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tRNA-dependent alanylation of diacylglycerol and phosphatidylglycerol in *Corynebacterium glutamicum*

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Summary

Aminoacyl-phosphatidylglycerol synthases (aaPGSs) are membrane proteins that utilize aminoacylated tRNAs to modify membrane lipids with amino acids. Aminoacylation of membrane lipids alters the biochemical properties of the cytoplasmic membrane, and enables bacteria to adapt to changes in environmental conditions. aaPGSs utilize alanine, lysine, and arginine as modifying amino acids, and the primary lipid recipients have heretofore been defined as phosphatidylglycerol (PG) and cardiolipin. Here we identify a new pathway for lipid aminoacylation, conserved in many Actinobacteria, which results in formation of Ala-PG and a novel alanylated lipid, Ala-diacylglycerol (Ala-DAG). Ala-DAG formation in *Corynebacterium glutamicum* is dependent on the activity of an aaPGS homolog, while formation of Ala-PG requires the same enzyme acting in concert with a putative esterase encoded upstream. The presence of alanylated lipids is sufficient to enhance the bacterial fitness of *C. glutamicum* cultured in the presence of certain antimicrobial agents, and elucidation of this system expands the known repertoire of membrane lipids acting as substrates for amino acid modification in bacterial cells.

Keywords

phospholipids; multiple peptide resistance factor (MprF); Actinobacteria; diacylglycerol; phosphatidylglycerol; aminoacyl-tRNA synthetase; transfer RNA (tRNA); amino acid; lipids; membrane proteins

Introduction

Aminoacyl-phosphatidylglycerol synthases (aaPGSs) are integral membrane proteins responsible for the synthesis of aminoacyl-phosphatidylglycerol (aa-PG) in the cytoplasmic membranes of bacteria. The hydrophilic domain of aaPGS, located in the cytoplasm, bears

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the active site of the transferase activity and utilizes aminoacyl-tRNAs (aa-tRNAs) as amino acid (aa) donors, and phosphatidylglycerol (PG) as an aa acceptor (Peschel *et al.*, 2001). A separate domain, integral to the membrane, is required for translocation of newly formed aminoacylated phospholipids from the inner leaflet of the membrane to the outer leaflet (Ernst *et al.*, 2009, Ernst & Peschel, 2011, Slavetinsky *et al.*, 2013, Ernst *et al.*, 2015). Initially discovered in *Staphylococcus aureus*, the gene *mprF* encodes a LysPGS, which attaches lysine to PG, increasing the organism's virulence, as well as resistance to various classes of antibacterial agents (*i.e.*, cationic antimicrobial peptides (Peschel *et al.*, 2001), beta-lactams (Komatsuzawa *et al.*, 2001), glycopeptides, and lipopeptides (Hachmann *et al.*, 2009)). Investigation of aaPGS homologs in other species allowed for discovery of enzymes that exhibit different aa-tRNA specificities. For example, two aaPGS paralogs in *C. perfringens*, LysPGS and AlaPGS, are responsible for the synthesis of Lys-PG and Ala-PG, respectively (Roy & Ibba, 2008b). Investigation of an aaPGS homolog from *Enterococcus faecium* revealed a triple specific enzyme (*i.e.*, RakPGS) that enables formation of Lys-PG, Ala-PG and Arg-PG. The function of a second paralog in this species remains to be determined (Roy & Ibba, 2009). To date, the specificity of a total of 23 aaPGSs has been characterized either directly *in vitro*, or by analyzing aminoacylated lipids produced *in vivo* (Arendt *et al.*, 2012). Lys-tRNA^{Lys}, Ala-tRNA^{Ala}, and Arg-tRNA^{Arg} are currently the only known aa donors for aa-PG synthesis, while PG is the only known naturally occurring aa acceptor, with the exception of di-phosphatidylglycerol (*i.e.*, cardiolipin), which acts as an aa acceptor (in addition to PG) in *Listeria monocytogenes* (Thedieck *et al.*, 2006). Besides the different aa and lipid substrate specificities, some more distant aaPGS homologs exhibit functions that are quite different than those of traditional aaPGSs. One example is the protein VlmA that is found in various Actinobacteria, and which was the focus of a recent study carried out in *Streptomyces viridifaciens*. VlmA was found to be unable to aminoacylate any membrane lipids. Instead, this protein was shown to utilize Ser-tRNA^{Ser} for serine addition to an intermediate in the pathway for biosynthesis of the antibiotic valanimycin (Garg *et al.*, 2008). Thus, the aaPGS family of proteins is a heterogeneous family of tRNA-dependent transferases that exhibit diverse substrate specificities. Despite recent progress, no relationship between the structure and function of aaPGS homologs has been established, and it is not possible to predict the function of individual enzymes based solely on primary sequence (Arendt *et al.*, 2012). Moreover, it is not known whether the aaPGS family of proteins contains additional enzymes with functions that are distinct from those described to date.

A recent analysis to identify potential functional associations between *aaPGSs* and other genes revealed three conserved families of proteins encoded at *aaPGS* loci. Each of the three families, α/β-hydrolases (Pfam: PF12697), esterases (Pfam: PF00756), and VirJ proteins (Pfam: PF06057), represents a distinct class of putative hydrolytic enzymes distributed within specific bacterial phyla (Smith *et al.*, 2013). Enzymes belonging to the VirJ family of proteins are found exclusively in Gram-negative bacteria, whereas proteins from the α/β-hydrolases family are found in both Gram-positive and Gram-negative species. Proteins from the esterase group, on the other hand, are found mostly in Actinobacteria and Cyanobacteria. Two of these putative hydrolytic enzymes, AhyD from *E.* faecium and PA0919 from *P. aeruginosa*, representing the α/β-hydrolase class of enzymes and the VirJ

proteins, respectively, were recently the focus of independent functional studies (Smith *et al.*, 2013, Arendt *et al.*, 2013). Both of these proteins were shown to be aa-PG hydrolases that modulate overall levels of aa-PG in the bacterial membrane, and that are necessary for providing optimal resistance to antibiotics and other stress conditions. The function of proteins belonging to the third group of hydrolytic enzymes (*i.e.*, the esterases) has not yet been determined.

During the course of this study, we performed an analysis on aaPGS sequences and identified a new type of aaPGS homolog found exclusively in Actinobacteria. We studied the role of one representative protein (Cg1103) from *Corynebacterium glutamicum*, a Gram-positive, non-motile, soil dwelling bacterium, and showed that this enzyme displays a tRNA-dependent alanyl-diacylglycerol synthase (AlaDAGS) activity, which synthesizes Ala-DAG using Ala-tRNA^{Ala} as an aa donor and diacylglycerol (DAG) as an aa acceptor. Although Cg1103 was unable to synthesize Ala-PG *in vitro* or *in vivo*, we found that Ala-PG synthesis could be reconstituted by co-expression of Cg1103 with PesT, a conserved putative esterase (Cg1104) encoded upstream and adjacent to *cg1103*. These results suggest that the *cg1103*/*pesT* system generates two distinct alanylated lipids, expanding the known range of lipid components utilized for aa modification. Growth curve analysis demonstrated that expression of *cg1103*/*pesT* moderately enhances the fitness of *C. glutamicum* grown in the presence of lactic acid and the antimicrobial agents protamine sulfate and polymyxin B.

Results

aaPGS homologs consist of seven types of proteins

To determine whether aaPGSs can be categorized into distinct structural types, and whether, in light of recent functional studies, these structural types can be correlated to function, 257 representative sequences of aaPGSs were aligned and compared. The set of sequences used in this analysis originated from 174 bacterial species, each containing one or more aaPGS homolog. A phylogenetic analysis was performed on 160 degapped positions of the aminoacyl-transferase domain (in the C-terminus) of the proteins. Based on the topology of the resulting phylogenetic tree, aaPGS homologs can be delineated into seven main types of proteins (I–VII, with nodes exhibiting local support values >0.75; Fig. 1). To understand whether the seven types correlate to functions observed in aaPGS homologs, additional information such as substrate specificities of characterized enzymes, origin of the sequences, and information regarding the architecture of the proteins and loci, was added to the analysis. This method revealed that each of the aaPGS structural types are confined to specific bacterial phyla, suggesting that the dichotomy between types may reflect phylogenetic origin rather than functional differences. Type I and type III enzymes are found mostly in Proteobacteria, type II are found in Firmicutes, type IV and type VII are found in Actinobacteria, and type VI are found in Actinobacteria and some Proteobacteria.

Type I and II homologs include numerous aaPGSs that have been well characterized including LysPGSs, AlaPGSs, and the enterococcal triple specific RakPGS. The actinobacterial type IV homologs exhibit a putative membrane domain that is shorter than that of type I and type II proteins, while some type IV proteins exhibit an additional C-terminal domain containing a class II lysyl-tRNA synthetase (Fig. 1). Characterization of

one type IV homolog, the protein LysX from *Mycobacterium tuberculosis*, showed that this enzyme is responsible for synthesis of Lys-PG (Maloney *et al.*, 2009). Type VI homologs include the VlmA protein, which catalyzes a tRNA-dependent aa addition during biosynthesis of valanimycin (Garg *et al.*, 2008). This activity correlates with the lack of a membrane domain, suggesting that type VI proteins are probably not associated with the membrane. There is currently no evidence regarding the function of type III, V, and VII homologs. Type III and type V homologs, which are found primarily in Gram-negative species, tend to display shorter transmembrane domains than the other types. Type VII enzymes, on the other hand, display (like most type I and type II aaPGSs) a membrane domain composed of 14 predicted transmembrane helices.

Some bacterial species exhibit multiple aaPGS homologs with distinct functions (*i.e.*, paralogs). In general, paralogs arise through a gene duplication event and acquire distinct functions that are mechanistically related during the course of evolution (Koonin, 2005). To gain additional clues into the functional roles of aaPGSs, we considered the co-occurrence of multiple paralogs in individual species (Table 1 and Fig. S1). Multiple aaPGS paralogs are encountered most frequently in actinobacterial species (Fig. S1). For example, certain Actinobacteria contain up to five distinct aaPGS paralogs, whereas Firmicutes display a maximum of two paralogs in a given species. About 50% of the actinobacterial species included in our dataset displayed two or more aaPGS paralogs, whereas only 20% of the species belonging to other phyla exhibited two paralogs. Table 1 summarizes the co-occurrence of aaPGS paralogs in relation to the seven main types of aaPGSs. In some species, all the aaPGS paralogs belong to a single type (*e.g.*, both the AlaPGS and LysPGS from *C. perfringens* are type II enzymes). In other cases, multiple types of aaPGSs co-occur in a single species (*e.g.*, *Corynebacterium pseudotuberculosis* contains a type IV and a type VII homolog), suggesting that these types may exhibit distinct functionalities.

Our sequence analysis also included the three families of putative hydrolytic enzymes that are encoded within the aaPGS loci in many bacterial species (Smith *et al.*, 2013). While enzymes belonging to the α/β-hydrolase and VirJ families of proteins have been shown to be aa-PG hydrolases (Smith *et al.*, 2013, Arendt *et al.*, 2013), the function of proteins belonging to the esterase family (PF00756) is still unknown. To determine whether the putative esterases correlate with a certain type of aaPGS homolog, the distribution of these enzymes was considered. The putative esterases were found to be primarily associated with type VII and type IV aaPGS homologs. Based on the observations that type VII and type IV homologs sometimes co-occur in a single species (Fig. S1), and that some type IV homologs exhibit a LysPGS activity, we hypothesized that type VII paralogs might bear an alternate functionality. We selected the type VII enzyme Cg1103 from *C. glutamicum* (accession number CAF19673) for further analysis. The genomic context of the *cg1103* gene, along with the putative esterase *pesT*, is depicted in Fig. S2.

aaPGS from *C. glutamicum* aminoacylates a lipid other than PG

To determine whether type VII homologs exhibit aaPGS activity, the gene *cg1103* from *C. glutamicum* was cloned and expressed in *Escherichia coli*, which does not harbor an endogenous aaPGS activity. Cg1103 was expressed as a full-length protein, or as an N-

terminal 6xHis-tagged variant deprived of the membrane domain and encompassing only the last 350 residues of the C-terminus (residues 483–832). The membrane domains of several aaPGSs have been shown to be dispensable for reconstituting enzymatic activity *in vitro* (Roy & Ibba, 2008b), and so the latter construct ($Cg1103_{483–832}$) was included in our studies to facilitate expression of this large membrane protein. Indeed, we were able to isolate heterologously expressed $Cg1103_{483–832}$ as a soluble, pure protein after a single step of affinity chromatography. $Cg1103_{483–832}$ was tested for tRNA-dependent transfer of aa using [^{14}C]Lys-$tRNA^{Lys}$ or [^{14}C]Ala-$tRNA^{Ala}$ as aa donors, and commercial egg PG, or total lipids extracted from *C. glutamicum*, as aa acceptors. $Cg1103_{483–832}$ was unable to modify any of the lipid sources with lysine (data not shown). However, $Cg1103_{483–832}$ was capable of alanylation of an unknown lipid (UL) when *C. glutamicum* lipid extract was used as the aa acceptor (Fig. 2, lane 1). The alanylated lipid (Ala-UL) exhibited a chromatographic migration pattern different from that of an Ala-PG control, formed using the RakPGS from *E. faecium* and *C. glutamicum* lipids as aa acceptors (lane 5). Ala-UL was not observed when commercial PG was substituted as a lipid source (lane 2), or when tRNA or enzyme was eliminated from the reaction (lane 3, 4). Membrane extracts from the *E. coli* strain expressing full-length Cg1103 did not yield activity with any of the substrates tested, suggesting there may have been a problem with expression of this construct (data not shown).

To verify that synthesis of Ala-UL by $Cg1103_{483–832}$ was not due to lack of the membrane domain in the truncated protein, the full-length ORF encoding Cg1103 was cloned into the expression vector pEKEx2-GFP (yielding the construct pEKEx2-Cg1103), and expressed in *C. glutamicum*. Activity in a membrane extract isolated from this strain was tested using commercial PG, or lipids extracted from *C. glutamicum*, as potential aa acceptors. A single product with chromatographic mobility identical to that of the Ala-UL formed by the truncated enzyme was observed in reactions containing *C. glutamicum* lipid extract as aa acceptors (Fig. 2, lane 6). This product was not observed in reactions containing commercial PG as a lipid source (lane 7). These findings show that both $Cg1103_{483–832}$ and the full-length enzyme are able to utilize [^{14}C]Ala-$tRNA^{Ala}$ to form [^{14}C]Ala-UL (but not [^{14}C]Ala-PG) *in vitro.*

Identification of diacylglycerol as a substrate of Cg1103

For identification, UL was purified from a lipid extract, prepared from the wild-type strain of *C. glutamicum*, and characterized by mass spectrometry. Total lipids were extracted using the Bligh and Dyer procedure (Bligh & Dyer, 1959), and non-lipid contaminants were eliminated using the Folch method (Folch *et al.*, 1957). Lipid classes were fractionated by flash chromatography on silica gel to yield three fractions containing non-polar lipids (fraction F1), glycolipids (fraction F2), and phospholipids (fraction F3, Fig. 3A). The presence or absence of UL in each fraction was determined based on the enzymatic activity observed when $Cg1103_{483–832}$ was added to an aliquot from each fraction (data not shown). UL was only detected in fraction F1 containing the non-polar lipids. A second step of flash chromatography on silica gel was performed to further purify UL, yielding a single fraction containing UL at approximately 90% purity as estimated by TLC analysis (Fig. 3B, Fraction F6).

Figure 3C shows the ESI/TOF mass spectrum of fraction F6 containing UL. The main peak at a m/z ratio of 617.5128, and the isotopic distribution of the corresponding compound, are characteristic of diacylglycerol (DAG) substituted with palmitoyl and oleoyl fatty acid chains (16:0–18:1 DAG). The second most abundant compound present in the preparation exhibited a m/z value of 643.5272, corresponding to dioleyl-glycerol (18:1,18:1 DAG). The observation of these acyl chains is consistent with those that are found in several species of *Corynebacterium* (see (Crellin *et al.*, 2013) for review). To confirm that DAG is a substrate of Cg1103, we tested a commercial preparation of 16:0–18:1 DAG as a [^{14}C]-Ala acceptor. Alanylated DAG (Ala-DAG) was formed *in vitro* and displayed a chromatographic mobility identical to that of the Ala-UL formed when total lipids from *C. glutamicum* were used as aa acceptors (Fig. 3D). These findings demonstrate that Cg1103 exhibits an alanyl-diacylglycerol synthase (AlaDAGS) activity, which uses DAG and Ala-tRNA^{Ala} as substrates to form Ala-DAG.

Expression of Cg1103 in *C. glutamicum* produces Ala-DAG and Ala-PG

To verify whether Ala-DAG is formed by Cg1103 *in vivo*, *C. glutamicum* lipids from the wild-type strain, or from the strain harboring the expression plasmid pEKEx2-Cg1103, were analyzed by TLC. Total lipids were visualized using iodine vapor, and amine-containing lipids (ACL) were revealed by treatment with ninhydrin. No ACLs were detected in the wild-type strain, suggesting that aminoacylated lipids are not abundant in cells grown in the standard laboratory conditions used here. However, two ACLs were detected in the lipid preparation isolated from the strain expressing Cg1103 (Fig. 4A). One ACL (ACL1) exhibited mobility consistent with Ala-DAG, whereas the second one (ACL2) displayed a slower migration pattern similar to Ala-PG. Additional insight into the identities of ACL1 and ACL2 was gained using Dittmer dye (Dittmer & Lester, 1964), which confirmed that ACL2 contains phosphorous, while ACL1 does not (Fig. 4A, lane 4). To verify the identities of ACL1 and ACL2, both lipids were purified from a *C. glutamicum* total lipid extract isolated from cells expressing Cg1103. Lipid classes were fractionated by flash chromatography on silica gel as described above. Non-polar lipids and ACL1 were sequentially eluted with chloroform, and ACL2 was eluted, along with other phospholipids, using methanol (Figure S4A). Fractions containing each ACL were subjected to a second round of flash chromatography yielding ACL1 at > 90% purity (Fig. S4B, Fraction 3), and ACL2 mixed with a small amount (~10%) of contaminating PG (Fig. S4C, Fraction 3). Both lipids were characterized by ESI/TOF and ESI/MS/MS. Exact mass, isotopic distribution (Fig. S5), and fragmentation patterns of the major peaks confirmed the identities of ACL1 and ACL2 as being Ala-DAG and Ala-PG, respectively (Fig. 4B and 4C). Acyl moieties observed in the purified Ala-DAG and Ala-PG (*i.e.*, oleoyl and palmitoyl) were the same as those found in the non-alanylated DAG characterized above. These findings demonstrate that expression of Cg1103 in wild-type *C. glutamicum* yields synthesis of Ala-DAG and Ala-PG *in vivo*. These findings only partially corroborate the *in vitro* results, which showed that Cg1103 is able to alanylate DAG, but not PG.

Ala-PG synthesis is dependent on a putative esterase encoded upstream of *cg1103*

The ORFs encoding the putative esterase *pesT* (accession CAF19674) and *cg1103* are separated by only 2 nucleotides, and potentially constitute a single operon. PesT is a 426 aa

protein, belonging to a family of putative hydrolytic enzymes (Pfam: PF00756) that are frequently encoded at the same loci as type VII aaPGS homologs (Fig. 1). 32 putative esterase sequences, encoded in organisms that also harbor a type VII aaPGS homolog, were subjected to a membrane domain prediction analysis using the program TOPCONS. This analysis revealed that these proteins are made up of two domains. The N-terminal portion contains a membrane domain consisting of four transmembrane helices, and the C-terminal portion, which is more hydrophilic, contains a putative esterase signature exhibiting a conserved serine residue (Pfam: PF00756). In 26 of the proteins, the C-terminal domain of the protein was predicted to be localized to the outer side of the cytoplasmic membrane. These observations suggested that PesT might work in concert with Cg1103 to affect the modification of lipids in *C. glutamicum*.

To gain insight into the possible role of *pesT* in lipid aminoacylation, two constructs were cloned into the pEKEx2-GFP expression vector, and transformed into wild-type *C. glutamicum*. The first construct included the entire *pesT-cg1103* operon, and the second harbored the *pesT* ORF alone. Figure 5A shows that expression of PesT by itself did not induce synthesis of any ACLs *in vivo*, whereas expression of PesT and Cg1103 together resulted in synthesis of Ala-PG, but not Ala-DAG. These results, together with the observation that Cg1103 uses DAG, but not PG as a substrate *in vitro* (Fig. 2), led to the hypothesis that PesT is required for formation of Ala-PG. This hypothesis satisfies the initially confounding observation that expression of Cg1103 in wild-type *C. glutamicum*, which contains *pesT* on the chromosome, was capable of synthesizing both Ala-DAG and Ala-PG (Fig. 5A). To further test this hypothesis we constructed a *C. glutamicum* mutant strain in which the region encompassing *pesT* and *cg1103* was deleted (Δ*pesT*Δ*cg1103*). The Δ*pesT*Δ*cg1103* strain was transformed with the different pEKEx2 constructs harboring GFP (as a negative control), *cg1103*, or both *cg1103* and *pesT*. Similar to wild-type *C. glutamicum*, no lipid aminoacylation was measured in strain Δ*pesT*Δ*cg1103* transformed with either pEKEx2-GFP or pEKEx2-*pesT* (data not shown). However, expression of Cg1103 in the double knockout strain resulted in formation of Ala-DAG, while co-expression of Cg1103 and PesT yielded Ala-PG and, to a lesser extent, Ala-DAG (Fig. 5B). These results support the hypothesis that synthesis of Ala-PG is dependent on the presence of both *pesT* and *cg1103*, whereas synthesis of Ala-DAG is catalyzed by *cg1103* alone.

cg1103 confers a fitness advantage to *C. glutamicum* grown in certain conditions of stress

To date, synthesis of aa-PG in some organisms has been linked to an increase in the minimum inhibitory concentrations (MICs) of various drugs such as certain cationic antimicrobial peptides (CAMPs), lipopetides and glycopetides (*e.g.*, vancomycin and daptomycin), and to some osmolytes such as lactic acid (Klein *et al.*, 2009, Dare *et al.*, 2014) (for review see (Roy, 2009)). To determine whether *cg1103* or *pesT* provides *C. glutamicum* with resistance to some of these compounds, markerless deletion strains lacking the two genes were constructed, and the MICs for various CAMPs and other types of inhibitors were measured. No difference was observed between the MICs for wild-type *C. glutamicum* and the mutant strains, Δ*cg1103* and Δ*pesT*, when tested in the presence of nisin (20 μg ml^{-1}), bacitracin (0.5 μg ml^{-1}), polymyxin B (1.6 μg ml^{-1}), protamine sulfate (40 μg ml^{-1}), daptomycin (3.3 μg ml^{-1}), vancomycin (0.2 μg ml^{-1}), and lactic acid (0.2 % v/v).

To determine whether lipid alanylation affects *C. glutamicum* fitness (*i.e.*, the ability of the organism to adapt to conditions in its environment), growth of the wild-type strain was compared to that of the Δ*cg1103* and Δ*pesT* strains in the presence or absence of the compounds listed above. Growth was monitored continuously in BHI broth, and the maximum growth rate (μ_{max}), which is commonly used for evaluating bacterial fitness ((Walkiewicz *et al.*, 2012, Andersson & Hughes, 2010)), was calculated using the *grofit* R package (Kahm *et al.*, 2010). In the absence of drugs, growth of *C. glutamicum* was not affected by the loss of either *cg1103* or *pesT* in the knockout strains (Fig. 6). Among the compounds tested, only lactic acid, protamine sulfate, and polymyxin B had a markedly higher inhibitory effect on the Δ*cg1103* strain than on wild-type cells. Inhibition of growth of the Δ*cg1103* strain and wild-type *C. glutamicum*, in the presence of lactic acid, is shown as an example in Figure 6A, and the fitness of each strain (*i.e.*, the μ_{max}) is plotted relative to variable concentrations of each of the three compounds in Figure 6B. The fitness advantage of the wild-type strain over the Δ*cg1103* mutant was observed in a narrow range of concentrations for each compound. For example, upon addition of 0.13 % lactic acid, or 20 mg l^{-1} of protamine sulfate, the Δ*cg1103* cell growth rate was ~30% lower than that of the wild-type strain. At higher concentrations of each of these two compounds, no growth was recorded for the Δ*cg1103* mutant, whereas the wild-type strain exhibited some growth within 20 h. Likewise, for polymyxin B, the Δ*cg1103* strain was unable to thrive with drug concentrations at or above 1.3 mg l^{-1} while the wild-type strain continued to grow. The inhibited growth phenotype of the Δ*cg1103* strain was completely reverted back to wild-type levels in the complementation strain, Δ*cg1103-c*, in which the *cg1103* gene was replaced in the genome at the original locus (Fig. 6B).

While a difference was observed in the growth of the Δ*cg1103* strain (relative to wild-type) in the presence of the three compounds, no similar effect was observed with the Δ*pesT* strain (Fig. 6B). Both the wild-type strain and the Δ*pesT* strain grew equally well in the presence of all of the compounds tested. Altogether, these results suggest that Ala-DAG alone is able to confer a fitness advantage to *C. glutamicum* in the presence of certain inhibitors, and the physiological relevance of Ala-PG is not immediately apparent based on these experiments.

Discussion

An AlaDAGS and a putative esterase are responsible for synthesis of two alanylated lipids in *C. glutamicum*

aaPGSs are a family of enzymes capable of using various aa-tRNAs as aa donors for aminoacylation of phospholipids. The most common lipid that acts as an aa acceptor is PG, while di-PG (*i.e.*, cardiolipin) has also been reported to be an efficient substrate for LysPGS in *L. monocytogenes* (Thedieck *et al.*, 2006). Here we identify a novel pathway for membrane lipid aminoacylation. This pathway, which is present in various Actinobacteria species, involves an aaPGS homolog (Cg1103), and a putative esterase (PesT) that is encoded upstream. *In vitro* and *in vivo* data demonstrated that Cg1103 is able to alanylate the membrane lipid DAG, while synthesis of a second aminoacylated lipid, Ala-PG, was found to be dependent on co-expression of Cg1103 and PesT.

Additional work is required to clarify the precise role of PesT in this process. One possibility is that Cg1103 alone synthesizes Ala-DAG, but the presence of PesT modulates the specificity of Cg1103 to allow formation of Ala-PG. In this scenario, PesT may or may not exhibit an Ala-DAG hydrolytic activity. In an alternative model, Cg1103 synthesizes Ala-DAG, and PesT supports a transferase activity (instead of a hydrolytic one) that transfers the Ala born by DAG, to PG, to form Ala-PG. This latter scenario is supported by the predicted membrane topology of PesT, which places the location of the active site of the protein on the outer surface of the cytoplasmic membrane. The periplasmic localization of the active site of PesT would allow for synthesis of Ala-PG on the outer face of the membrane, using Ala-DAG as an aa donor, without depleting PG from the inner leaflet.

Although the putative esterase PesT exhibits a conserved Ser residue for potential catalysis of a hydrolytic reaction, this residue could instead be involved in an acyltransferase reaction. Esterases and acyltransferases are mechanistically and evolutionary related enzymes, which both frequently use a catalytic Ser in their active sites. Both types of enzymes use the catalytic residue to produce an acyl-enzyme intermediate, which is released by a second nucleophilic attack of either a water molecule (in the case of esterases), or an acceptor molecule (in the case of transferases). The distinct functions of the two proteins are due to subtle differences in the structures of their active sites that determine which substrate is used for the second nucleophilic attack. These differences cannot be detected based on sequence comparison alone (Jiang *et al.*, 2011). If PesT supports a transferase activity, DAG, which has the particularity of flipping spontaneously from one lipid leaflet to the other, would act as an Ala carrier for the synthesis of Ala-PG in the periplasm. Similar strategies, where functional groups are transferred from carrier lipids to acceptor groups in the periplasm, are used for synthesis of peptidoglycan, acylation of lipoproteins, and decoration of oligosaccharides and lipoteichoic acids (for review see (Zhang & Rock, 2008)).

Ala-DAG provides a fitness advantage to *C. glutamicum* and increases the range of aminoacylated components available for membrane remodeling

The observations presented above show that lipid alanylation confers a modest fitness advantage to *C. glutamicum* cultured in the presence of protamine sulfate or polymyxin B. This effect was dependent on *cg1103*, but not *pesT*, suggesting that Ala-DAG is sufficient to provide a fitness advantage against these compounds. Synthesis of aa-PG is a common strategy used by bacteria to reduce the net negative charge of the cytoplasmic membrane, thus decreasing its affinity for positively charged antimicrobial compounds (Peschel *et al.*, 2001, Klein *et al.*, 2009). Studies have shown that PG alanylation or lysylation is correlated in some species with resistance to protamine sulfate (Klein *et al.*, 2009, Samant *et al.*, 2009) and polymyxin B (Sohlenkamp *et al.*, 2007, Maloney *et al.*, 2009, Maloney *et al.*, 2011). Both of these compounds are CAMPs with a broad-spectrum antibiotic activity affecting both Gram-negative and Gram-positive species. Therefore, it is not surprising that we observe an Ala-DAG dependent increase in the fitness of *C. glutamicum* in the presence of these CAMPs, since Ala-DAG is expected to alter the net charge of the cellular membrane similarly to Ala-PG.

In *Pseudomonas aeruginosa*, Ala-PG formation confers enhanced resistance to lactic acid (Klein *et al.*, 2009). In the current study, we similarly observed an increase in the fitness of *C. glutamicum* cells grown in the presence of this compound. This phenotype was dependent on *cg1103*, but not *pesT*, suggesting that Ala-DAG alone is sufficient to enhance fitness. Lactic acid (in its protonated form) can cross the lipid bilayer by passive diffusion, without the aid of transport proteins (Finkelstein, 1976, Rubin *et al.*, 1982). PG aminoacylation has been shown to impact the biophysical properties of lipid bilayers by altering the molecular packing and interactions between lipid head groups, which in turn affects the passive diffusion of solutes through the membrane (Tocanne *et al.*, 1974, Haest *et al.*, 1972, Sacre *et al.*, 1977). For now, it is unclear whether similar alterations to the biophysical properties of the bacterial membrane, resulting from Ala-DAG synthesis, are responsible for the increased fitness of *C. glutamicum* grown in the presence of lactic acid.

The effects we observed on the fitness of *C. glutamicum* cells capable of producing alanylated lipids were modest. This may be due to low levels of Ala-PG and Ala-DAG present in the membranes of cells grown using the culture conditions described here. Indeed, we were unable to detect any amine-containing lipids (by TLC) in wild-type *C. glutamicum* cells grown in BHI, and the levels of these components in cells grown in the presence of inhibitory compounds was not determined. Different culture conditions may result in increased synthesis of alanylated lipids, which may significantly alter the associated resistance phenotypes.

A system producing two distinct aminoacylated lipids (*e.g.*, Ala-DAG and Ala-PG) may provide an advantage over a system utilizing only PG. For example, a dual system may confer an advantage, in peculiar metabolic conditions, during which PG may not be abundant (*e.g.*, (Zavaleta-Pastor *et al.*, 2010, Lopez-Lara *et al.*, 2003)). Some organisms are able to, during phosphorus limiting conditions, replace constitutive phospholipids in the membrane with phosphorus-free lipids. During this adaptation, liberated phosphate can then be re-routed for synthesis of essential molecules such as nucleic acids. Ala-DAG and Ala-PG may also differentially affect the basic biophysical properties of the cell wall, and be expressed in variable amounts (even when conditions are not limiting) to best suit a given cellular environment. Since no alanylated lipids were detected in *C. glutamicum* cells cultured in rich media, it is apparent that they do not always constitute major components of the cytoplasmic membrane, but may be produced by a system that is induced only in peculiar metabolic or stress conditions. Investigations into the regulatory networks controlling levels of alanylated lipids in *C. glutamicum* will provide much needed insight into the role of this system in general membrane homeostasis, as well as bacterial fitness and adaptability.

Experimental Procedures

Bacterial strains and growth conditions

C. glutamicum (ATCC 13032) was obtained from the American Type Culture Collection, and grown in BHI broth at 30 °C with aeration by shaking. Alternatively, cells were grown on plates of the same medium containing 1.5% (w/v) agar and supplemented with 25 mg l^{-1}

of kanamycin, as needed. Electrocompetent cells of *C. glutamicum* were prepared and used according to established methods (Ming *et al.*, 2010).

Cloning and gene expression

Bacterial strains, plasmids, and oligonucleotide sequences are reported in Supplementary Tables S1 and S2. The DNA sequence encoding amino acids 483–832 of Cg1103 (accession number CAF19673) from *C. glutamicum* was amplified by PCR (using oligonucleotides 998 and 999), and cloned between the NdeI and XhoI restriction sites of a modified version of the pET-28a vector (*kan*^{R}, Novagen), in which the thrombin cleavage site was replaced with an HRV 3C cleavage site. The construct was over-expressed in *E. coli* strain C41 as an N-terminally His-tagged protein using an autoinduction medium as described (Roy & Ibba, 2008a). Cg1103 (483–832) was purified by cobalt-affinity chromatography according to standard procedures. After dialysis and concentration, the protein was stored at a concentration of 17 mg ml^{-1} in a buffer containing 50 mM Tris•HCl, pH 8.1, 100 mM NaCl, 2 mM β-mercaptoethanol (β-ME), and 50% glycerol. For gene expression in *C. glutamicum*, the shuttle plasmid pEKEx2-GFP (*kan*^{R}), for expression of GFP in *C. glutamicum* (Lausberg *et al.*, 2012), was used. The vectors pEKEx2-Cg1103 and pEKEx2-PesT-Cg1103 were obtained by substituting the fragment between the PstI and SalI restriction sites (encoding GFP in pEKEx2-GFP) by the PCR fragments encoding either *cg1103* (amplified with oligonucleotides 1004 and 978), or *cg1103* and *pesT* (amplified with oligonucleotides 1000 and 978). A plasmid expressing *pesT* (pEKEx2-PesT) alone was obtained by inactivating *cg1103* in the pEKEx-2-PesT-Cg1103 construct by removing the EcoRI fragment encoding the 258 aa of the C-terminal end of Cg1103. Constructs were electrotransformed into *C. glutamicum* as described (Ming *et al.*, 2010), and gene expression was achieved by incubation overnight in liquid BHI. Membrane extracts from recombinant *C. glutamicum* and *E. coli* strains were prepared as described previously (Roy & Ibba, 2009).

Markerless deletion of *cg1103* and *pesT* from *C. glutamicum*

Markerless inactivation of *C. glutamicum* genes was achieved using allelic exchange by homologous recombination in a classic two step approach with the vector pK19mobsacB (Schäfer *et al.*, 1994). Briefly, the upstream and downstream regions (1 kb each) flanking the targeted genes were PCR amplified using *C. glutamicum* ATCC13032 genomic DNA as template. Resulting PCR products were stitched together by overlap extension during PCR using oligonucleotides as indicated in Figure S2 and Table S2. The resulting cassettes, containing the upstream and downstream regions fused together, were cloned into the suicide vector pK19mobsacB, following digestion with the restriction enzymes HindIII and XbaI. Thus, *pesT*, *cg1103*, or both genes were inactivated using the vectors pK19mobsacB-Δ*pesT*, pK19mobsacB-Δ*cg1103*, *or* pK19mobsacB-Δ*cg1103*Δ*pesT*, respectively. The genetic environments, upstream and downstream regions, and location of oligonucleotides are indicated in Fig. S2. Plasmid constructs were transformed into *C. glutamicum* by electroporation as described (Ming *et al.*, 2010), and cells were plated on BHI supplemented with 9% (w/v) sorbitol and kanamycin (25 mg l^{-1}). Strains containing plasmid integrated at the targeted loci (pop-in) were subjected to one passage in BHI broth supplemented with kanamycin, and two passages without antibiotic, before being plated on BHI containing 10% (w/v) sucrose. Sucrose-resistant and kanamycin-sensitive clones, having lost the

pK19mobsacB constructs (pop-out), were identified by replica plating on BHI alone and BHI supplemented with kanamycin. Positive clones were subjected to PCR screening and sequence analysis to verify removal of the targeted genes.

Extraction, fractionation, and purification of lipids from *C. glutamicum*

For analytical purposes, lipids from as little as 10 OD of *C. glutamicum* cells (*i.e.*, 2ml of culture exhibiting an OD=5) were prepared and analyzed by TLC using the Bligh-Dyer procedure (Bligh & Dyer, 1959) with modifications as described (Smith *et al.*, 2013). TLC solvent systems consisted of chloroform:methanol:water (14:6:1, solvent A), and hexane:diethylether:acetic acid (7:3:0.2, solvent B). Extraction and purification of DAG, Ala-DAG, and Ala-PG (*i.e.*, also referred to as UL, ACL1, and ACL2, respectively) for mass spectrometry characterization was performed in glassware as follows. Cells from an overnight culture grown in 1 l of BHI (OD~10) were washed in a buffer containing 30 mM Tris•HCl and 3 mM β-ME, followed by resuspension in 40 ml of the same buffer and disruption by sonication. Lipids were extracted according to the Bligh-Dyer procedure (Bligh & Dyer, 1959) by addition of 100 ml of methanol and 50 ml of chloroform. After 1 h of extraction under vigorous agitation, phase separation was carried out by addition of 50 ml of chloroform and 50 ml of water. Lipids in the organic phase were dried using a rotavapor, and hydrosoluble contaminants were removed using the Folch procedure (Folch *et al.*, 1957) by addition of 15 ml of chloroform:methanol:water (8:4:3). Lipids in the organic phase were dried again and resuspended in 1 ml of chloroform. Lipid classes were separated by flash chromatography on 10 ml of silica gel (mesh 220–440, Alfa Aesar) under nitrogen gas as described previously (Christie & Han, 2010). Non-polar and slightly polar lipids were eluted with chloroform. Specifically, DAG was eluted with 40 ml of chloroform, and Ala-DAG was eluted with an additional 80 ml of chloroform. After two successive wash steps (with 40 ml of chloroform followed by 50 ml of acetone) Ala-PG and other phospholipids were eluted using 50 ml of methanol collected in 2 ml fractions (Fig. S4A).

Fractions containing DAG were identified using the tRNA-dependent lipid aminoacylation assay (see below), and those containing Ala-PG and Ala-DAG were revealed by ninhydrine treatment of an aliquot subjected to TLC. A second step of flash chromatography on silica gel was used to remove contaminants. Fractions containing lipids of interest from the first chromatography step were dried and resuspended in 1 ml of the solvent used to equilibrate the matrix in the second column. DAG was loaded onto 6 ml of matrix equilibrated with hexane:diethylether:acetic acid (7:3:0.2) and was eluted in 6 ml fractions by application of the same solvent. Ala-DAG was loaded onto 4.5 ml of silica gel equilibrated with diethylether:acetic acid (10:0.2). After washing with 6 volumes of the same solvent, Ala-DAG was eluted with solvent A (Fig. S4B). Ala-PG was loaded onto 4.5 ml of matrix equilibrated with chloroform:methanol:water (14:3:0.2), and eluted with the same solvent (Fig. S4C).

Lipid analysis using mass spectrometry

Lipid fractions were dried and resuspended in acidified methanol (0.1% formic acid). Mass spectrometry analyses were performed at the University of Florida Department of Chemistry Mass Spectrometry Services (Gainesville, FL). Accurate mass experiments were conducted

using an Agilent 6220 electrospray ionization-time of flight (ESI/TOF) mass spectrometer equipped with an electrospray DART source. ESI/MS/MS experiments were performed on a ThermoScientific LCQ Deca ion trap mass spectrometer equipped with an electrospray source. Both devices were operated in positive ion mode. Optimal accurate mass determination conditions were as follows: injection rate 100 μl min^{-1}, capillary voltage 4000 V, source temperature 350°C, and cone voltage 60 V. The TOF analyzer was scanned over a m/z range of 100–1000 with a 1 s integration time. Optimal conditions for MS/MS were: injection rate 5 μl min^{-1}, spray voltage 4.0 kV, and capillary temperature 250°C, with nitrogen as the nebulizer gas (8 psi). Collision-induced dissociation was performed by isolating the m/z of interest and fragmenting at a collision energy required for ~50% beam reduction.

tRNA-Dependent lipid aminoacylation assay

tRNA-dependent lipid aminoacylation was measured according to established methods (Roy & Ibba, 2009). Activity was assayed using purified aaPGSs (or homologs), or enzymes present in a crude membrane extract isolated from *C. glutamicum* as previously described (Roy & Ibba, 2009). Aminoacylation reaction media contained 200 mM Hepes•NaOH, pH 7.2, 60 mM KCl, 20 mM $MgCl_2$, 4 mM ATP, 2 mg ml^{-1} total tRNA (from *E. coli* MRE600, Roche Applied Science), 20 μM [^{14}C]Ala (75 Ci/mole, Perkin Elmer), 0.1 μM alanyl-tRNA synthetase, and either 0.5 mg ml^{-1} membrane extract, or 1 μM purified $Cg1103_{483–832}$. Lipids were dried under vacuum and resuspended (by low power sonication) in a buffer containing 100 mM Hepes, 30 mM KCl, and 10 mM $MgCl_2$. Lipids added to the reaction mixture consisted of 2 mg ml^{-1} egg-PG (Avanti), with or without 0.5 mg ml^{-1} of DAG (Avanti), or lipids isolated from 5 OD of *C. glutamicum* cells. Lipids purified by TLC were eluted from plates using chloroform:methanol (1:1). After incubation of the complete reaction mixture for 30 min at 37°C, lipids were extracted using the Bligh and Dyer procedure (22), dried, and resuspended in 20 μl of chloroform:methanol (2:1). Reaction products were subjected to analysis by TLC in chloroform:methanol:water (14:6:1), and [^{14}C]-alanylated lipids were visualized by phosphorimaging.

Phylogenetic analysis of aaPGS homologs

Protein sequences were retrieved from the non-redundant database at the National Center for Biotechnology Information (NIH, Bethesda). The database was searched using the BLAST program (with default parameters) (Altschul *et al.*, 1997) with the LysPGS from *B. subtilis* being used as a query sequence. Redundant sequences were removed using a Perl script to eliminate sequences displaying >90% identity with another sequence from the same species. In this process, species exhibiting paralogous aaPGSs were preferentially kept in the dataset. Sequences were aligned using the ClustalO program with default parameters (Sievers *et al.*, 2011); columns of the alignment containing gaps and non-homogenous columns (exhibiting an entropy >2.2) were removed from the alignment with BioEdit software (Hall, 1999). The resulting 160-column alignment was used to construct a maximum likelihood phylogenetic tree using the FastTree program (Price *et al.*, 2009) with default parameters (JTT evolutionary model, discrete gamma model with 20 rate categories). Transmembrane helices were predicted using the TOPCONS program (Bernsel *et al.*, 2009), and phylogenetic tree graphics were generated using iTol (Letunic & Bork, 2007).

Determination of the maximal growth rate (μ_{max}) of *C. glutamicum*

Growth kinetics were performed in 96-well plates with each well containing 10 µl of inhibitor solution, or inhibitor solvent (water) alone as a negative control, and 140 µl of BHI inoculated with 14,000 cfu of *C. glutamicum* (1 OD = 185,000 cfu μl^{-1}). Plates were sealed with gas permeable film and incubated in an Elx800 Microplate Reader (BioTek) at 30°C with constant, linear agitation. The A630 was measured at 6 min intervals over 20 h. The maximal growth rates were determined with the R package *grofit* using the model-free spline method. Each growth condition was tested in duplicate in two sets of independent experiments (n=4).

Determination of MICs

MICs were measured in BHI broth by microdilution of inhibitory compounds (diluted serially 1:1) using a final *C. glutamicum* inoculum of 10^5 cfu ml^{-1}. The MIC was defined as the lowest drug concentration preventing visible turbidity after 20 h of incubation at 30°C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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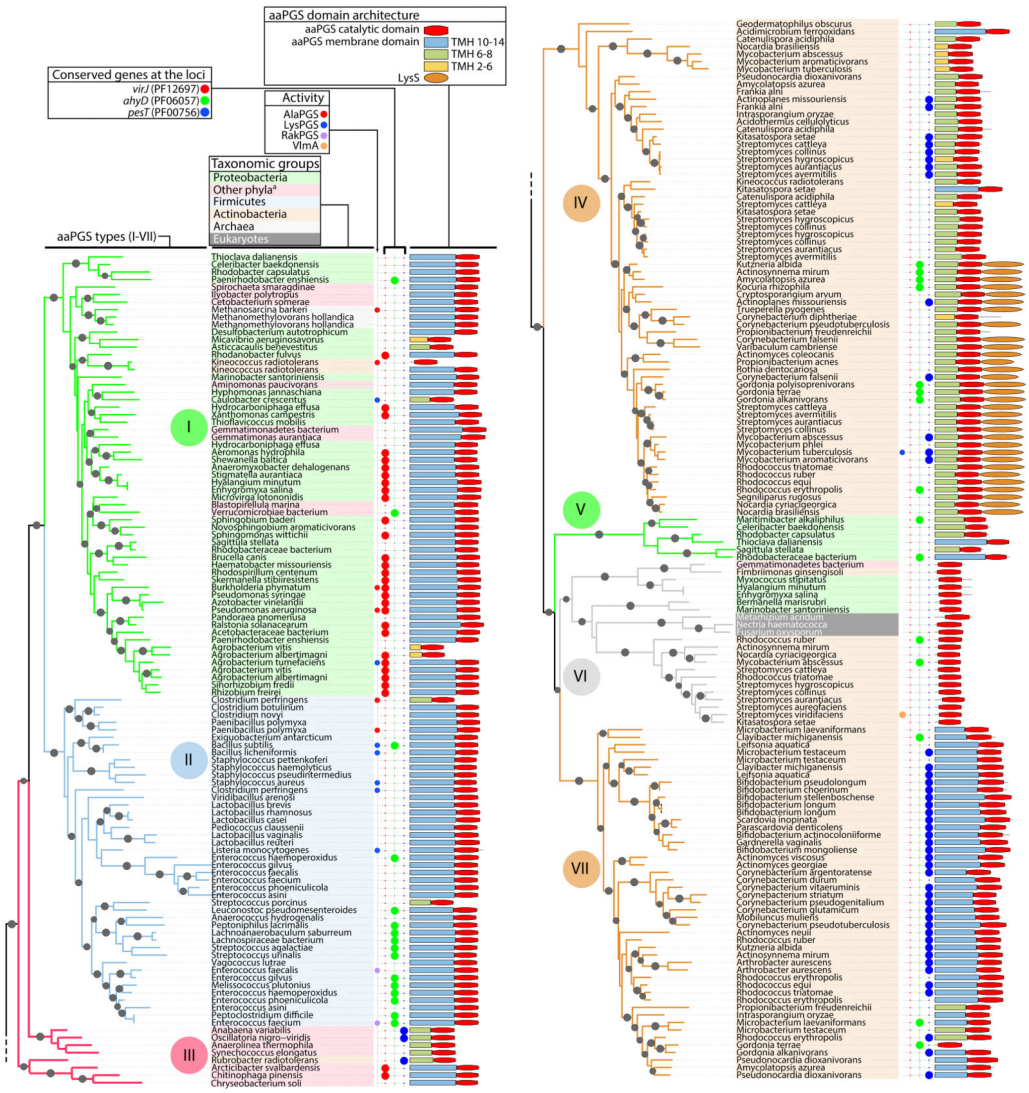

Fig. 1. Phylogenetic analysis of aaPGS homologs

Phylogenetic tree was constructed based on alignment of 160 residues in the C-terminal domain of 253 aaPGS homolog sequences using the program FastTree. Organism names are shown on a color background delineating the major taxonomic groups. The functions of characterized homologs (LysPGS, AlaPGS, RakPGS, VlmA), occurrence of adjacently encoded hydrolytic enzymes (VirJ, AhyD, PesT), and aaPGS domain architectures are indicated for each organism. When present, the number of predicted transmembrane helices (TMH 2–14) in the membrane domain is color-coded. Sequence identification (GI) numbers are shown in Fig. S3. [a]Bacteria from these phyla belong to one of the following: Fusobacteria, Gemmatimonadetes, Planctomycetes, Bacteroidetes, Chloroflexi, Cyanobacteria, Spirochaetes, Synergistetes, Verrucomicrobia.

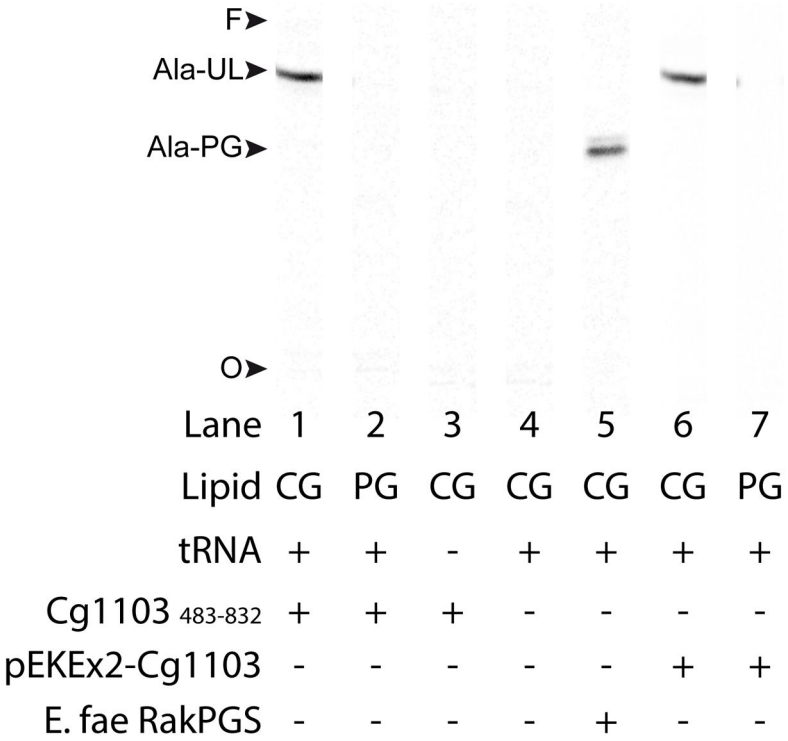

Fig. 2. tRNA-dependent lipid alanylation by Cg1103

tRNA-dependent lipid alanylation activity was measured *in vitro* using either the purified construct $Cg1103_{483–832}$ (lanes 1–3), or a membrane preparation from *C. glutamicum* harboring pEKEx2-Cg1103 (lanes 6 and 7). The lipids used as aa acceptors were a total lipid preparation isolated from *C. glutamicum* (CG), or commercial egg-PG (PG). Lipid products were analyzed by TLC using solvent A for the mobile phase. Activity with a membrane extract isolated from *E. coli* cells expressing RakPGS from *E. faecium* is shown as a positive control for [^{14}C]Ala-PG synthesis (lane 5). O: TLC origin, F: solvent front, Ala-UL: alanylated unknown lipid.

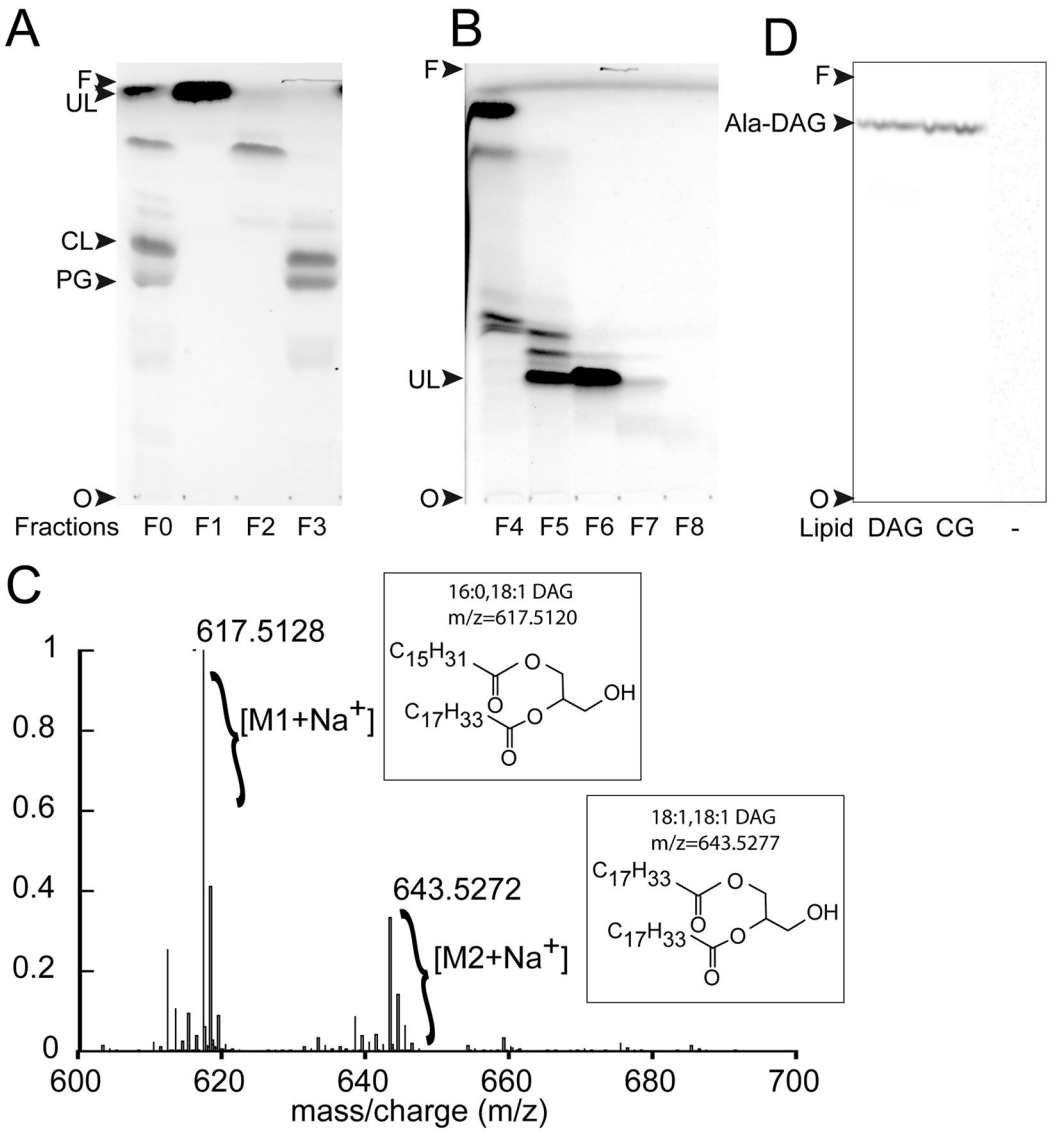

Fig. 3. Identification of DAG as a lipid substrate of Cg1103

(A) TLC analysis (using solvent A for the mobile phase) of lipids extracted from the wild-type strain of *C. glutamicum*, before (F0) and after (F1–F3) fractionation of lipid classes by flash chromatography. TLC spots were isolated and tested for tRNA-dependent lipid alanylation activity to identify the unknown lipid (UL) substrate of Cg1103. (B) Lipids in fraction F1 were further separated using a second step of flash chromatography on silica gel. Resulting fractions (F4–F8) were analyzed by TLC using solvent B for the mobile phase. (C) ESI/TOF mass spectrum of purified UL from fraction F6. (D) tRNA-dependent lipid alanylation activity of $Cg1103_{483–832}$ was assayed in the presence of commercial 18:1-16:0 DAG, lipids extracted from *C. glutamicum* (CG), or in the absence of lipids (−). Lipids modified with [^{14}C]Ala were analyzed by TLC (in solvent A), and visualized by phosphorimaging. Ala-DAG: alanyl-diacylglycerol, CL: cardiolipin, PG: phosphatidylglycerol, O: TLC origin, F: solvent front.

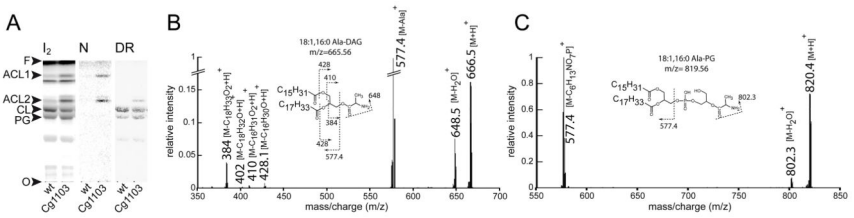

Fig. 4. ESI/MS/MS analysis of amine-containing lipids in *C. glutamicum*

(A) Lipids from wild-type *C. glutamicum* (wt), and a strain harboring the plasmid pEKEx2-Cg1103, were analyzed by TLC using solvent A as the mobile phase. Total lipids were revealed using iodine vapor (I_2), and amine and phosphorus containing lipids were revealed using ninhydrin (N) and Dittmer reagent (DR), respectively. Amine-containing lipids (ACL1 and ACL2) were purified using a two-step flash chromatography procedure as described in methods (see Fig. S4 for fractionation data) and analyzed by mass spectrometry. ESI/MS/MS spectra of fractions containing ACL1 (B) and ACL2 (C) revealed molecular ion and fragmentation peaks consistent with m/z values for Ala-DAG (665.56) and Ala-PG (810.56), respectively. CL: cardiolipin, PG: phosphatidylglycerol, O: TLC origin, F: solvent front.

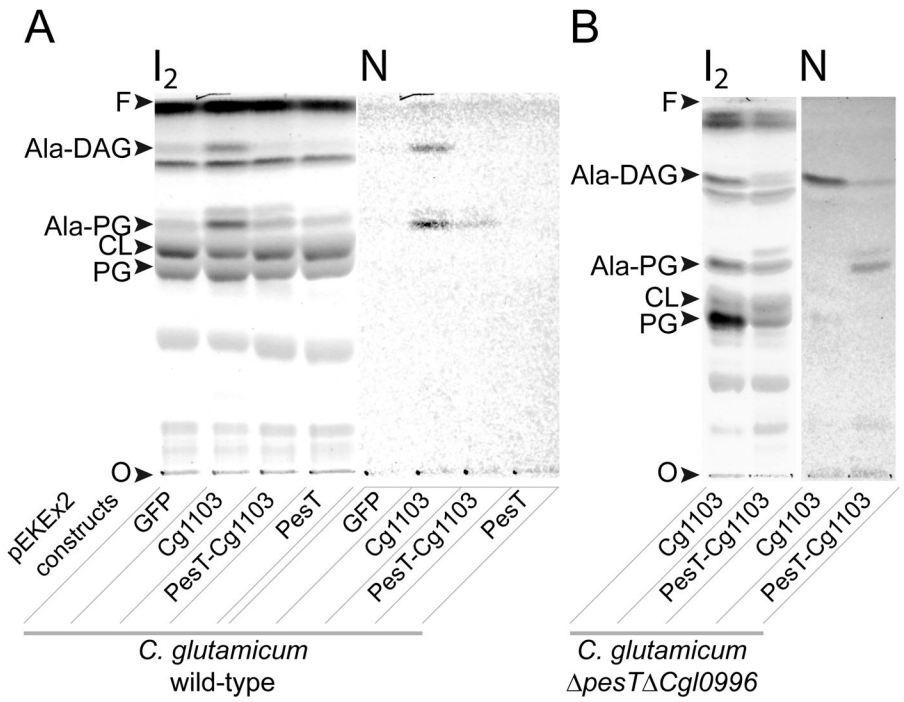

Fig. 5. Expression of Cg1103 and PesT in *C. glutamicum*.

TLC analysis of lipids isolated from wild-type *C. glutamicum* (A), and a deletion strain Δ*cg1103*Δ*pesT* (B), transformed with the expression vector pEKEx2 harboring GFP (negative control) *cg1103*, *pesT*, or both genes. TLCs were developed in solvent A. Total lipids were visualized with iodine vapor (I_2), and Ala-DAG and Ala-PG were detected with ninhydrine (N). CL: cardiolipin, PG: phosphatidylglycerol, O: TLC origin, F: solvent front.

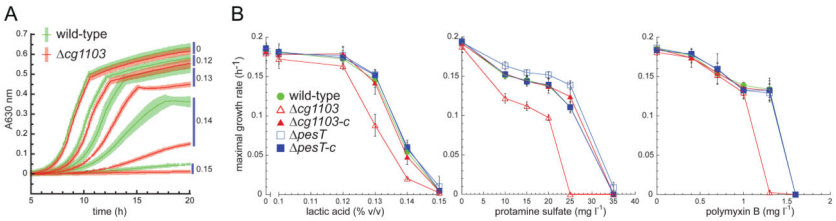

Fig. 6. Fitness of *C. glutamicum* in the presence of selected antimicrobial compounds

(A) Effect of different concentrations of lactic acid on the growth of wild-type *C. glutamicum* (green) and the Δ*cg1103* mutant strain (red). Percent of lactic acid (v/v) is indicated on the right. For each time point, the average value (n=4) of the A630 is indicated by a dark line, and the standard deviation is represented by a light colored area. (B) Derived dose-response curves from growth experiments performed in the presence of lactic acid, protamine sulfate, or polymyxin B. μ_{max} values were calculated for each growth curve using the *grofit* R package. Averages from four experiments are shown (n=4), and error bars represent standard deviations. Δ*cg1103*-c and Δ*pesT*-c represent complementation strains in which the deleted genes have been replaced back into the original loci. A growth rate of 0 indicates that no growth was recorded after an incubation period of 20 h.

TABLE 1

Co-occurrence of aaPGS paralogs according to type defined in Fig. 1

Paralog type	I	II	III	IV	V	VI	VII
I	+			+	+	+	
II		+					
III							
IV	+			+		+	+
V	+						
VI	+			+			+
VII				+		+	+

The co-occurrence of aaPGS paralogs was analyzed according to the seven aaPGS types defined in Fig. 1. For instance, some organisms harboring a type VI homolog also harbor a type I, type IV, and/or type VII aaPGS homolog. A comprehensive depiction of distribution and co-occurrence of each type is shown in Fig. S1.