Phosphatidylglycerol Is the Lipid Donor for Synthesis of Phospholipid-Linked Enterobacterial Common Antigen

Kinsey N. Morris,[a] Angela M. Mitchell[a]

[a]Department of Biology, Texas A&M University, College Station, Texas, USA

ABSTRACT The Gram-negative outer membrane (OM) is an asymmetric bilayer with phospholipids in its inner leaflet and mainly lipopolysaccharide (LPS) in its outer leaflet and is largely impermeable to many antibiotics. In *Enterobacterales* (e.g., *Escherichia*, *Salmonella*, *Klebsiella*, and *Yersinia*), the outer leaflet of the OM also contains phosphoglyceride-linked enterobacterial common antigen (ECA$_{PG}$). This molecule consists of the conserved ECA carbohydrate linked to diacylglycerol-phosphate (DAG-P) through a phosphodiester bond. ECA$_{PG}$ contributes to the OM permeability barrier and modeling suggests that it may alter the packing of LPS molecules in the OM. Here, we investigate, in *Escherichia coli* K-12, the reaction synthesizing ECA$_{PG}$ from ECA precursor linked to an isoprenoid carrier to identify the lipid donor that provides the DAG-P moiety to ECA$_{PG}$. Through overexpression of phospholipid biosynthesis genes, we observed alterations expected to increase levels of phosphatidylglycerol (PG) increased the synthesis of ECA$_{PG}$, whereas alterations expected to decrease levels of PG decreased the synthesis of ECA$_{PG}$. We discovered depletion of PG levels in strains that could synthesize ECA$_{PG}$, but not other forms of ECA, causes additional growth defects, likely due to the buildup of ECA precursor on the isoprenoid carrier inhibiting peptidoglycan biosynthesis. Our results demonstrate ECA$_{PG}$ can be synthesized in the absence of the other major phospholipids (phosphatidylethanolamine and cardiolipin). Overall, these results conclusively demonstrate PG is the lipid donor for the synthesis of ECA$_{PG}$ and provide a key insight into the reaction producing ECA$_{PG}$. In addition, these results provide an interesting parallel to lipoprotein acylation, which also uses PG as its DAG donor.

IMPORTANCE The Gram-negative outer membrane is a permeability barrier preventing cellular entry of antibiotics. However, outer membrane biogenesis pathways are targets for small molecule development. Here, we investigate the synthesis of a form of enterobacterial common antigen (ECA), ECA$_{PG}$, found in the outer membrane of *Enterobacterales* (e.g., *Escherichia*, *Salmonella*, and *Klebsiella*). ECA$_{PG}$ consists of the conserved ECA carbohydrate unit linked to diacylglycerol-phosphate—ECA is a phospholipid headgroup. The details of the reaction forming this molecule from polymerized ECA precursor are unknown. We determined the lipid donor providing the phospholipid moiety is phosphatidylglycerol. Understanding the synthesis of outer membrane constituents such as ECA$_{PG}$ provides the opportunity for development of molecules to increase outer membrane permeability, expanding the antibiotics available to treat Gram-negative infections.

KEYWORDS cell envelope biogenesis, enterobacterial common antigen, glycolipids, outer membrane, phospholipids

G ram-negative bacteria have a cell envelope that consists of an inner membrane (IM), aqueous periplasm containing the peptidoglycan cell wall, and an outer membrane (OM). In contrast to the IM and most biological membranes, the OM is an asymmetrical membrane with phospholipids in the inner leaflet and mainly lipopolysaccharide (LPS) in the outer leaflet (1). Beyond its lipid components, the OM is heavily
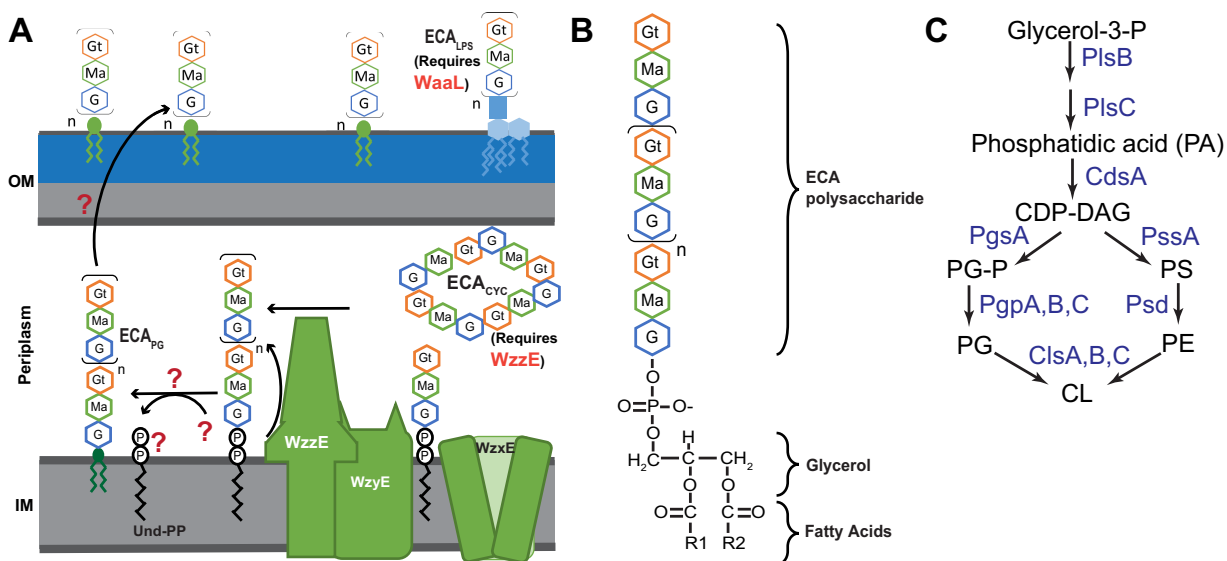
FIG 1 Structure and synthesis of ECA_{PG}. (A) Subunits of ECA are assembled on the cytoplasmic face of the inner membrane, attached to an isoprenoid carrier, undecaprenyl-pyrophosphate (Und-PP). The subunits are then flipped across the membrane by WzxE and polymerized by WzyE. WzzE controls the chain length of the final ECA molecules. Polymerized ECA can be made into three forms: cyclic ECA (ECA_{CYC}), LPS-linked ECA (ECA_{LPS}), and ECA_{PG} (see below). ECA_{LPS} synthesis requires the O-antigen ligase, WaaL, for synthesis, while ECA_{CYC} synthesis requires WzzE. The steps and genes required for the formation of ECA_{PG} from polymerized ECA on Und-PP and for its subsequent surface exposure are unknown. (B) Structure of ECA_{PG} glycolipid. The ECA carbohydrate chain is linked to diacylglycerol-phosphate (DAG-P) through a phosphodiester bond. Thus, ECA is linked to the phospholipid backbone in place of a headgroup. (C) *E. coli* phospholipid synthesis begins with the addition of acyl chains to glycerol-3-phosphate by PlsB and PlsC, forming phosphatidic acid (PA). Then, CdsA activates PA with CDP for form CDP-DAG, the precursor for all phospholipid synthesis. From this point, PgsA and PgpA, PgpB, or PgpC synthesize phosphatidylglycerol (PG), while PssA and Psd synthesize phosphatidylethanolamine (PE). Cardiolipin (CL) is synthesized by ClsA or ClsB from two PG molecules, while ClsC synthesizes CL from one PG and one PE molecule. PS, phosphatidylserine; PG-P, phosphatidylglycerol phosphate.

populated with outer membrane proteins and OM lipoproteins (1–3). In addition, the OM contains lower abundance constituents, such as enterobacterial common antigen (ECA) (4), which is found throughout *Enterobacterales*.

The OM presents a strong permeability barrier capable of excluding both large molecules and hydrophobic molecules, including many antibiotics (1, 5, 6). Thus, the biogenesis pathways for OM components are potential targets for the development of small molecules to increase outer membrane permeability and the cell's susceptibility to antibiotics (7, 8). In fact, several potential antimicrobials targeting biosynthesis of LPS and OM protein biosynthesis have recently been developed (8–15). Although these pathways are found throughout Gram-negative bacteria, the biosynthesis of ECA is a pathway that could allow development of small molecules to increase permeability in an order-specific manner, limiting off-target effects on bystander species during treatment.

ECA is an invariant carbohydrate moiety that is found in all *Enterobacterales* (e.g., *Escherichia*, *Salmonella*, *Klebsiella*, *Shigella*, *Enterobacter*, and *Yersinia*), with the exception of endosymbionts with greatly reduced genomes (reviewed in reference 4). The carbohydrate moiety consists of GlcNAc (*N*-acetylglucosamine), ManNAcA (*N*-acetyl-D-mannosaminuronic acid), and Fuc4NAc (4-acetamido-4,6-dideoxy-D-galactose) which form repeating units of →3)-α-Fuc4NAc-(1→4)-β-ManNAcA-(1→4)-α-GlcNAc-(1→ (Fig. 1A) (16, 17). ECA can be found in three forms: ECA_{CYC}, a cyclic form found soluble in the periplasm; ECA_{PG} (ECA phosphoglyceride), a surface-exposed phospholipid form of ECA (see below); and ECA_{LPS}, LPS with ECA attached in place of O antigen (18–25). We have observed that ECA_{CYC} and ECA_{PG} play roles in maintaining the OM permeability barrier in *Escherichia coli* K-12 (26). Modeling through molecular dynamics has suggested that the presence of ECA_{PG} in the outer leaflet of the OM can lead to changes in packing of LPS with more space allotted per molecule (27, 28). In addition, work in *Salmonella enterica* serovar Typhimurium shows that ECA-deficient strains are nonvirulent and establish low-level persistent infections (29). Beyond its direct role in pathogenesis, exposure to ECA can lead to the development of

antibodies that recognize species throughout *Enterobacterales* (30–32), likely playing an important protective or pathogenic role in *Enterobacterales* infection. These antibodies are most easily generated by exposure to rough *Enterobacterales* strains, which have high levels of ECA_{LPS}.

Given their differences in structure, localization, and phenotypes, it is likely that each form of ECA plays distinct roles in the cells. However, lack of understanding of the steps that differentiate the biosynthesis of the three forms of ECA impedes experimental approaches to investigate their function. ECA is synthesized in a Wzy pathway analogous to that of many O antigens (Fig. 1A) (33). ECA trisaccharide units are first assembled on undecaprenyl-pyrophosphate (Und-PP), an isoprenoid carrier, and then flipped to the outer leaflet of the inner membrane by WzxE (4, 34–36). ECA units are polymerized by WzyE with the chain length of the final ECA unit controlled by WzzE (37, 38). Once a polymerized ECA precursor is formed on Und-PP, it can be made into ECA_{PG}, ECA_{LPS}, or ECA_{CYC}. Formation of ECA_{CYC} requires the chain length regulator, WzzE (24), while formation of ECA_{LPS} requires the O-antigen ligase WaaL (18). However, the details of the reaction forming ECA_{PG} and leading to ECA_{PG} surface exposure are completely unknown.

Here, we set out to identify, in *E. coli* K-12, the lipid donor(s) that provides the "phospholipid base" to ECA_{PG}. ECA_{PG} consists of the ECA carbohydrate moiety attached to diacylglycerol-phosphate (DAG-P; phosphatidic acid) through a phosphodiester bond (Fig. 1B) (20, 25). In essence, ECA is a large phospholipid headgroup. It seems biochemically likely that ECA is removed from the isoprenoid carrier freeing Und-PP, as occurs when O antigen and other forms of ECA are synthesized, and transferred to a specific subset of phospholipids or phospholipid precursors releasing the headgroup in favor of ECA (Fig. 1A).

In actively growing *E. coli*, the cell's phospholipid composition is 75% phosphatidylethanolamine (PE), 20% phosphatidylglycerol (PG), and 5% cardiolipin (CL) with the amount of CL increasing in stationary phase at the expense of PG (reviewed in reference 39). The distribution of phospholipids in the IM is asymmetric with higher PE levels in the inner leaflet than the outer leaflet (40). Phospholipid synthesis begins with the sequential addition of fatty acids to glycerol-3-P by PlsB and PlsC to form phosphatidic acid (PA) (Fig. 1C) (41). Subsequently, CdsA activates PA through addition of CMP to form CDP-DAG, the precursor for phospholipid biosynthesis (42). From this point, the pathway splits between synthesis of PG and PE. PgsA exchanges CMP for glycerol-3-phosphate to form phosphatidylglycerol phosphate (PG-P) (43). Then, PG-P is dephosphorylated by PgpA, PgpB, or PgpC to form PG (44–46). While PgpA and PgpC are specific to PG-P dephosphorylation (45, 46), PgpB can also dephosphorylate DAG-PP, PA, lyso-PA, and Und-PP (44, 47). For PE synthesis, PssA (Pss) first synthesizes phosphatidylserine (PS) from CDP-DAG (48). Then, Psd decarboxylates PS to form PE (49). *E. coli* has three CL synthase ClsA, ClsB, and ClsC (50–53). ClsA and ClsB form CL from two molecules of PG (50, 54), while ClsC synthesizes CL from one PG molecule and one PE molecule (50). ClsA is mainly responsible for CL synthesis in exponential phase, while all three synthases contribute to CL synthesis in stationary phase.

We examined the effects of alterations in expression of phospholipid biosynthesis on levels of ECA_{PG}. Our overexpression data suggest that increasing PG synthesis increases production of ECA_{PG}. We determined that the donor for ECA_{PG} synthesis is PG since ECA_{PG} is still synthesized in the absence of CL or PE and depletion of PG leads to inhibited cell growth even in a strain where PG is normally not essential, likely due to the accumulation of ECA precursor on Und-PP inhibiting peptidoglycan biosynthesis. These data are the first to characterize the reaction(s) forming ECA_{PG} and suggest a common approach to use of PG as a lipid donor.

RESULTS

Genetic alterations in phospholipid synthesis do not affect ECA_{LPS} levels. The kinetics of biochemical reactions, and so the amount of product produced, often
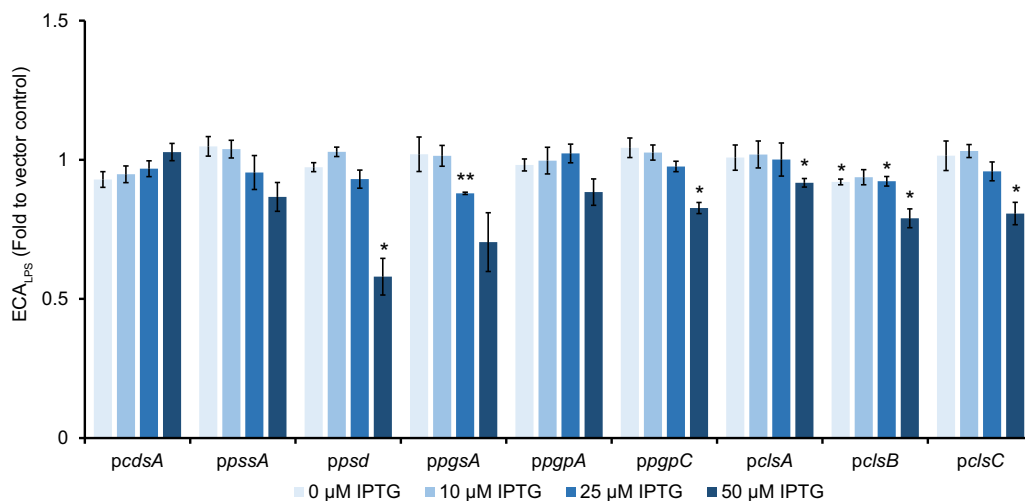
FIG 2 Alterations in phospholipid synthesis cause only minor changes in ECA_{LPS} levels. The levels of ECA_{LPS} were assayed by WGA staining in strains overexpressing the indicated genes in phospholipid biosynthesis. The data are displayed as fold values relative to the vector control. Only minor decreases and no significant increases in ECA_{LPS} levels were observed. The data are means of three biological replicates $\pm$ the standard errors of the mean (SEM). *, $P < 0.05$ (by paired t test); **, $P < 0.005$ (by paired t test).

depend on the amounts of available substrates for the reaction. For ECA_{PG} to be formed, at least two substrates are necessary: the polymerized ECA precursor on Und-PP and the donor phospholipid or phospholipid precursor. Thus, we hypothesized that changing the availability of the donor lipid would alter the kinetics of the reaction(s) producing ECA_{PG} and so the amount of ECA_{PG} produced. However, changes in phospholipid composition can alter the folding and topology of membrane proteins (reviewed in reference 55). Since the ECA biosynthesis pathway contains many membrane proteins (4), we sought to determine whether alteration of phospholipid composition would affect ECA synthesis in a manner unrelated to the ECA_{PG} lipid donor. Therefore, we tested the effect of overexpressing genes in phospholipid biosynthesis on levels of ECA_{LPS} to identify any off-target effects of phospholipid level alteration on this form of ECA. Using our previously described WGA-staining protocol (56), we found that overexpression of genes in phospholipid biosynthesis caused only minor decreases in the levels of ECA_{LPS} (Fig. 2). This result is consistent with our previous observation that ECA_{LPS} levels are largely dependent on the availability of WaaL (56) and confirms that ECA can be successfully synthesized with our tested alterations in phospholipid biosynthesis.

The ECA_{PG} lipid donor is not PA. We investigated the effect of phospholipid gene overexpression on the accumulation of ECA_{PG} and on activity of the P_{wec} promoter region responsible for expression of the *wec* operon that contains the genes necessary for synthesis of the polymerized ECA precursor. We reasoned overexpression of genes encoding enzymes functioning upstream of synthesis of the lipid donor in the phospholipid biosynthesis pathway would increase the amount of the lipid donor and so the amount of ECA_{PG}. Conversely, overexpression of genes whose products lead to consumption of the lipid donor or which are on a different branch of the pathway would decrease the amount of the donor and so the amount of ECA_{PG}. Changes in P_{wec} activity suggest changes to ECA precursor levels that may be occurring, in addition to any changes in lipid donor levels.

The lipid to which ECA is attached in ECA_{PG} is DAG-P and so is most similar to PA. Therefore, we assayed the effect on ECA_{PG} levels of overexpression under an IPTG inducible promoter of a His-tagged *cdsA* construct, whose product converts PA into CDP-DAG. Accumulation of CdsA was confirmed by immunoblot (see Fig. S1 in the supplemental material, lane 1 versus lanes 2 to 5). To allow specific detection of ECA_{PG}, we tested ECA_{PG} levels in a $\Delta wzzE \, \Delta waaL$ strain that only produces ECA_{PG} and not the
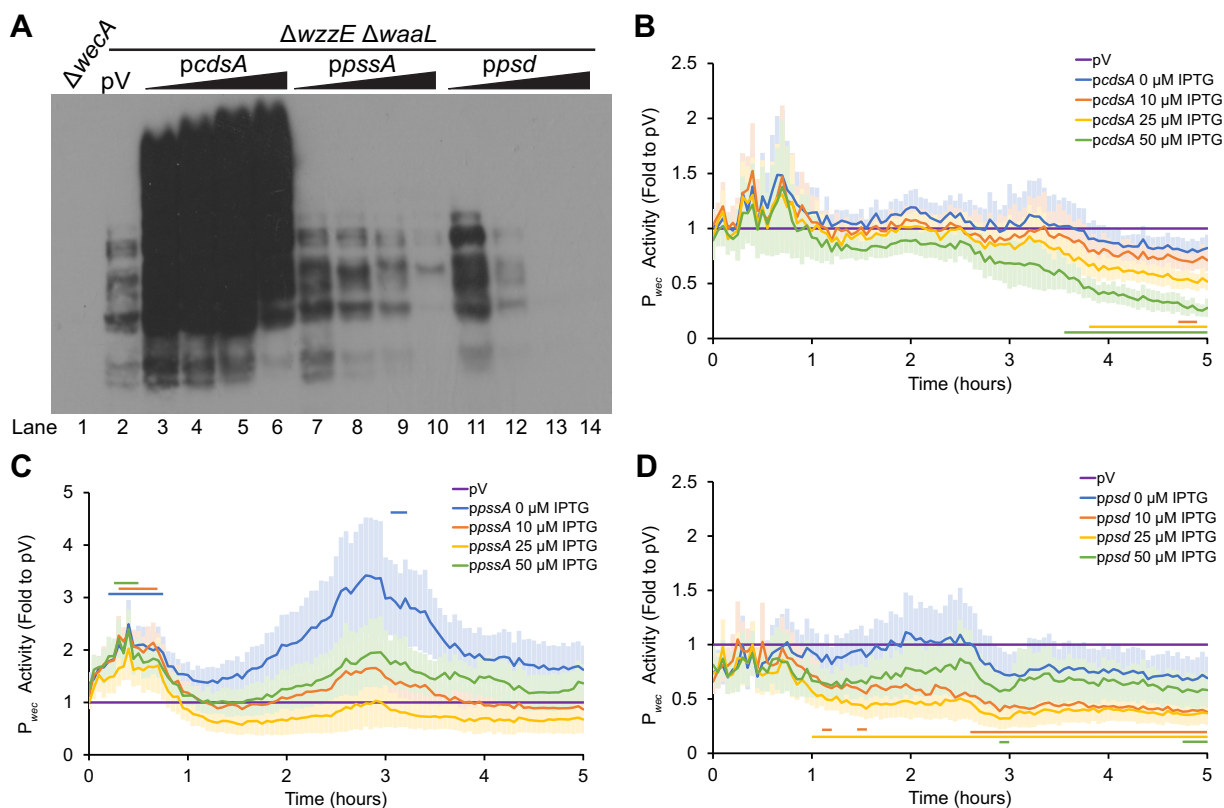
FIG 3 A phospholipid is the substrate for ECA_{PG} synthesis. (A) The levels of ECA_{PG} were assayed by immunoblotting in a strain that produces only ECA_{PG} and not the other two forms of ECA ($\Delta wzzE\ \Delta waaL$). The vector control (pV) treated with 50 μM IPTG is compared to strains overexpressing the indicated genes in phospholipid biosynthesis with increasing concentrations of IPTG (0, 10, 25, and 50 μM). A strain with a deletion of *wecA*, the first gene in the ECA biosynthesis pathway, serves as a negative control. Overexpression of *cdsA* causes large increases in the amounts of ECA_{PG}, while overexpression of *pssA* and *psd* decrease ECA_{PG} levels. Image is representative of three independent experiments. (B to D) Strains overexpressing genes in phospholipid biosynthesis and carrying a luciferase reporter for *wec* operon promoter (P_{wec}) activity were assayed for luminescence and OD_{600}. The data are shown as fold value of the relative luminescence to the vector control and are means from six biological replicates $\pm$ the SEM. The empty vector (pV) sample contained the empty vector for phospholipid gene overexpression and the P_{wec} reporter and was treated with 50 μM IPTG. Horizontal bars: $P < 0.05$ by *t* test consistently for three or more time points. (B) Strains overexpressing *cdsA* have very similar P_{wec} activity to that of the empty vector control, with a decrease later in growth. (C) Strains overexpressing *pssA* have some increase in P_{wec} activity. (D) Overexpression of *psd* decreases P_{wec} activity.

other two forms of ECA. At all tested IPTG (isopropyl-β-D-thiogalactopyranoside) concentrations, we observed a large increase in the level of ECA_{PG} (Fig. 3A, lanes 2 versus lanes 3 to 6). In contrast, there were no significant increases in P_{wec} activity when *cdsA* was overexpressed (Fig. 3B). These data indicate that the lipid donor for ECA_{PG} synthesis is CDP-DAG and/or a phospholipid but is not PA or an earlier biosynthetic intermediate.

PE synthesis is off the pathway of the lipid donor. To determine whether PE could serve as the donor for ECA_{PG} synthesis, we then overexpressed the genes in PE synthesis pathway, *pssA* and *psd*. We confirmed accumulation of PssA and Psd by immunoblot (see Fig. S1, lane 1 versus lanes 6 to 9 and 10 to 13, respectively). Overexpression of these genes caused a decrease in the accumulation of ECA_{PG} in a manner that correlates with the concentration of IPTG (Fig. 3A, lane 2 versus lanes 7 to 10 and 11 to 14). Overexpression of *pssA* caused some significant increases in P_{wec} activity at early time points after induction but no decreases in activity (Fig. 3C). However, *psd* overexpression caused decreased P_{wec} activity at higher IPTG concentrations (Fig. 3D), confounding interpretations of decreased ECA_{PG} accumulation observed with *psd* overexpression. Nevertheless, the decreased ECA_{PG} levels without changes in P_{wec} activity with *pssA* overexpression demonstrate the pathway for synthesis of PE is not involved in synthesis of the ECA_{PG} lipid donor.

Overexpression suggests the ECA_{PG} lipid donor is part of the PG/CL synthesis pathway. Since PE synthesis appeared off pathway for the synthesis of the ECA_{PG} lipid donor, we then examined the effects of gene overexpression in the PG synthesis
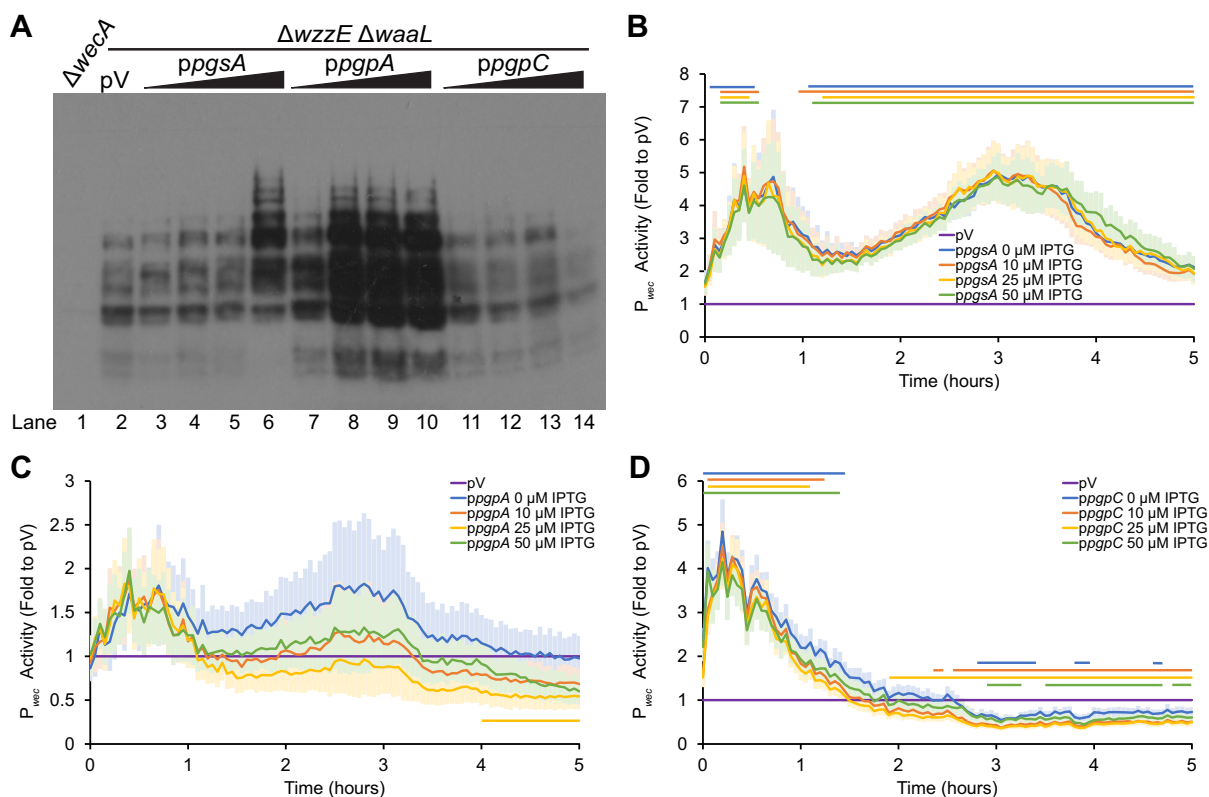
FIG 4 The substrate for ECA$_{PG}$ biosynthesis is in the PG/CL biosynthesis pathway. (A) ECA$_{PG}$ levels were assayed by immunoblot analysis in strains overexpressing the indicated genes in phospholipid biosynthesis. The ΔwzzE ΔwaaL strain background allows the production of ECA$_{PG}$ without the other forms of ECA. For phospholipid synthesis gene overexpression, cultures were treated with 0, 10, 25, and 50 μM IPTG. The vector control (pV) was treated with 50 μM IPTG. A ΔwecA strain serves as a negative control. The image is representative of three independent experiments. Overexpression of both pgsA and pgpA increase accumulation of ECA$_{PG}$. Overexpression of pgpC causes a small decrease in ECA$_{PG}$ levels. (B to D) P$_{wec}$ activity was assayed in strains overexpressing genes in phospholipid biosynthesis as in Fig. 3. The means ± the SEM for the relative luminescence as a fold value to the vector control are shown for six biological replicates. pV, empty vector sample carrying the P$_{wec}$ reporter and the empty vector for the phospholipid synthesis gene overexpression and treated with 50 μM IPTG. Horizontal bars: $P < 0.05$ by t test consistently for three or more time points. (B) Overexpressing pgsA consistently increased P$_{wec}$ activity. (C) Overexpression of pgpA did not change P$_{wec}$ activity at most time points and IPTG concentrations. (D) Overexpressing pgpC causes early increases and later decreases in P$_{wec}$ activity.

pathway. As PG is necessary for synthesis of CL, overexpression of these genes would also be expected to increase the synthesis of both PG and CL (50, 54). We confirmed the accumulation of proteins from the overexpression constructs by immunoblot (see Fig. S2). Overexpression of pgsA, the first dedicated gene in PG synthesis, caused an increase in ECA$_{PG}$ levels at the highest IPTG concentration we tested (Fig. 4A, lane 2 versus lanes 3 to 6). However, pgsA overexpression caused a consistent increase in P$_{wec}$ activity (Fig. 4B), making these data difficult to interpret. Overexpression of pgpA led to higher levels of ECA$_{PG}$ at all IPTG concentrations assayed (Fig. 4A, lane 2 versus lanes 7 to 10), despite very little significant change in P$_{wec}$ activity (Fig. 4C). This result suggests that the lipid donor for ECA$_{PG}$ synthesis may be downstream of PgpA in the biosynthesis pathway.

Interestingly, when we overexpressed pgpC, we observed the opposite phenotype, a decrease in ECA$_{PG}$ levels at the highest IPTG concentration (Fig. 4A, lane 2 versus lanes 11 to 14). There are two likely explanations for the contradictory results between pgpA and pgpC overexpression. The first is that the decrease in ECA$_{PG}$ levels with pgpC is due to the lower P$_{wec}$ activity observed several hours after induction of pgpC overexpression (Fig. 4D). The second is due to the relative activities of PgpA, PgpB, and PgpC. Of the three enzymes, PgpA has the highest activity and the most effect on the levels of PG-P and PG (46). Thus, it is possible that overexpression of pgpC actually decreases flux through the PG/CL synthesis pathway. We did not overexpress pgpB, as PgpB is
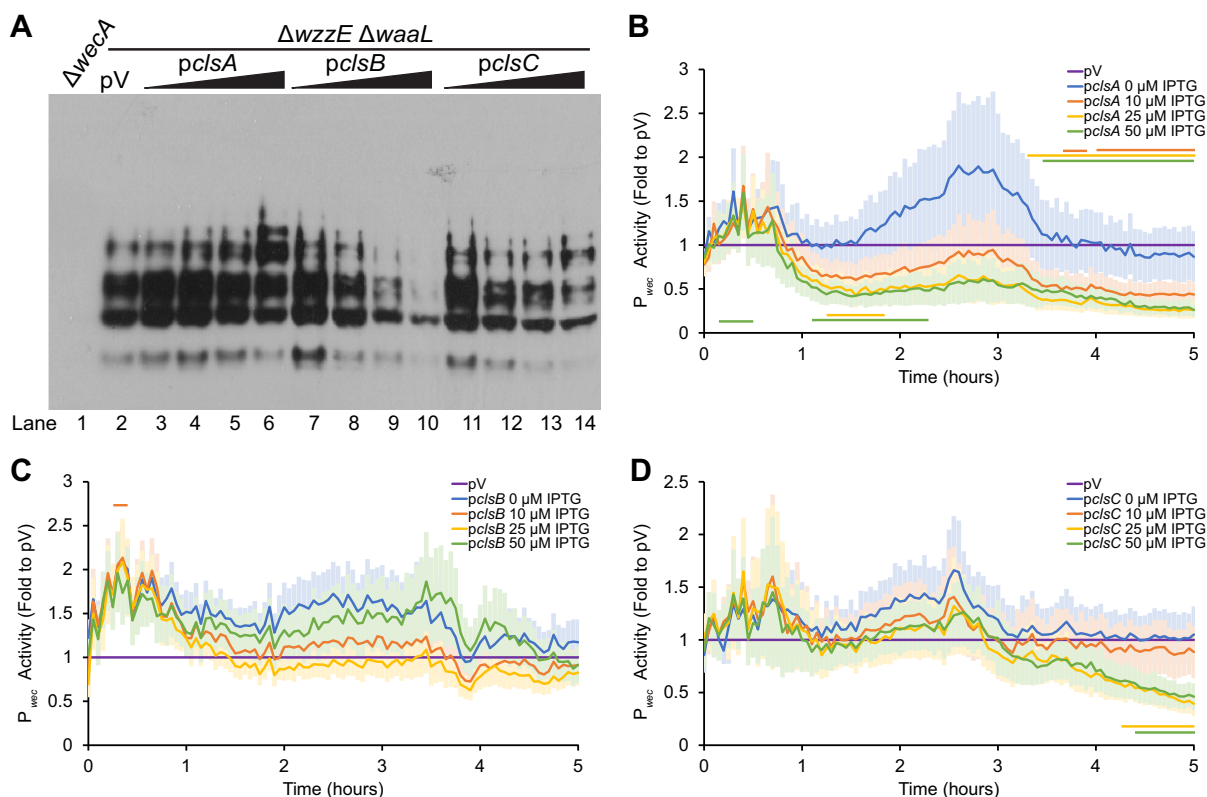
FIG 5 CL synthase overexpression decreases ECA_{PG} levels. (A) ECA_{PG} levels were assayed by immunoblot in strains overexpressing genes in CL biosynthesis. The $\Delta wzzE$ $\Delta waaL$ strain produces only ECA_{PG} and not the other two forms of ECA. The vector control (pV) was treated with 50 μM IPTG, while overexpression strains were treated with 0, 10, 25, and 50 μM IPTG. The $\Delta wecA$ strain serves as a negative control. The image is representative of three independent experiments. Overexpression of both $clsB$ and $clsC$ decreases ECA_{PG} levels, while the levels of ECA_{PG} are constant with $clsA$ overexpression. (B to D) The activity of P_{wec} was assayed as in Fig. 3. The data are shown as the fold of the relative luminescence to the vector control (pV) and are means of 5 to 6 biological replicates $\pm$ the SEM. pV control carried the P_{wec} reporter and an empty vector for overexpression and was treated with 50 μM IPTG. Horizontal bars: $P < 0.05$ by t test consistently for three or more time points. (B) Overexpression of $clsA$ causes some significant increases and decreases in P_{wec} activity at higher IPTG concentrations. (C) Overexpression of $clsB$ does not change P_{wec} activity. (D) Overexpression of $clsC$ causes some decrease in P_{wec} activity at higher IPTG concentrations and later time points.

active on several different substrates including Und-PP (47). Overall, these results suggest that PG or CL may be the donor for ECA_{PG} biosynthesis.

CL synthesis is downstream from the ECA_{PG} lipid donor. There are three CL synthases: ClsA, ClsB, and ClsC (50–53). When we overexpressed the gene for the CL synthase active in exponentially growing cells, $clsA$, we observed very little change in either ECA_{PG} levels (Fig. 5A, lane 2 versus lanes 3 to 6) or in P_{wec} activity (Fig. 5B). This may be due to feedback inhibition of CL on ClsA activity (57), due to the low levels of ClsA from overexpression we observed compared to ClsB and ClsC (see Fig. S3), or due to inhibition of activity from His-tagging. However, when we overexpressed either $clsB$ or $clsC$, which are normally active in stationary phase when CL levels increase, we observed a decrease in ECA_{PG} levels that was more severe at higher IPTG concentrations (Fig. 5A, lane 2 versus lanes 7 to 10 and 11 to 14). Overexpression of $clsB$ did not cause significant changes in P_{wec} activity (Fig. 5C), while overexpression of $clsC$ caused some decreases in P_{wec} activity at higher IPTG concentrations (Fig. 5D). These data suggest that CL is downstream of the lipid donor for ECA_{PG} synthesis.

PG is the substrate for ECA_{PG} biosynthesis. Taken as a whole, our overexpression data point to PG as the lipid donor for ECA_{PG} synthesis: Overexpression of genes expected to increase levels of PE and CL at the expense of PG decreases levels of ECA_{PG}, while overexpression of genes expected to increase levels of PG increases levels of ECA_{PG}. Therefore, we decided to examine the growth of strains where genes in the PG/CL biosynthesis pathway could be depleted. ECA and peptidoglycan are both

synthesized on Und-P (58, 59), and so interruption of ECA biosynthesis can lead to the buildup of Und-PP-linked intermediates, disrupting peptidoglycan biosynthesis (24, 56, 60–64). The severity of the peptidoglycan defect depends on the stage in biosynthesis interrupted. Defects in ECA biosynthesis leading to the accumulation of Und-PP-GlcNAc-ManNAcA (Lipid II^{ECA}) produce cell shape defects, envelope permeability, and envelope stress response activation (60, 61, 63). Disruption of the flippases capable of flipping Und-PP-GlcNAc-ManNAcA-Fuc4NAc (Lipid III^{ECA}) ECA across the IM or of the ECA polymerase, *wzyE*, is lethal in *E. coli* K-12 (24). We have speculated that, in a strain that makes only ECA$_{PG}$, disruption of the next step in ECA$_{PG}$ biosynthesis would be lethal as well (56). In fact, we have shown that dysregulation of ECA$_{PG}$ biosynthesis in a strain making only ECA$_{PG}$ is lethal (56). Therefore, we hypothesized that loss of the lipid donor for ECA$_{PG}$ biosynthesis would cause peptidoglycan synthesis defects, and so growth phenotypes, specifically when ECA$_{PG}$ is made without the other forms of ECA.

Our depletion strategy involved cloning phospholipid biosynthesis genes under the control of the P$_{BAD}$ promoter, which is arabinose inducible and subject to catabolite repression (65). We then deleted the chromosomal copies of the affected genes while maintaining expression from the plasmid-borne copy in either the wild-type background, the Δ*wzzE* Δ*waaL* strain that produces ECA$_{PG}$ without the other two forms of ECA, or an isogenic background without ECA (Δ*wecA-wzzE* Δ*waaL*) to control of effects of loss of ECA envelope strength. Treating cells with arabinose or glucose in the presence of the empty plasmid produced similar growth curves in the wild-type and Δ*wzzE* Δ*waaL* strains (see Fig. S4A).

pgsA is essential due to the necessity for PG for lipoprotein processing (66–69). The essentiality of *pgsA* can be circumvented by deletion of *lpp* (66). Lpp (Braun's lipoprotein) is highly abundant and IM localization is lethal due to cross-linking of the peptidoglycan to the IM (70). Δ*lpp* Δ*pgsA* strains are still temperature sensitive, however (66). This temperature sensitivity can be relieved by inactivating the Rcs stress response (67), which is overactivated by localization of RcsF in the inner membrane (71). Therefore, we built PgsA depletion strains in Δ*rcsF* Δ*lpp* and Δ*rcsF* Δ*lpp* Δ*wzzE* Δ*waaL* backgrounds to test the effect of PgsA depletion in conditions where PG is normally not essential. In the Δ*rcsF* Δ*lpp* background, the PgsA depletion strain grew equally with arabinose or glucose, confirming PG is not essential in this strain (Fig. 6A). However, the Δ*rcsF* Δ*lpp* Δ*wecA-wzzE* Δ*waaL* background strain ceased growth when depleted, suggesting that the presence of ECA is important for the integrity of the cell envelope in the absence of PG. In contrast, the Δ*rcsF* Δ*lpp* Δ*wzzE* Δ*waaL* background strain grew to near stationary phase and then abruptly lysed, demonstrating a different phenotype than either the wild type and no ECA strains.

Depletion of the phosphatidylglycerophosphatases (PgpA, PgpB, and PgpC) (see Fig. S4B) or of PgsA with Lpp and RcsF intact (see Fig. S4C) also lead to increased lysis in the ECA$_{PG}$ only (Δ*wzzE* Δ*waaL*) background. These data confirm a second growth defect in this strain beyond that observed in the wild-type background, likely due to the buildup ECA intermediates on Und-PP. Depletion of either PgpABC or PgsA will reduce the level of both PG and CL. Thus, to confirm that the results we observed with depletion were the result of loss of PG and not loss of CL, we assayed the growth of a ClsA depletion strain in a Δ*clsA* Δ*clsB* Δ*clsC* background. CL is not essential and this strain grew fully when depleted in both the wild type and the Δ*wzzE* Δ*waaL* strain (Fig. 6B). These data confirm that it is loss of PG not CL that causes increased lysis with PgsA and PgpABC depletion.

Depletion of PG in a background where it is normally nonessential leads to lysis in an ECA$_{PG}$ only background but not a background wild type for ECA, suggesting accumulation of ECA precursors on an isoprenoid carrier. As accumulation of Lipid III^{ECA} due to Δ*wzyE* or deletion of all the flippases capable of flipping ECA across the IM is lethal in *E. coli* K-12 (24), we can exclude complete inhibition of WzyE and/or flippase activity as the cause of the lysis since cells wild type for ECA synthesis survive depletion. However, it is possible that partial inhibition, due to improper membrane protein
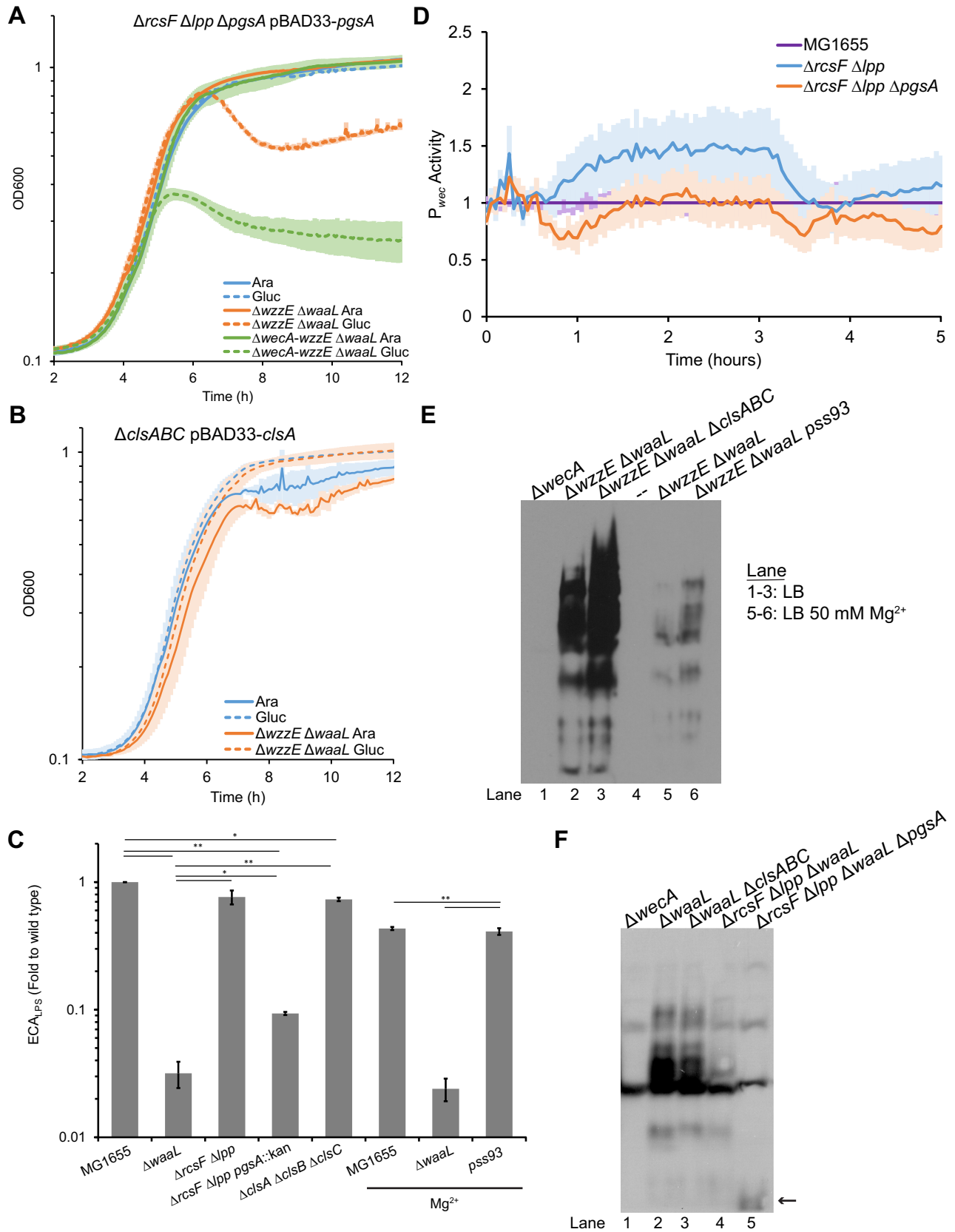
FIG 6 Phosphatidylglycerol is the substrate for the synthesis of ECA$_{PG}$. (A) PG is not essential in strains where pressure on lipoprotein maturation is relieved ($\Delta rcsF$ Δlpp). Growth curves were performed with PgsA depletion strains in this background alone (blue), with $wzzE$ and $waaL$ deletions

folding, plays a role in the lysis observed in the ECA$_{PG}$ only strain, in addition to specific inhibition of ECA$_{PG}$ synthesis.

To investigate whether the shared steps in ECA synthesis are intact in strains without specific phospholipids, we assayed ECA$_{LPS}$ levels in strains lacking either PG, CL, or PE. Preventing synthesis of CL ($\Delta clsA$ $\Delta clsB$ $\Delta clsC$) or PE (pss93) caused only minimal decreases in ECA$_{LPS}$ levels (Fig. 6C); however, preventing PG synthesis ($\Delta rcsF$ Δlpp $\Delta pgsA$) caused a large decrease in ECA$_{LPS}$ levels. ECA$_{LPS}$ levels were, nevertheless, detectable. P$_{wec}$ activity was not changed in this strain, suggesting that the decrease is posttranscriptional (Fig. 6D) and may be due to regulation of synthesis or membrane protein misfolding in the absence of PG leading to decreased efficiency in ECA or LPS synthesis.

To finalize our conclusion that PG was the lipid donor for the synthesis of ECA$_{PG}$, we attempted to build deletion strains for the PG, PE, and CL synthesis genes in the $\Delta wzzE$ $\Delta waaL$ background. Although PE makes up 75% of the membrane in wild-type cells (39), strains with disruption of pssA are viable as long as they are maintained with 20 to 50 mM Ca^{2+} or Mg^{2+} (72). We were able to build strains with a pssA disruption allele (pss93) and with a $\Delta clsA$ $\Delta clsB$ $\Delta clsC$ background. When we assayed these strains, we found that both strains had increased ECA$_{PG}$ levels compared to the $\Delta wzzE$ $\Delta waaL$ strain (Fig. 6E, lane 2 versus lane 3 and lane 5 versus lane 6). We also observed a large decrease in ECA$_{PG}$ levels with Mg^{2+} treatment. Consistent with the growth defects we observed in depletion strains (Fig. 6A), we were not successful in building a $\Delta rcsF$ Δlpp $\Delta wzzE$ $\Delta waaL$ $\Delta pgsA$ background and reviving it from frozen stocks. Therefore, we investigated the effect of loss of PG and CL in a $\Delta waaL$ background. ECA$_{CYC}$ cannot be detected through immunoblot and so ECA$_{PG}$ levels can be assayed in this strain. However, posttranscriptional regulation of ECA$_{PG}$ synthesis occurs in with $\Delta waaL$ that decreases ECA$_{PG}$ synthesis (56). ECA$_{PG}$ was observed in the strain lacking CL and the $\Delta rcsF$ Δlpp background strain (Fig. 6F, lanes 2 to 4). However, in the strain without PG, only one very low molecular weight band was observed other than those present in the $\Delta wecA$ strain (Fig. 6F, lane 1 versus lane 5). This band may represent an Und-PP-linked ECA precursor or a degradation product thereof. Together, our data demonstrate that PG is the lipid donor for the synthesis of ECA$_{PG}$.

DISCUSSION

In this work, we investigated the identity of the lipid donor for synthesis of ECA$_{PG}$. Our data demonstrate through several lines of evidence that this donor is PG and that other phospholipids cannot efficiently substitute when PG is lost. (i) Changes in flux through phospholipid biosynthesis expected to increase PG production increase ECA$_{PG}$ levels, while changes expected to decrease PG production decrease ECA$_{PG}$ levels. (ii) Depletion of PG levels when ECA precursors cannot be made into other forms of ECA

FIG 6 Legend (Continued)

(orange), or an isogenic strain with a wecA deletion (green) to investigate growth phenotypes caused by loss of ECA and disruptions to peptidoglycan biosynthesis caused by accumulation of ECA precursor. Cultures were diluted 1:10,000 and treated with arabinose to induce expression from the P$_{BAD}$ promoter (solid lines) or glucose to repress expression (hashed lines). Depletion of PgsA in the $\Delta rcsF$ Δlpp background allowed full growth. Cells without ECA ($\Delta wecA$-wzzE $\Delta waaL$) stopped growing when PG was depleted and then showed a slow decrease in OD$_{600}$. However, cells making only ECA$_{PG}$ ($\Delta wzzE$ $\Delta waaL$) grew to stationary phase and then quickly lysed. The data are means of four biological replicates ± the SEM. (B) CL is not essential. Cultures for growth curves were grown as in panel A. Depletion of ClsA in strains with deletions of clsA, clsB, and clsC did not cause lysis either alone or when combined with $\Delta wzzE$ $\Delta waaL$. The data are means of three biological replicates ± the SEM. (C) ECA$_{LPS}$ levels were assayed by WGA staining of strains with deletions in the indicated genes. Where indicated, cultures were grown with 50 mM Mg^{2+} to allow survival of strains lacking PE (pss93). Strains lacking CL and PE had small but significant decreases in ECA$_{LPS}$ levels. The strain lacking PG had a large decrease in ECA$_{LPS}$; however, ECA$_{LPS}$ levels remained detectable. The data are the mean of three biological replicates ± the SEM. *, $P < 0.05$ (by paired t test); **, $P < 0.005$ (by paired t test). (D) The activity of P$_{wec}$ was assayed as in Fig. 3. The data are shown as the fold of the relative luminescence to the wild type and are means of six biological replicates ± the SEM. No significant changes determined by t test were observed for contiguous data points. (E) ECA$_{PG}$ levels in strains without CL ($\Delta clsABC$) or without PE (pss93) were assayed in the $\Delta wzzE$ $\Delta waaL$ (ECA$_{PG}$ only) strain background. Cultures for lanes 5 and 6 were grown with 50 mM Mg^{2+}. The $\Delta wecA$ strain serves as a negative control. Both strains lacking CL and strains lacking PE retained production of ECA$_{PG}$. The images are representative of two independent experiments. (F) Since depletion of PG causes lysis in an ECA$_{PG}$ only background, the effect of the $\Delta pgsA$ mutation was assayed in a $\Delta waaL$ background where only ECA$_{PG}$ can be observed by immunoblotting. Immunoblot analysis was performed to assay ECA$_{PG}$ levels in strains without CL ($\Delta clsABC$) or without PG ($\Delta rcsF$ Δlpp $\Delta pgsA$). The $\Delta wecA$ strain serves as a negative control, indicating nonspecific bands. Deletion of waaL in the presence of ECA$_{CYC}$ causes inhibition of ECA$_{PG}$ synthesis (56), leading to lower ECA levels overall. ECA$_{PG}$ is observed in the strain without CL. However, only a low-abundance, low-molecular-weight band (arrow) is observed in the strain without PG. The images are representative of three independent experiments.

causes cell lysis, even where PG is otherwise nonessential. (iii) ECA_{PG} is produced in the absence of PE or CL and is not produced in the absence of PG. These results also suggest preventing transfer of ECA from Und-PP to the lipid donor may cause sufficient peptidoglycan stress to inhibit growth despite the Und-PP released when ECA subunits are polymerized.

In our previous investigations (56), we used this peptidoglycan stress phenotype to identify a system that regulates the production of ECA_{PG}. ElyC and ECA_{CYC} work together to posttranscriptionally inhibit the production of ECA during normal growth conditions (56). We envision this system as a feedback pathway that regulates ECA_{PG} production based on levels of ECA_{CYC}. We can now add the identity of the lipid donor for ECA_{PG} synthesis to our model of ECA_{PG} synthesis. In our model, polymerized ECA is removed from Und-PP and transferred to the DAG-P portion of a PG molecule, releasing glycerol into the periplasm and Und-PP into the outer leaflet of the IM. The protein(s) responsible for this reaction would be regulated by ElyC in a manner controlled by ECA_{CYC}. This model provides a framework for further investigations of the synthesis of ECA_{PG} including the following: identification of the gene(s) involved in synthesizing ECA_{PG} from PG and the ECA precursor, determining whether the synthesizing enzyme(s) have specific preferences for PG fatty acid composition, the details of the reaction forming ECA_{PG} and of the mechanism of regulation by ElyC and ECA_{CYC}, and the pathway leading to ECA_{PG} surface exposure.

In addition to regulation of ECA_{PG} production by ElyC and ECA_{CYC} (56), our results have revealed other mechanisms through which ECA_{PG} production can be regulated, specifically in strains lacking ECA_{CYC} and so full activity of the ElyC-ECA_{CYC} pathway. We relied in our overexpression experiments on the ability of ECA_{PG} production to be altered by lipid donor availability and found the changes in ECA_{PG} production to be robust, with both quite large increases and decreases in ECA_{PG} levels (for example see Fig. 3A, lane 6 versus lane 10). These data are especially interesting given that the ratio of PG to CL varies based on growth phase with the amount of CL increasing in stationary phase at the expense of PG levels (39, 73). Our data suggest that this change may have a direct effect on the synthesis of ECA_{PG}.

Beyond regulation by substrate availability, we observed several changes in phospholipid-synthesis gene expression that led to changes in the activity of the promoter region of the *wec* operon that encodes the genes necessary to synthesize the ECA precursor. The largest of these changes was the 4- to 5-fold increase in P_{wec} activity we observed when we overexpressed *pgsA* (Fig. 4B). These data demonstrate an interplay between phospholipid synthesis and ECA synthesis and suggest that there may be feedback from membrane conditions that affects ECA synthesis. We also observed a large decrease in ECA_{PG} levels when cells were grown with a high concentration of Mg^{2+} (Fig. 6E). This decrease was present in both the strain with normal phospholipid composition and the strain lacking PE. It is possible that this decrease could be due to changes in Mg^{2+} sensing transcriptional regulation pathways, due to changes in OM order due to increase bridging between LPS molecules, or to changes in the activity of enzymes in the ECA synthesis pathway. Experiments investigating these phenotypes are ongoing in our lab.

As mentioned above, our results demonstrate, while cells producing only ECA_{PG} and not the other forms of ECA lyse in the absence of PG, cells without ECA deplete earlier and do not continue to grow even when PG is nonessential (Fig. 6A). The lack of growth in the strain without ECA and the longer growth before lysis in the ECA_{PG}-only strain are intriguing. Both of these strains lack ECA_{CYC}, which is important for maintaining the OM permeability barrier (26); however, it may be that ECA_{PG} plays an important role in maintaining envelope integrity and that loss of ECA_{PG} before depletion is the reason that the cells without ECA cease growth earlier. Alternatively, it may be that accumulation of Und-PP-linked ECA precursors lead to further growth before lysis either through envelope stress response activation (60, 61, 63) or through a direct role of ECA precursors in envelope integrity (74). These questions warrant future investigation.

It is interesting to compare the synthesis of ECA_{PG} to another glycolipid with a very similar structure, membrane protein intergrase (MPIase) (reviewed in reference 75). MPIase consists of the same carbohydrate unit as ECA_{PG} attached to DAG through a pyrophosphate diester bond (76). This molecule is essential and has been implicated in insertion of proteins into the IM in a Sec-independent manner and in Sec-dependent protein translocation (75–81). Unlike ECA_{PG}, the MPIase sugar subunit is built on DAG-PP with the first step being attachment of GlcNAc-P to DAG-P, a reaction catalyzed by CdsA and YnbB (77). The lipid donor for this reaction is CDP-DAG. The synthesis of MPIase has previously been shown to be independent of ECA synthesis (82), and our results confirm that these molecules use distinct lipid donors. The ECA genes necessary for biosynthesis of the ECA sugars and the formation of the ECA chain has also been shown to be dispensable for MPIase formation (82), although later results suggest they may contribute to some extent (74). Exploring the differences and similarities between these biosynthetic pathways will provide interesting insights into both pathways.

Our identification of PG as the lipid donor for ECA_{PG} biosynthesis provides an interesting parallel with lipoprotein synthesis: Lgt uses PG as the donor to transfer DAG to nascent lipoproteins (69). Despite this, PE is the most abundant phospholipid in the *E. coli* membrane. It is possible that these pathways use PG as the lipid donor due to the asymmetry of the IM, which contains more PE in the inner leaflet than the outer leaflet (40). It is also possible that it is advantageous to the cell to use a less abundant membrane constituent for lipid modification as it can be more easily regulated, such as by decreasing PG levels in favor of CL during stationary phase (39, 73). Finally, the use of PG as a donor may be due to relative ease of recycling the glycerol headgroup compared to recycling of ethanolamine. These are intriguing questions for future study.

MATERIALS AND METHODS

Strains and growth conditions. The strains used in these experiments are listed in Table 1. Cultures were grown in LB Lennox at 37°C unless otherwise noted. Where necessary for plasmid maintenance, medium was supplemented with 20 mg/L chloramphenicol and/or 25 mg/L kanamycin. Where indicated, cultures were supplemented with 0.2% glucose, 0.2% arabinose, or 50 mM $MgSO_4$. Unless otherwise noted, deletion alleles are from the Keio collection and were moved into our strains using P1*vir* transduction (83, 84). A deletion allele for *pgsA* was constructed using λ-Red recombineering and the primers listed in Table S1 (85). Kanamycin resistance cassettes were flipped out using the Flp recombinase-FRT system as has been described (85). Mikhail Bogdanov and William Dowhan (McGovern Medical School, University of Texas—Houston) provided us with the kind gift of a previously published *pssA* disruption allele, *pss93::kan* (72). Strains with the *pss93::kan* allele were constructed by transforming strains with pDD72GM (86), which carries a wild-type *pssA* allele, transducing in the *pss93::kan* allele, and then curing the temperature sensitive pDD72GM plasmid.

IPTG inducible overexpression constructs were derived from the non-GFP-tagged version of the ASKA collection of *E. coli* gene overexpression plasmids and were transformed into the indicated strains using standard molecular biology techniques. For construction of PgsA, PgpC, and ClsA depletion strains, *pgsA*, *pgpC*, and *clsA* were cloned into pBAD33 using Gibson Assembly (65). Briefly, pBAD33 was linearized by PCR using the pBAD33 F and R primers (see Table S1). The inserts amplified from MG1655 genomic DNA using the primers listed in Table S1 from 30 bp upstream of the open reading frame (ORF) to the end of the ORF. The PCR fragments were assembled using HiFi Assembly Master Mix (New England Biolabs) according to the manufacturer's instructions.

Quantification of ECA levels. ECA_{LPS} levels were quantified by staining cells with Alexa Fluor 488-conjugated WGA (Thermo Fisher Scientific) as we have described earlier (56). Briefly, 250-μL aliquots of washed overnight cultures were combined with 10 μg/mL wheat germ agglutinin (WGA) in phosphate-buffered saline (PBS) and then incubated for 10 min at room temperature. Cells were washed twice and resuspended in PBS. The fluorescence (excitation, 485 nm; emission, 519 nm) and the optical density at 600 nm (OD_{600}) were read in a BioTek Synergy H1 plate reader. Fluorescence relative to the OD was calculated.

ECA_{PG} was detected through immunoblot analysis as we have described (26, 56). Briefly, samples from overnight cultures were normalized to equal OD_{600} and resuspended in BugBuster protein extraction reagent (Millipore Sigma), followed by an equal volume of Laemmli sample buffer (Bio-Rad). Samples were run on 12% acrylamide gels and transferred to nitrocellulose. Blots were probed with α-ECA antibody at a 1:30,000 dilution and a goat α-rabbit-HRP secondary antibody (Prometheus) at a 1:100,000 dilution. α-ECA antibody was kindly provided by Renato Morona (University of Adelaide). Detection was performed using Prosignal Pico ECL (Prometheus) using Prosignal ECL-blotting film (Prometheus).

Luciferase reporter assays. Activity of the P_{wec} promoter was assayed with a plasmid-based P_{wec}: *luxCDABE* reporter as we have described (56) with minor modifications. Cultures were grown overnight in media without IPTG. For reporter assays, strains were subcultured 1:100 into media with the indicated concentration of IPTG. The luminescence and the OD_{600} were assayed in technical quadruplicate in a

TABLE 1 Strains used in this study

Strain	Genotype	Source or reference	Plasmids and alleles (source reference)
MG1655	K-12 F⁻ λ⁻ rph-1	87	
AM334	MG1655 ΔwecA	26	
AM366	MG1655 ΔwaaL	26	
AM395	MG1655 ΔwzzE ΔwaaL	26	
AM1357	MG1655 ΔclsA ΔclsB ΔclsC	This study	
AM1453	MG1655 ΔclsA ΔclsB ΔclsC ΔwaaL::Kanʳ	This study	
AM1358	MG1655 ΔwzzE ΔwaaL ΔclsA ΔclsB ΔclsC	This study	
KM031	MG1655 pss93::Kanʳ	This study	pssA93::Kanʳ allele (72)
KM032	MG1655 ΔwzzE ΔwaaL pss93::Kanʳ	This study	
AM1340	MG1655 ΔrcsF Δlpp	This study	
AM1421	MG1655 ΔrcsF Δlpp ΔpgsA::Kanʳ	This study	
AM1352	MG1655 ΔrcsF Δlpp ΔwaaL	This study	
AM1452	MG1655 ΔrcsF Δlpp ΔwaaL ΔpgsA::Kanʳ	This study	
AM1138	MG1655 pCA24N	56	Plasmid is ASKA collection empty vector (88); Cmʳ
AM1144	MG1655 ΔwzzE ΔwaaL pCA24N	This study	Plasmid is ASKA collection empty vector (88); Cmʳ
AM1220	MG1655 pCA24N-cdsA	This study	Plasmid (88)
AM1286	MG1655 ΔwzzE ΔwaaL pCA24N-cdsA	This study	
AM1326	MG1655 pCA24N-pssA	This study	Plasmid (88)
AM1327	MG1655 ΔwzzE ΔwaaL pCA24N-pssA	This study	
AM1176	MG1655 pCA24N-psd	This study	Plasmid (88)
AM1283	MG1655 ΔwzzE ΔwaaL pCA24N-psd	This study	
AM1324	MG1655 pCA24N-pgsA	This study	Plasmid (88)
AM1325	MG1655 ΔwzzE ΔwaaL pCA24N-pgsA	This study	
AM1174	MG1655 pCA24N-pgpA	This study	Plasmid (88)
AM1282	MG1655 ΔwzzE ΔwaaL pCA24N-pgpA	This study	
AM1328	MG1655 pCA24N-pgpC	This study	Plasmid (88)
AM1329	MG1655 ΔwzzE ΔwaaL pCA24N-pgpC	This study	
AM1330	MG1655 pCA24N-clsA	This study	Plasmid (88)
AM1331	MG1655 ΔwzzE ΔwaaL pCA24N-clsA	This study	
AM1177	MG1655 pCA24N-clsB	This study	Plasmid (88)
AM1284	MG1655 ΔwzzE ΔwaaL pCA24N-clsB	This study	
AM1332	MG1655 pCA24N-clsC	This study	Plasmid (88)
AM1333	MG1655 ΔwzzE ΔwaaL pCA24N-clsC	This study	
KM014	MG1655 ΔwzzE ΔwaaL pCA24N pJW15	This study	pJW15 is an empty lux reporter (89); Kanʳ
KM015	MG1655 ΔwzzE ΔwaaL pCA24N pJW15-P$_{wec}$	This study	pJW15-P$_{wec}$ reporter plasmid (88)
KM011	MG1655 ΔwzzE ΔwaaL pCA24N-cdsA pJW15-P$_{wec}$	This study	
KM008	MG1655 ΔwzzE ΔwaaL pCA24N-pssA pJW15-P$_{wec}$	This study	
KM009	MG1655 ΔwzzE ΔwaaL pCA24N-psd pJW15-P$_{wec}$	This study	
KM005	MG1655 ΔwzzE ΔwaaL pCA24N-pgsA pJW15-P$_{wec}$	This study	
KM006	MG1655 ΔwzzE ΔwaaL pCA24N-pgpA pJW15-P$_{wec}$	This study	
KM012	MG1655 ΔwzzE ΔwaaL pCA24N-pgpC pJW15-P$_{wec}$	This study	
KM007	MG1655 ΔwzzE ΔwaaL pCA24N-clsA pJW15-P$_{wec}$	This study	
KM013	MG1655 ΔwzzE ΔwaaL pCA24N-clsB pJW15-P$_{wec}$	This study	
KM010	MG1655 ΔwzzE ΔwaaL pCA24N-clsC pJW15-P$_{wec}$	This study	
AM1225	MG1655 pJW15	56	
AM1455	MG1655 ΔrcsF Δlpp pJW15	This study	
AM1456	MG1655 ΔrcsF Δlpp ΔpgsA pJW15	This study	
AM1162	MG1655 pBAD33	56	Plasmid (65); Cmʳ
AM1158	MG1655 ΔwzzE ΔwaaL pBAD33	56	
AM1425	MG1655 ΔpgpB ΔpgpA pBAD33-pgpC ΔpgpC	This study	
AM1426	MG1655 ΔwzzE ΔwaaL ΔpgpB ΔpgpA pBAD33-pgpC ΔpgpC	This study	
AM1407	MG1655 pBAD33-pgsA ΔpgsA	This study	
AM1408	MG1655 ΔwzzE ΔwaaL pBAD33-pgsA ΔpgsA	This study	
AM1409	MG1655 ΔrcsF Δlpp pBAD33-pgsA ΔpgsA	This study	
AM1412	MG1655 ΔrcsF Δlpp ΔwzzE ΔwaaL pBAD33-pgsA ΔpgsA	This study	
AM1459	MG1655 ΔrcsF Δlpp ΔwaaL ΔwecA-wzzE pBAD33-pgsA ΔpgsA	This study	ΔwecA-wzzE allele (56)
AM1413	MG1655 ΔclsA ΔclsB pBAD33-clsA ΔclsC	This study	
AM1414	MG1655 ΔwzzE ΔwaaL ΔclsA ΔclsB pBAD33-clsA ΔclsC	This study	

BioTek Synergy H1 plate reader every 3 min for 5 h. The luminescence relative to OD_{600} was calculated, and technical replicates were averaged. Then, the fold value of each sample to the empty vector control for each time point was calculated.

Depletion growth curves. For depletion experiments, the indicated strains were grown overnight with arabinose. Then, cultures were washed once in plain Luria-Bertani medium and diluted 1:500 or 1:10,000, as indicated, into media with either arabinose to induce expression from the P_{BAD} plasmid or glucose to repress expression. For strains where the phospholipid was nonessential (i.e., CL, PG in a Δlpp $\Delta rcsF$ background), cultures were maintained with chloramphenicol during the course of the growth curve. Cultures were transferred to a 24-well plate and were incubated shaking at 37°C in a BioTek Synergy H1 plate reader. The OD_{600} was assayed every 5 min for 12 h.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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