of the cell. The ability to adapt to particular environments may be facilitated further by regulation of aaPGSs of differing specificities in a single organism, such as in *C. perfringens* that encodes both an Ala-PGS and a Lys-PGS (9). Future studies will focus on understanding in more detail how the full range of aaPGS activities alters cellular physiology, in particular with respect to determining the roles of aaPGs in defining a wide spectrum of antibiotic resistances.

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