Phosphatidylglycerol (PG) makes up 5–20% of the phospholipids of *Escherichia coli* and is essential for growth in wild-type cells. PG is synthesized from the dephosphorylation of its immediate precursor, phosphatidylglycerol phosphate (PGP) whose synthase in *E. coli* is PgsA. Using genetic, biochemical, and highly sensitive mass spectrometric approaches, we identified an alternative mechanism for PG synthesis in *E. coli* that is PgsA independent. The reaction of synthesis involves the conversion of phosphatidylethanolamine and glycerol into PG and is catalyzed by ClsB, a phospholipase D-type cardiolipin synthase. This enzymatic reaction is demonstrated herein both in vivo and in vitro as well as by using the purified ClsB protein. When the growth medium was supplemented with glycerol, the expression of *E. coli* ClsB significantly increased PG and cardiolipin levels, with the growth deficiency of *pgsA* null strain also being complemented under such conditions. Identification of this alternative mechanism for PG synthesis not only expands our knowledge of bacterial anionic phospholipid biosynthesis, but also sheds light on the biochemical functions of the *cls* gene redundancy in *E. coli* and other bacteria. Finally, the PGP-independent PG synthesis in *E. coli* may also have important implications for the understanding of PG biosynthesis in eukaryotes that remains incomplete.

In *Escherichia coli*, depending on growth phase and conditions, phosphatidylglycerol (PG) can account for 5–20% of the total phospholipid content, with the remainder mainly comprising phosphatidylethanolamine (PE) and cardiolipin (CL) (1, 2). Besides serving as a basic membrane component (2, 3), PG also plays critical roles in SecA-dependent protein translocation, is involved in the initiation of DNA replication at oriC, and is required for the proper location of the division septum at mid-cell (4–7). In *E. coli*, PG is synthesized by a system of enzymes first described by Eugene Kennedy and colleagues (1) (Fig. 1). In the initial steps of the pathway, the intermediate cytidine diphosphate-diacylglycerol (CDP-DAG) is formed from a condensation reaction between phosphatidic acid (PA) and cytidine triphosphate. The enzyme phosphatidylglycerol phosphate (PGP) synthase (PgsA) then catalyzes a reaction between CDP-DAG and glycerol-3-phosphate to yield PGP, which in turn is dephosphorylated to PG by three PGP phosphatases (PGpA, PGpB, and PGpC) (8). PG is a direct precursor of CL, whose synthesis is mediated by three CL synthase genes in *E. coli* (2).

Deletion of the *pgsA* gene in *E. coli* blocks the synthesis of PG and CL (5, 9, 10). The Δ*pgsA* mutant strain is not viable unless it also harbors mutations in the *lpp* and *rcsF* genes (10). The *lpp* mutation prevents accumulation of a nascent major outer membrane lipoprotein that requires PG for its modification (5, 9, 11), whereas the *rcsF* mutation prevents the lysis of Δ*pgsA* cells at the temperature of 37–42 °C (12). Thin-layer chromatography (TLC) and direct injection MS analysis have shown that the Δ*pgsA* strain (UE54) lacks detectable PG and CL, but accumulates phosphatidic acid, CDP-diacylglycerol, and N-acetylphosphatidylethanolamine (N-acetyl-PE) (13).

In this study, using highly sensitive normal phase liquid chromatography/mass spectrometry (LC/MS) (2, 14), we unexpectedly detected small amounts of PG species in Δ*pgsA* cells. We found that the formation of PG species in these mutant cells is catalyzed by cardiolipin synthases (Cls) with ClsB playing a dominant role. Furthermore, we purified the ClsB protein and demonstrated its PG synthesis activity using PE and glycerol as substrates. Elucidation of this PGP-independent PG synthesis not only expands our knowledge of anionic phospholipid biosynthesis and metabolism but also sheds light on the biochemical functions of the multiple *cls* genes found in *E. coli* and many other bacteria.

**Results**

*LC/MS Reveals pgsA-independent and cls-dependent PG Formation in *E. coli*—*Under the normal phase LC conditions used in this study, the major *E. coli* glycerophospholipids elute in the following order: PG (~11–12 min), CL (~12–14 min), PE (~16–17 min), and PA (~20–21 min). As identified by the exact mass measurement and MS/MS analysis, wild-type *E. coli* cells contain mainly PG (32:1), PG (33:1), PG (34:2), and PG (34:1), where the numbers of acyl chain carbon atoms and double bonds or double bond equivalents (such as cyclopropane) appear in parentheses (Fig. 24). Surprisingly, when the total lipid extract of Δ*pgsA* (BKT25) cells was subjected to the same*
three cls genes was not deleted, indicating that each of the cls genes might contribute to in vivo PG synthesis in the ΔpgsA mutant. However, the relative contributions of the three cls genes to PG synthesis remain to be delineated (for reasons that the relative PG levels in E. coli vary drastically with growth conditions).

To further assess whether Cls proteins are involved in the synthesis of PG in vivo, we cloned each of the cls genes into the arabinose-inducible pBAD30 expression vector (15) and introduced the plasmids into the ΔpgsA ΔclsABC (BKT29) mutant strain. The transformants were grown to stationary phase (A600 of 1~2) in medium supplemented with carbenicillin and 0.02% arabinose. LC/MS/MS analysis of the total lipids extracted from these cells indicated that PG levels were increased upon ClsB expression but not when ClsA or ClsC were expressed (data not shown). In all cases, neither PG nor CL production was discernible by TLC analysis (Fig. 3A).

Synthesis of PG by ClsB Requires Glycerol—All three E. coli Cls belong to the phospholipase D (PLD) superfamily, with each containing two HKD motifs (Fig. 4) (2, 16). PLD catalyzes the hydrolysis of phospholipids into PA and the corresponding headgroup (17). It is also known that in the presence of a primary alcohol (e.g. butanol), PLD can catalyze a trans-phosphatidylation reaction to yield a phosphatidyl alcohol (17, 18). We speculated that any PG formed by Cls would result from such a trans-phosphatidylation reaction, and thus supplemented the growth medium with glycerol to a final concentration of 0.4% (v/v). As shown by TLC analysis of the total lipids extracted from stationary phase cells (Fig. 3B), CIsB expression in conjunction with glycerol supplementation restored the production of PG and CL in the ΔpgsA ΔclsABC (BKT29) mutant to wild-type levels. The identities of CIsB-produced PG and CL species were further confirmed by normal phase LC/MS/MS analysis of total lipid extracts from cells expressing this protein (Fig. 3, C and D). In contrast, the levels of PG and CL upon expression of ClsA or ClsC remain very low (Fig. 3B). As such, we chose to focus on the role of CIsB in the cls-mediated PG formation. The glycerol-dependent PG synthesis by CIsB was also demonstrated by an in vitro assay using CIsB-expressing BKT29 membranes (supplemental Fig. S1).

CIsB Synthesizes PG Using PE as a Substrate—To identify the substrate (or phosphatidyl donor) used for PG synthesis by CIsB, we paid special attention to PE as it was noted that the composition of PG acyl chains (Fig. 3C) very closely matched those of PE (Fig. 3E), suggesting that the phosphatidyl groups of the PG species might be directly transferred from PE species in the ΔpgsA ΔclsABC (BKT29) mutant. To test whether PG could indeed be formed from PE by CIsB, 32P-labeled PE was prepared from BKT29 E. coli cells by supplementing the growth medium with [32P]PO4. Following fractionation on a DEAE column, the partially purified 32P-labeled PE substrate was incubated with cell membranes derived from ΔpgsA ΔclsABC (BKT29) cells expressing a vector control (pBAD30), wild-type CIsB, or a CIsB-H113A mutant. As shown by TLC and phosphorimaging analysis (Fig. 5), a significant amount of 32P-labeled PG was generated by membranes expressing wild-type CIsB, indicating that PE was indeed converted into PG by CIsB. In contrast, no

In conclusion, our results indicate that PG is synthesized by E. coli Cls proteins and that PG synthesis is not only dependent on glycerol but also requires PE as the phosphatidyl donor. This study provides new insights into the role of PG in E. coli and the mechanisms involved in its synthesis.
PG was formed when membranes expressing the vector control or the ClsB-H113A mutant were used (Fig. 5).

To definitively demonstrate that ClsB catalyzes the conversion of PE to PG in vitro, we carried out this purification of ClsB protein. For this, pET vector constructs encoding C terminally FLAG-tagged wild-type ClsB or the protein containing mutations in either of the two HKD motifs (H113A and H291A) were transformed into E. coli strain C41 (DE3). The FLAG-tagged
proteins were purified to homogeneity using anti-FLAG M2 affinity gel resin (Fig. 6A). To perform in vitro enzyme activity assays, synthetic PE (17:0/14:1) and penta-deuterated glycerol (d₅-glycerol) were mixed with each of three recombinant ClsB proteins (Fig. 6B). After incubation, the reaction solutions were subjected to lipid extraction followed by normal phase LC/MS/MS analysis. As shown in Fig. 6C, the wild-type ClsB-FLAG protein produced the expected product, d₅-PG whose [M-H]- ion is observed at m/z 710.5. In contrast, the two catalytically inactive HKD mutant proteins, ClsBH113A-FLAG and ClsBH291A-FLAG, were unable to convert PE into PG (Fig. 6, D and E).

MS/MS analysis further confirmed the m/z 710.5 species produced by ClsB (Fig. 6C) as the [M-H]- ion of d₅-PG derived from PE (17:0/14:1) and d₅-glycerol. As shown in Fig. 6F, the m/z 158.03 product ion derived is from the cyclic phosphodiester of penta-deuterated glycerol; the corresponding m/z 153.0 ion is a signature product ion in the MS/MS spectra of non-deuterated glycerophospholipids (19). The m/z 225.190 and 269.252 ions correspond to the carboxylic anions of the C17:0 and C14:1 fatty acids, respectively, with both being derived from the synthetic PE (17:0/14:1) substrate. The MS/MS spectrum of the [M-H]- ion of PE (17:0/14:1) and its fragmentation scheme are shown in Fig. 6G.

Other phospholipids were tested as potential substrates of ClsB, including synthetic standards of CDP-DAG, PA, and PS. None of these was utilized by ClsB to synthesize PG.

ClsB Uses Two PG Molecules to Synthesize CL—Although the role of ClsB as a cardiolipin synthase has been well established (2, 16), characterization of its substrate(s) remains incomplete. Previously, PG was identified as a substrate for ClsB, however, this finding was based on an in vitro study using membrane preparations (16), and as such could not rule out the possible use of other membrane-derived species as substrate(s). The availability of purified ClsB protein allowed us to address this question. Using synthetic PG (17:0/14:1) as substrate, ClsB-FLAG produced a single CL species whose molecular weight is consistent with the condensation of two synthetic PG (17:0/14:1) molecules (Fig. 7). This result provides the first experimental evidence that ClsB employs the classical “prokaryotic” mechanism to synthesize CL (3).

ClsB Complements Deficiencies of a ΔpgsA Mutant—An E. coli pgsA null mutant requires mutations in the lpp and rcsF genes to be viable (9, 10). Given that over-expression of ClsB leads to the production of near wild-type levels of PG and CL in the BKT29 mutant (ΔpgsA ΔclsABC, derived from UE54) when the growth medium was supplemented with glycerol, we tested whether ClsB expression could functionally complement the growth deficiency of a pgsA::KanR deletion in a wild-type background (i.e. without requiring additional suppressor mutations). Accordingly, two pBAD30 constructs containing either pgsA or clsB were individually transformed into the wild-type E. coli strain (W3110), followed by deletion of the pgsA gene from the chromosome by P1::vir transduction, as described previously (2).

As demonstrated by a spot assay (Fig. 8A), the pBAD-clsB pgsA::KanR strain (CL15) required the addition of both arabinose and glycerol for growth. For comparison, the growth of a pBAD-pgsA pgsA::KanR strain (CL14) required neither additional arabinose nor glycerol, suggesting that a minimal level of pgsA expression is sufficient to maintain cell viability. In fact, over-expression of pgsA seems detrimental to cells, as reflected by the smaller colonies that appeared upon induction with arabinose (Fig. 8A).
Total lipid analysis by TLC shows that expression of ClsB produced significant levels of PG and CL in the \textit{pgsA}\textsuperscript{::}Kan\textsuperscript{R} strain (CL15), comparable with those in the wild-type or the \textit{pgsA}-covered /H9004\textit{pgsA} strains (Fig. 8B). These results demonstrated that with glycerol supplementation, ClsB expression could sufficiently complement the deficiencies of the /H9004\textit{pgsA} mutant in cell growth (without additional suppressor mutations), as well as in PG and CL synthesis.

**Discussion**

Pathways for the biosynthesis of the major \textit{E. coli} glycerophospholipids (including PE, PG, and CL) were first described by Hirschberg and Kennedy (3) over 40 years ago. The key technique employed for their investigations was radioisotopic labeling (3), primarily involving the use of \textsuperscript{32}P-labeled phosphate and \textsuperscript{14}C-labeled glycerol or fatty acids as tracing substances. Radiolabeling techniques also played critical roles in the discovery and characterization of the biosynthetic enzymes of these glycerophospholipids (1). Although the power of radiolabeling techniques is unquestionable, such approaches are limited in terms of molecular specificity and in detecting minor species in a complex mixture.

The past decade has seen a dramatic increase in the application of MS to lipid research (4, 20–22). Lipid MS, like all other biological applications of MS, was revolutionized by the introduction of soft ionization techniques, most notably electrospray ionization (ESI) (23) and matrix-assisted laser desorption ionization (MALDI) (24). Furthermore, the combination of online chromatographic separation with ESI/MS detection has greatly improved the applicability of MS to the analysis of complex mixtures. In particular, LC/MS has greatly improved the detection of a minor species by separating them from major species, thus reducing or eliminating ion signal suppressions from the latter. Signal suppression is a common issue in direct injection MS, which is often responsible for the failure to detect the minor species in a mixture. As demonstrated in this study, normal phase LC/MS is particularly well suited for the detection and characterization of glycerophospholipids as these lipid species can be separated based on their headgroups (or charges). Our detection of residual PG species in \textit{E. coli} /H9004\textit{pgsA} mutant cells benefited from the application of normal phase LC/MS. Indeed, the exceptional sensitivity and specificity afforded by modern high-resolution LC/MS/MS instruments have facilitated the discovery and characterization of numerous minor, novel lipid species (25–27), as well as functional elucidation of novel genes involved in lipid biosynthesis and metabolism (8, 28, 29), including the recent discovery of the first mammalian PGP phosphatase (29). In this study, the LC/MS-based approach was critical in revealing the PE to PG conversion previously unknown in \textit{E. coli}, extending the \textit{s+}udies on the inter-conversion of phospholipids observed in other bacteria (30–32). For example, Lombardi and Fulco (31) reported that PE could be directly made from PG in \textit{Bacillus megaterium}. Walton and Goldfine (32) reported \textit{in vitro} trans-phosphatidylation activities that allowed remodeling of the headgroups of \textit{Clostridium butyricum} membrane phospholipids, including the formation of PE from PG without the formation of PS intermediates. These observations were, however, all based exclusively on radioisotope-labeling techniques and lacked genetic and detailed biochemical characterization.
The physiological significance of the alternative mechanism of PG synthesis mediated by a CL synthase reported here is unknown. The ability to remodel pre-existing phospholipids may allow bacteria to adapt to changing environmental conditions without resorting to de novo phospholipid synthesis (32). The recent revelation that multiple paralogous cls genes exist in E. coli and many other bacteria calls for the study of their biochemical and physiological functions (1, 2, 33, 34). Given the dual functions of ClsB in E. coli phospholipid synthesis as described above, it is possible that some bacterial cls genes may be involved in inter-converting zwitterionic lipids (e.g. PE) to anionic phospholipids (e.g. PG) under certain environmental stress conditions.

Finally, the identification of an PGP-independent PG synthesis in E. coli may have important implications for fully understanding PG synthesis in eukaryotes (35). For example, the first yeast PGPase (Gep4) and mammalian PGPase (PTPMT1) were identified only during the last few years (29, 36). However, as reported in both studies, considerable amounts of PG species were still present in the PGPase-null yeast (ΔGep4) (36) and mammalian cell (ΔPTPMT1) mutants (29), indicating the existence of additional PGPase(s) or alternative mechanism(s) for PG synthesis in these eukaryotic cells. It is particularly intriguing that the acyl chain compositions of the PG species in ptpmt1-KO mouse embryonic fibroblasts differed from those of the accumulated PGP species, strongly implying that not all PG species were derived from PGP in such cells (29). Given that no PTPMT1 homologous genes have been identified in the mouse genome, it is tempting to speculate that some of these residual PG species in the ptpmt1-KO mouse embryonic fibroblast cells might be formed via a PLD-like trans-phosphatidylation activity. In a preliminary study, we demonstrated the conversion of PE into PG in yeast by overexpressing clsB in the ΔGep4 mutant cells (supplemental Fig. S2).

Experimental Procedures

Materials—Silica Gel 60 TLC plates, l-(-)-arabinose, and glycerol were obtained from EMD Chemicals (Gibbstown, NJ). Agar, tryptone, and yeast extract were purchased from Difco. Sodium chloride and HEPES were from VWR International (West Chester, PA). Tween 20 and the bicinechonic acid protein concentration determination kit were from Thermo Fisher Scientific (Waltham, MA). DEAE-cellulose (type DE52) was from Whatman (Florham Park, NJ). Isopropyl 1-thio-β-D-galactopyranoside was from Invitrogen. Reagent grade chloroform, methanol, hydrochloric acid, sulfuric acid, ethanol, and d5-glycerol were from Sigma. Synthetic PG, PA, PE, and CDP-DAG were from Avanti Polar Lipids (Alabaster, AL). [32P]PO4

FIGURE 7. Purified ClsB-FLAG protein uses two PG molecules to synthesize CL (the classical prokaryotic mode). After incubating purified ClsB-FLAG protein with synthetic PG (17:0/14:1) in a reaction buffer, the solution was subjected to lipid extraction and analysis by normal phase-LC/MS/MS. A, a single molecular species of CL was produced, and is detected as the [M-2H]2+ ion at m/z 659.449. This corresponds to a molecular weight of 1320.914, consistent with CL formed from the condensation of two synthetic PG (17:0/14:1) molecules. B, MS/MS analysis further supports that the CL product was synthesized from the condensation of two synthetic PG (17:0/14:1) molecules (C). The fragmentation scheme of major product ions observed in the MS/MS spectrum is illustrated (C).
was from PerkinElmer Life & Analytical Sciences (Waltham, MA).

**Bacterial Strains and Growth Conditions**—All *E. coli* strains used in this study are listed in Table 1. The *E. coli* MG1655 and W3110 strains are designated as the wild-type strains with respect to their glycerophospholipid composition. Liquid LB medium (10 g/liter of tryptone, 5 g/liter of yeast extract, and 10 g/liter of NaCl) was used to culture *E. coli*. Solid medium consisted of LB medium with the addition of 15 g/liter of agar. For strain selection purposes, cell cultures were supplemented with kanamycin (50 mg/liter) or carbenicillin (100 mg/liter). All strains were grown at 30 or 37 °C as noted, and cell density was measured as absorption at 600 nm (A600) using a DU spectrophotometer (Beckman Coulter, Brea, CA).

**Construction of Chromosomal Mutants Lacking cls Genes**—Chromosomal deletions of *cls* genes were constructed using the Keio Collection (the *E. coli* K12 single-gene deletion library), which served as the mutation donor (37). A gene of interest was replaced by a kanamycin cassette with flanking FLP sequences in each Keio mutant. P1<sub>vir</sub> transduction and subsequent kanamycin incision were performed according to a previous description (2). All strains with *cls* deletions were further verified by PCR.

**DNA Manipulations and Plasmid Constructions**—All plasmids used in this study are listed in Table 2. Plasmids were isolated using the Qiagen Spin Miniprep kit, and DNA fragments were isolated with the Qiaquick Spin Kits (Qiagen, Valencia, CA). Phusion High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA), T<sub>4</sub> DNA ligase (Invitrogen), and restriction endonucleases (New England Biolabs) were used according to the manufacturer’s instructions. Expression of *clsB* from the low-copy pBAD30 plasmid was carried out as described previously, using pBAD-B (2). Site-directed mutagenesis was used to mutate His residues in each HKD motif of ClsB to Ala (Fig. 1). For protein expression and purification purposes, the *clsB* gene and DNA encoding the corresponding His-Ala mutants were also cloned into pET vectors. DNA encoding a C-terminal FLAG epitope was fused to each gene, which were cloned into the XbaI and HindIII sites of vector pET21b. The resulting FLAG-tagged ClsB constructs were designated as pET21b-B, pET21b-B-H113A, and pET21b-B-H291A, respectively. To generate the pBAD-pgsA vector, the *pgsA* gene from W3110 cells was amplified and cloned into the XbaI and HindIII sites of plasmid pBAD30. All constructs were verified by DNA sequencing at the Duke DNA Sequencing Facility.

**FIGURE 8. Expression of ClsB complements the defects of a ΔpgsA::Kan<sup>R</sup> mutant in cell growth and in PG and CL synthesis.** A, a spot assay was conducted with a 10-fold series dilution of *E. coli* ΔpgsA::Kan<sup>R</sup> strains covered by plasmids expressing either *pgsA* or *clsB*. The strains were grown on LB plates in the presence or absence of 0.4% glycerol and/or 0.02% arabinose. The growth of the pBAD-clsB ΔpgsA::Kan<sup>R</sup> strain required both arabinose induction and glycerol supplementation. In contrast, the pBAD-pgsA ΔpgsA::Kan<sup>R</sup> strain required neither. B, TLC analysis of lipid extracts from wild-type, and pBAD-pgsA and pBAD-clsB ΔpgsA::Kan<sup>R</sup> *E. coli* strains.
**Synthesis of PG by an E. coli Cardiolipin Synthase**

**TABLE 1**

| Strain | Genotype | Source or Ref. |
|--------|-----------|----------------|
| W3110  | Wild-type | 2              |
| MG1655 | Wild-type | 2              |
| JW1241 | BW25113 ΔclsA::Kan<sup>+</sup> | 1              |
| JW0772 | BW25113 ΔclsB::Kan<sup>+</sup> | 1              |
| JW3150 | BW25113 ΔclsC::Kan<sup>+</sup> | 1              |
| UE54   | MG1655 Δpp2 ara714S rcsF:miniTn10Kam ΔpgsA::FRT-Kan-FRT | 2              |
| BKT25  | BKT25 ΔclsA, ΔclsB, ΔclsC, ΔymdB::Kan<sup>+</sup> | 2              |
| BKT29  | BKT25 ΔclsA, ΔclsB, ΔclsC, ΔymdB::Kan<sup>+</sup> | 2              |
| BKT30  | BKT25 ΔclsB::Kan<sup>+</sup> | 2              |
| CL01   | BKT25 ΔclsA::Kan<sup>+</sup> | This work      |
| CL02   | BKT25 ΔclsA (derived from CL01) | This work      |
| CL03   | BKT25 ΔclsA, ΔclsB::Kan<sup>+</sup> | This work      |
| CL04   | BKT25 ΔclsA, ΔclsB (derived from CL03) | This work      |
| CL06   | BKT25 ΔclsA, ΔclsC::Kan<sup>+</sup> | This work      |
| CL07   | BKT25 ΔclsA, ΔclsC (derived from CL06) | This work      |
| CL08   | BKT25 ΔclsB (derived from BKT30) | This work      |
| CL09   | BKT25 ΔclsB, ΔclsC::Kan<sup>+</sup> | This work      |
| CL10   | BKT25 ΔclsB, ΔclsC (derived from CL09) | This work      |
| CL11   | BKT25 ΔclsC::Kan<sup>+</sup> | This work      |
| CL12   | BKT25 ΔclsC (derived from CL11) | This work      |
| CL14   | W3110 pBAD30-ΔpgsA, ΔpgsA::Kan<sup>+</sup> | This work      |
| CL15   | W3110 pBAD30-Δ, ΔpgsA::Kan<sup>+</sup> | This work      |

**TABLE 2**

| Plasmid | Genotype | Source or Ref. |
|---------|-----------|----------------|
| pCP20   | FLP recombinase expression; AmpR CamR; temperature-sensitive replicon | 2              |
| pBAD30  | Low copy number expression plasmid | 2              |
| pBAD-A  | Expression of clsA on pBAD30 | 2              |
| pBAD-B  | Expression of clsB on pBAD30 | 2              |
| pBAD-B-H113A | Expression of clsB with H113A substitution on pBAD30 | 2              |
| pBAD-B-H291A | Expression of clsB with H291A substitution on pBAD30 | 2              |
| pBAD-C  | Expression of clsC on pBAD30 | 2              |
| pBAD-ΔpgsA | Expression of ΔpgsA on pBAD30 | This work      |
| pET21b  | Expression plasmid | Novagen        |
| pET21b-B | Expression of clsB-FLAG on pET21b | This work      |
| pET21b-B-H113A | Expression of clsB-FLAG with H113A substitution on pET21b | This work      |
| pET21b-B-H291A | Expression of clsB-FLAG with H291A substitution on pET21b | This work      |

**Lipid Extraction and TLC Analysis**—Lipid extraction was performed using a neutral Bligh-Dyer method (38). Cell pellets were washed twice with phosphate-buffered saline (PBS) before extraction. The washed pellets were then suspended in 1.9 ml of PBS, followed by the addition of 4.8 ml of methanol and 2.4 ml of chloroform to create a single-phase solution. The solution was incubated for 30 min at room temperature with intermittent mixing. After centrifugation at 3500 × g for 10 min, the supernatant was converted into a two-phase solution by adding 2.4 ml of PBS and 2.4 ml of chloroform. After centrifugation at 3500 × g for 10 min, the lower phase was recovered and dried under a stream of nitrogen gas. For TLC analysis, the dried lipid extracts were each dissolved in a 100 µl of chloroform/methanol (2:1, v/v). Approximately 1–5 µl of the solution was spotted onto a TLC plate. The TLC plate was developed in tanks equilibrated with chloroform/methanol/acetic acid (65:25:10, v/v). After drying the plate, lipids were visualized by spraying 10% sulfuric acid in ethanol (v/v), followed by charring on a hot plate (2).

**Isolation of [32P]PE**—To prepare radiolabeled [32P]PE, a 3-ml culture of E. coli BKT29 was grown overnight. Cells were harvested and washed three times by distilled water. The cells were diluted 100-fold with 10 ml of G56 medium (39) containing 100 µCi of [32P]orthophosphate and the cells were grown at 30 °C for 5 h. Lipid extraction and purification of PE using a DE52-cellulose column was carried out as previously described (40). To identify the PE-containing fractions, 10 µl of each fraction was loaded onto a silica TLC plate, which was developed using a solvent mixture consisting chloroform/methanol/acetic acid (65:25:10, v/v). After drying under hot air, the TLC plate was analyzed with a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). Fractions that contain [32P]PE were subjected to lipid extraction using a Bligh-Dyer method (38) as described above.

**Liquid Chromatography Mass Spectrometry of Lipids**—Normal phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column (5 µm, 25 cm × 2.1 mm) from Sigma. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v); mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v); mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. With a total flow rate of 300 µl/min, the LC eluent was injected into the ion spray source of a TripleTOF® 5600 quadrupole time-of-flight tandem mass spectrometer (AB SCIEX, Framingham, MA). Instrumen-
Synthesis of PG by an E. coli Cardiolipin Synthase

tal settings for negative ion ESI and MS/MS analysis of lipid species were as follows: electrospray ionization voltage (IS) = −4500 V; current gas (CUR) = 20 psi (pressure); gas-1 (GS1) = 20 p.s.i.; de-clustering potential (DP) = −55 V; and focusing potential (FP) = −150 V. The MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using Analyst TF1.5 software from AB SCEIX.

Complementation of the ΔpgsA::kanR Mutant—Plasmids pBAD-B and pBAD-pgsA were individually transformed into wild-type E. coli W3110 cells. The P1 phage was prepared from UE54, a strain containing a chromosomal ΔpgsA::FRT-kanR-ΔFRT cassette (Table 2). P1vir transductions of the W3110 strain containing either plasmid pBAD-B or pBAD-pgsA were performed as described above. After transduction, the recipient cells were incubated at 30 °C on LB agar plates containing 50 mg/liter of kanamycin, 100 mg/liter of carbencillin, 5 mM sodium citrate, arabinose (0, 0.002, 0.02, or 0.2%), and glycerol (0 or 0.4%). The surviving colonies were purified twice with the same growth condition and the chromosomal ΔpgsA::kanR cassette in each colony was verified by PCR. The ΔpgsA::kanR strains in the W3110 background complemented by plasmid pBAD-pgsA or pBAD-B were designated as CL14 and CL15, respectively.

To assess the cell viability of the CL14 and CL15 strains, different growth conditions were employed. The CL14 strain was grown in LB medium containing 100 mg/liter of carbencillin, whereas the CL15 strain was grown in LB medium containing 100 mg/liter of carbencillin, 0.2% arabinose, and 0.4% glycerol. For each strain, 100 μl of cell culture was harvested at an A600 of 1.0, washed twice with 1 ml of LB medium, and re-suspended in 100 μl of LB medium. A series of 10-fold dilutions were generated for each strain and 3 μl of each diluted solution were spotted onto LB agar plates containing 100 mg/liter of carbencillin, arabinose (0 and 0.02%), and glycerol (0 and 0.4%). Cells were grown at 30 °C overnight.

Expression and Purification of ClsB Proteins—For ClsB protein expression, the pET21b constructs were transformed into strain C41 (DE3) cells. 3-ml cultures were grown overnight at 37 °C and diluted 100-fold into 100 ml of LB medium containing 100 mg/liter of carbencillin. The cells were grown at 37 °C for an additional 6 h with shaking. For ClsB purification, the following procedures were conducted at 4 °C, unless otherwise noted. Cell pellets were washed twice with ice-cold PBS and suspended in 3 ml of lysis buffer containing 20 mM HEPES (pH 8.0) and 150 mM NaCl. The cells were lysed using a French press twice at 16,000 p.s.i., and spun at 10,000 × g for 30 min to remove cell debris. The supernatant was centrifuged at 200,000 × g for 1 h to collect membranes, which were homogenized and suspended in a buffer containing 20 mM HEPES (pH 8.0), 150 mM NaCl, 10% glycerol, and 0.5% Tween 20. The protein concentration from the resulting suspension was maintained at 1 mg/ml. To solubilize the membranes, the suspension was incubated with gentle rotation overnight, followed by another centrifugation at 200,000 × g for 1 h to remove insoluble materials. The resulting soluble fraction was loaded onto a 0.5-ml column of anti-FLAG M2 affinity gel (Sigma) and incubated at 4 °C with gentle rotation for 2 h. The column was washed twice by 10 ml of high salt buffer containing 20 mM HEPES (pH 8.0), 500 mM NaCl, and 0.008% Tween 20. ClsB-FLAG protein was eluted four times using 0.5 ml of elution buffer containing 20 mM HEPES (pH 8.0), 150 mM NaCl, 0.008% Tween 20, and 150 mg/liter of 3× FLAG peptide (Sigma). The eluted protein was dialyzed against low salt buffer (20 mM HEPES (pH 8.0), 150 mM NaCl, and 0.008% Tween 20). The purification of ClsB was assessed by SDS-PAGE analysis. Purified ClsB was stored at −80 °C until enzyme assay.

Enzyme Assay of ClsB—To analyze membrane PLD activity in vitro, crude membranes were prepared from BKT29 cells expressing the pBAD30 control vector, the pBAD-B vector, or the pBAD-B-H113A vector. Membranes were prepared as described previously (2). To perform the in vitro assay, 20-μl reaction mixtures consisting of PBS (pH 7.4), 10 μM β-mercaptoethanol, 2 mM MgCl2, 100 mM glycerol (Sigma), 0.008% Tween 20, 1,000 cpm/μl of [32P]PE, and 1 mg/ml of cell membranes were incubated at 30 °C for 30 min. After incubation, a 3-μl aliquot of each reaction was spotted onto a TLC plate, which was developed in a solvent mixture consisting chloroform/methanol/acetic acid (65:25:10, v/v). The TLC plate was visualized using a PhosphorImager (Molecular Dynamics). To assess the PG synthesis activity of purified ClsB, the reaction solutions consisted of PBS (pH 7.4), 10 μM β-mercaptoethanol, 2 mM MgCl2, 100 mM d5-glycerol (Sigma), 0.008% Tween 20, 5 μM synthetic PE (17:0/14:1) (Avanti Polar Lipids), and 20 ng/ml of purified proteins. After incubation at 30 °C for 30 min, 88 μl of PBS, 120 μl of chloroform, and 120 μl of methanol were added to generate a two-phase Bligh-Dyer solution. The mixture was vortexed for 2 min and spun at 10,000 × g for 1 min. 10 μl of the lower phase was analyzed by normal phase LC/MS/MS. For assaying in vitro enzymatic activity of purified ClsB, a reaction mixture containing 320 mM potassium phosphate (pH 7.0), 10 mM γ-mercaptoethanol, 0.008% Tween 20, 5 μM synthetic PG (17:0/14:1), and 20 ng/ml of protein was prepared. The reaction mixture was incubated at 30 °C for 30 min, followed by lipid extraction and analysis by normal phase LC/MS/MS, as described above.

Yeast—Wild-type BY4743 and gep4 KO yeast strains were ordered from ATCC. For protein expression in yeast, the E. coli ClsB gene was fused with N terminus su91–69 (36) by PCR and cloned onto p415GPD vector as described previously (29).

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