Supporting Information

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SI Materials and Methods

Reagents and Growth Conditions. Tryptone and yeast extract were purchased from Difco. Liquid LB media was made with 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per L (high-salt LB Miller medium) (1) and was the normal media for growth in all experiments not using [^{32}P]PO_4. Solid LB media consisted of the liquid broth with 20 g of agar per L. Unless otherwise indicated, growth media contained only 5 g/L of NaCl (low salt LB Lennox medium; Becton, Dickinson and Co.) in experiments using [^{32}P]PO_4. Antibiotics were added whenever necessary at concentrations of 50 μg/mL for kanamycin and 100 μg/mL for ampicillin. Strains were grown at 30°C or 37°C, and cell density was measured as absorption at 600 nm (A_{600}) by a DU spectrophotometer (Beckman Coulter). The pBAD30-derived (2) plasmids were grown with 0.2% L-(+)-arabinose unless indicated otherwise. Reagent grade chloroform, methanol, hydrochloric acid, sulfuric acid, isopropyl alcohol, and ethanol were purchased from Sigma. Synthetic phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylethanolamine (PE), and CDP-diacylglycerol (CDP-DAG) were obtained from Avanti Polar Lipids, Inc.

Bacterial Strain, Plasmids, and Molecular Biology Techniques. *Escherichia coli* W3110 is designated as the wild type (WT) with respect to its glycerophospholipid composition. All strains and plasmids with their corresponding genotypes are listed in Table S1.

Genetic material was amplified by polymerase chain reaction (PCR) with KOD Hot Start DNA Polymerase (Novagen) following the manufacturer's protocols. Primers were synthesized by Integrated DNA Technology. Colony PCR was used to screen for appropriate colonies, and then the PCR products were sequenced at the DNA Analysis Facility of Duke University Health System. Plasmid transformations were accomplished using electroporation (3) or chemical transformation (4). All genomic gene deletions and plasmid constructs were verified by PCR and DNA sequencing.

Construction of Chromosomal Mutants Lacking *cls* Genes. Mutants containing deletions of the *cls* genes were constructed using the Keio collection (5) of mutants (Table S1). In place of the gene of interest, Keio mutants harbor a kanamycin cassette flanked by two FLP recombinase sites, allowing for the excision of the cassette. $P1_{vir}$ transduction was used to transfer these cassettes from the mutants to other strains (1). Multiple nonadjacent deletions were accomplished by excision of the initial kanamycin cassette. Kanamycin cassette incision used the plasmid pCP20 (6), which contains an Flp recombinase. This recombinase mediates the recombination of the two flanking FLP sequences ejecting the kanamycin sequence leaving only a FLP scar sequence. Transformants for pCP20 were selected for ampicillin resistance at 30°C. Colonies were purified twice at 42°C without antibiotics to cure the pCP20 plasmid. Strains were replica plated on plates without antibiotics and plates with ampicillin or kanamycin. Colonies that did not grow on plates with antibiotics were verified as deletions using PCR and DNA sequencing. The derived strains were subjected to another round of $P1_{vir}$ transduction and elimination of the kanamycin marker to introduce additional null mutations. In the case of adjacent genes, PCR was used to synthesize a double-stranded DNA with a kanamycin cassette flanked by FLP regions, which are further flanked by 50 base pairs of the upstream and downstream regions of genes to be deleted. These linear DNA strands were transformed into strain DY330, which contains the λ red recombinase for efficient recombination (7). Once the deletions were confirmed in DY330, P1 transduction was used to move these deletions into the appropriate strains.

Strain BKT29 was derived from the strain of UE54 (Δ*pgsA*) in which PgsA (Fig. 1) is not expressed and additional mutations in *lpp* and *rcsF* are required for cell viability (8). UE54's kanamycin cassette was excised using the FLP recombinase found in pCP20. This yielded the strain BKT25 (MG1655 *lpp2* Δ*ara714* *rcsF*::mini-Tn10::Cam^R Δ*pgsA*), which was subjected to successive P1 transductions.

Construction of Plasmids Containing *cls* Genes. Regions from W3110 containing *clsA*, *clsB*, *clsC1*, *clsC1*, or *ymdB* were amplified using PCR. These PCR products were digested with XbaI, SphI, or HindIII restriction enzymes where appropriate and ligated into similarly digested pBAD30 vectors using T4 DNA ligase following the manufacture's protocols. These products were transferred into DH5α competent cells by means of chemical transformation. The transformants were grown overnight at 37°C on LB plates with ampicillin as the selection antibiotic. Colonies were purified twice on ampicillin plates. The placement of each gene under *araBAD* promoter regulation was verified by PCR and DNA sequencing.

Each of the two His residues in the two putative catalytic HKD motifs (Fig. S1) of ClsC were mutated to Ala. Plasmid pBAD-YC (carrying *ymdB-clsC*) was subjected to PCR mutagenesis to generate plasmids pBAD-YC H130A or pBAD-YC H369A.

Complementation of the Triple *cls* Mutant. Strain BKT12 (Δ*clsABC*) transformed with plasmid pBAD30, pBAD-A, pBAD-B, pBAD-C, or pBAD-YC was grown overnight and diluted to an A_{600} of 0.03 in LB media with 0.2% arabinose to induce *cls* gene expression. The strains were grown for 2 h to an A_{600} of ~0.5 for logarithmic growth phase cells or for 6 h to an A_{600} of ~2.0 for stationary phase cells. Cells were collected by centrifugation at 3,500 × *g* at 4°C and then washed with phosphate-buffered saline.

Lipid Extraction and TLC Analysis. Lipid extraction was accomplished through an acidic Bligh Dyer method (9). Each sample pellet from 100 mL of culture was brought up in 2 mL of 0.1 N HCl. Then 5 mL of methanol and 2.5 mL of chloroform were added to the aqueous solution to create a single-phase solution consisting of chloroform/methanol/0.1 N HCl [1:2:0.8 (vol/vol)]. This solution was incubated for 30 min at room temperature with intermittent mixing. Afterward, 2.5 mL of 0.1 N HCl and 2.5 mL of chloroform were added to convert the single phase to a two-phase solution consisting of chloroform/methanol/0.1 N HCl [2:2:1.8 (vol/vol)]. After centrifugation at 3000 × *g* for 25 min at room temperature, the lower phase was recovered and dried under a stream of nitrogen.

The dried lipid extract was resuspended in 100 μL of chloroform/methanol (2:1, vol/vol) and subjected to sonication for 1 min. Approximately 1–5 μL of sample was subjected to TLC using a TLC or HPTLC 60 plate (EMD) developed with solvent 4 (consisting of chloroform/methanol/acetic acid [65:25:5 (vol/vol)]). After drying the plate, lipids were visualized by spraying with 10% sulfuric acid in ethanol (vol/vol) followed by charring on a hot plate.

To determine the steady-state phospholipid composition by radiolabeling, cells were uniformly labeled with 5 μCi/mL of [^{32}P]PO_4 either during overnight growth and after dilution to A_{600} of 0.05 to initiate logarithmic growth or only after dilution of the overnight culture. Cells were harvested by centrifugation, and phospholipids were extracted as described above and analyzed after separation either by one-dimensional TLC on boric

acid–impregnated silica gel plates in solvent 1 (chloroform/methanol/water/ammonium hydroxide [60:37.5:3:1 (vol/vol)]) (10) or 2D TLC using solvent 2 (chloroform/methanol/ammonium hydroxide [130:60:8 (vol/vol)]) in the first dimension and solvent 3 (chloroform/methanol/acetic acid/water [170:25:25:6, (vol/vol)]) in the second dimension. Aqueous ammonia of 28–30% was used to make up solvents 1 and 2. Merck Kiesel gel 60 (0.25 mm) 20 × 20 cm TLC plates were used for 2D TLC. For one-dimensional TLC, 20 × 20 cm high-performance Partisil LK5 silica gel–precoated TLC plates with a concentration-zone (Whatman) were impregnated for 1 min in 1.2% boric acid in ethanol-water (1:1) as previously described (10). Radiolabeled lipids were visualized and quantified using a Personal Molecular Imager FX (Bio-Rad Laboratories). Stored images were processed and quantified using Quantity One software for scanning and analysis of the captured Phosphor images (Bio-Rad Laboratories). Phospholipid content is expressed as mol% of total phospholipid (correcting for two phosphates per molecule of CL) based on the intensity of the captured signal on Phosphor screen generated by the radiolabeled spots on the TLC plate. Values for quantified radiolabeled spots were rounded to two significant figures so totals are 100 ± 1%. Because many of the minor radiolabeled spots are unknown, they were not included in the quantification of phospholipid composition. The results presented are representative of two or more determinations.

Liquid Chromatography–Tandem Mass Spectrometry Analysis of *E. coli* Lipids. Normal phase liquid chromatography (LC) was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 μm, 25 cm × 2.1 mm (Sigma-Aldrich). Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide [800:195:5 (vol/vol)]; mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide [600:340:50:5 (vol/vol)]; and mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide [450:450:95:5 (vol/vol)]. 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. The total LC flow rate was 300 μL/min. To achieve optimum ESI efficiency, a postcolumn splitter was used to divert ∼10% of the LC effluent into the mass spectrometer, a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (MS/MS) (Applied Biosystems). Instrumental settings for negative ion ESI and MS/MS analysis of lipid species were as follows: ion spray voltage (IS) = −4500 V, curtain gas (CUR) = 20 psi, ion source gas 1 (GS1) = 20 psi, declustering potential (DP) = −55 V, and focusing potential (FP) = −150 V. MS/MS analysis used nitrogen as the collision gas. Data analysis was performed using analyst QS software (Applied Biosystem).

LC–Multiple Reaction Monitoring. For LC–multiple reaction monitoring (LC-MRM) analysis, the same LC conditions were used as above, but the effluent was analyzed with a 4000 Q-Trap hybrid triple quadrupole linear ion-trap mass spectrometer, equipped with a Turbo V ion source (Applied Biosystems). MRM was performed in the negative ion mode with MS settings as follows: curtain gas (CUR) = 20 psi, ion source gas 1 (GS1) = 20 psi, ion source gas 2 (GS2) = 30 psi, ion spray voltage (IS) = −4500 V, heater temperature (TEM) = 350°C, interface heater = ON, declustering potential (DP) = −40 V, entrance potential (EP) = −10 V, collision cell exit potential (CXP) = −5 V.

In Vitro Assay of CL Synthase. Strain BKT29 was created by introducing Δ*clsA*, Δ*clsB*, Δ*clsC,* and *ymdB::*Kan^R into UE54 (after removal of its Kan^R cassette), which is null for the *pgsA* gene (11) and therefore lacks the ability to make PG. The resulting BKT29 was transformed with plasmid pBAD30 as vector control or pBAD-A, -B, or -YC. These cells were grown to A_{600} of ∼0.5 at 37°C and then induced for the respective *cls* gene expression with 0.05% arabinose. After induction, cells were grown for two more h until harvesting by centrifugation at 3500 × *g* at 4°C. The cells were then washed with 1 × PBS (pH 7.4). Afterward, the pellet was resuspended in 2 mL of 1 × PBS and lysed by passing through a French press at 18,000 psi. Cell-free lysates were collected by centrifugation at 22,000 × *g* for 20 min at 4°C. The membrane fraction was collected by further ultracentrifugation of the cell-free lysates at 266,000 × *g* at 4°C for 1 h. Pellets were collected and solubilized using a PTFE tissue grinder (VWR) in PBS. Protein concentration for these membranes was determined using a Pierce BCA assay (Thermo Scienific).

The reaction mixture of 100 μL consisted of PBS, 0.1% Triton X-100, 10 mM β-mercaptoethanol, 5 μM of synthetic, and 1 mg/mL cell membranes with the following synthetic phospholipid substrates at 5 μM as indicated in the *Results* section: PG (17:0/14:1) or (12:0/13:0), PE (17:0/14:1), PA (17:0/14:1), or CDP-DAG (17:0/18:1). The final assay mixtures were incubated at 37°C for 30 min. To quench the reaction, 220 μL of chloroform/methanol [1:1 (vol/vol) was added to form a two-phase Bligh-Dyer solution. The resulting solution was vortexed and centrifuged at 5200 × *g* for 2 min. 20 μL of the lower phase was directly injected for normal phase LC/MS/MS analysis as described above.

1. Miller JR (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York).
2. Guzman L-M, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177:4121–4130.
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8. Shiba Y, et al. (2004) Activation of the Rcs signal transduction system is responsible for the thermosensitive growth defect of an *Escherichia coli* mutant lacking phosphatidylglycerol and cardiolipin. *J Bacteriol* 186:6526–6535.
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ClsA  201  EVVEALKVNLM---------RVFLRRMD--LRQHRKMIMIDNY  233
ClsB   79  EFVNELTAAGVVFRYYDPRPRLFGMRTNVFRRMHRKIVVIDAR  121
ClsC   96  PRIEVRLFNPFSFRLLRPL-GYITDFSRLNRRMHNKSFTVDGV  138

ClsA  400  ---GGLLHTKSVLVDGELSLVGTVNLDMRSLWLNFEITLAIDD  439
ClsB  286  ---RRPLHGKVALMDDHWATVGSSNLDPLSLSLNLEANVIIHD  325
ClsC  362  GNSGASLHAKTFSIDGKTVFIGSFNFDPRSTLLNTEMGFVIES  414
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Fig. S1. ClsC (YmdC) contains similar domains as canonical Cls. The three Cls of *E. coli* contain the two HKD motifs (*boxed* sequences) found in phospholipase D.

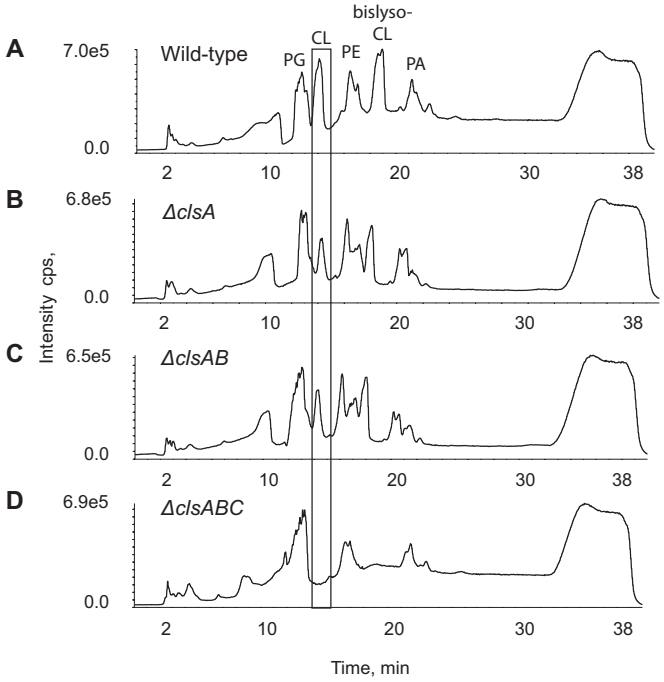

Fig. S2. Triple deletions of three Cls genes results in complete depletion of CL. Strains of *E. coli* were created lacking various *cls* genes. Cells were grown into stationary phase. Their lipid extracts were separated by LC and analyzed by MS/MS as shown in Fig. 2. Accumulation of CL was seen at ~14 min of the LC for the WT (*A*), Δ*clsA* (*B*), and Δ*clsAB* (*C*) strains. No CL was seen for the Δ*clsABC* (*D*) strain.

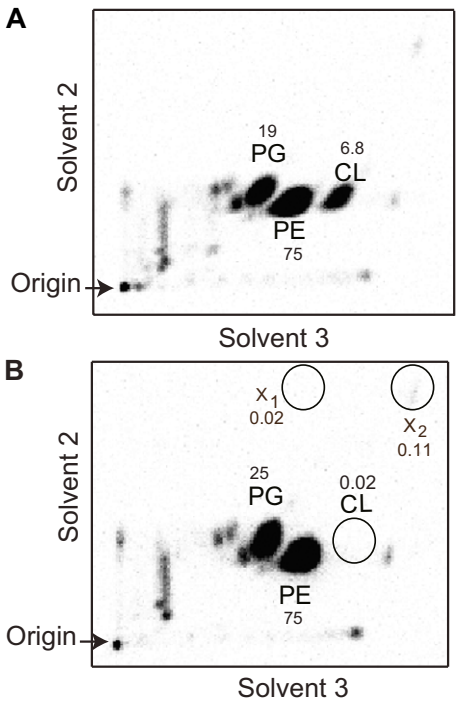

Fig. S3. Lack of CL formation in the Δ*clsABC* mutant. WT and Δ*clsABC* strains were grown to stationary phase (5 g/L NaCl) in the presence of [^{32}P]PO_4. (*A*) Two-dimensional TLC analysis of WT lipid fractions showed the major phospholipids (with their respective relative percent), as well as many minor phosphate-containing compounds. Quantification by imaging is described in the *SI Materials and Methods*. (*B*) The Δ*clsABC* strain showed only the background level (empty circle labeled X_1) at the position of CL (empty circle). The circle labeled X_2 contains a phosphate-positive spot near the lower limit of detection.

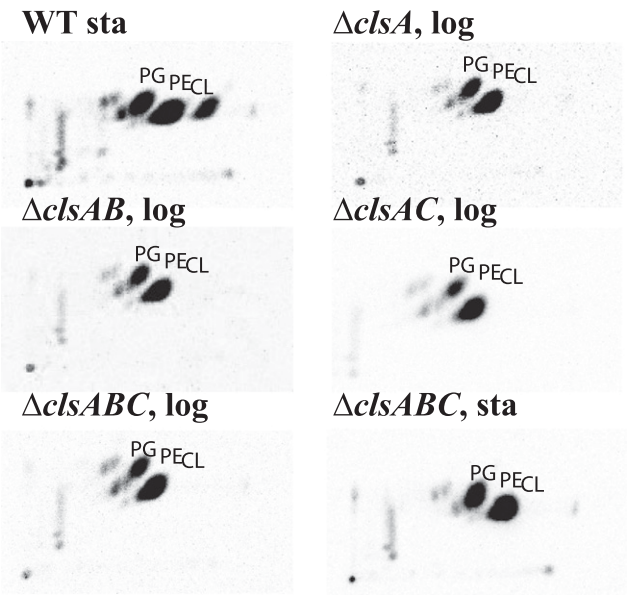

Fig. S4. Verification by 2D TLC of CL absence in strains displayed in Fig. 4. Selected phospholipid extracts from Fig. 3 and Table S2 were subjected to 2D TLC using solvent 2 in the vertical direction followed by solvent 3 in the horizontal direction. Growth phase of cells in LB with 5 g/L NaCl was either stationary (*sta*) or midlog (*log*). All Δ*clsA* strains lack CL in log phase and Δ*clsABC* lacks CL in log and stationary phase.

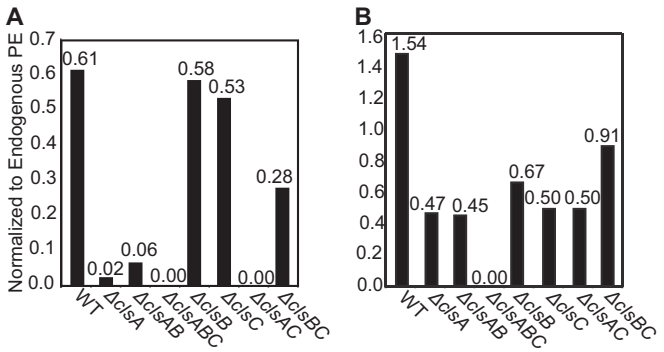

Fig. S5. Growth phase dependence of CL synthesis in *cls* mutants grown in 10 g/L NaCl. Cells were grown to midlog phase (*A*) or stationary phase (*B*) in LB medium containing 10 g/L NaCl followed by determining their relative CL content by LC/MS/MS by dividing the chromatographic peak areas of the most abundant CL (*m/z* 687.5) by the chromatographic peak area of the most abundant PE. At 10 g/L of NaCl, trace amounts of CL were observed only for Δ*clsA* and Δ*clsAB* mutants at midlog phase.

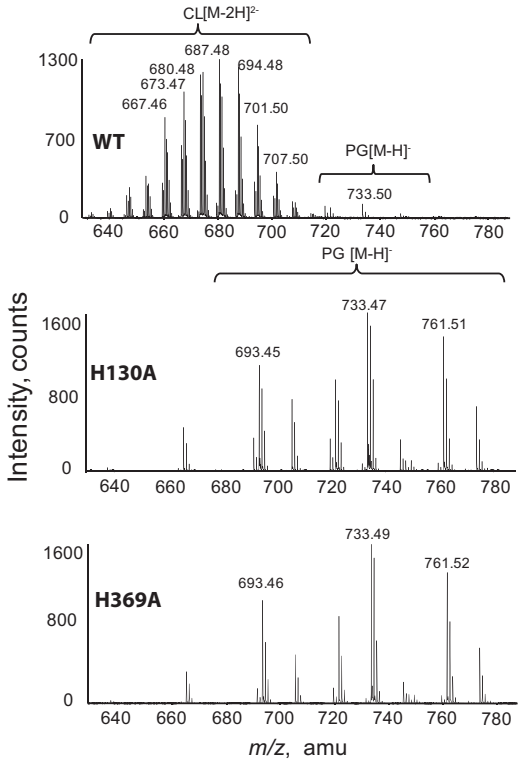

Fig. S6. Mutations in the HKD motifs of ClsC block CL synthesis. WT (*Upper*) or Δ*clsABC* strain expressing either pBAD-YC H130A (*Middle*) or pBAD-YC H369A (*Lower*) were grown to stationary phase in the presence of 0.2% arabinose. LC/MS analysis of lipid extracts showed no CL but elevated PG for ClsC in which either HKD motif was mutated (*Middle* and *Lower*).

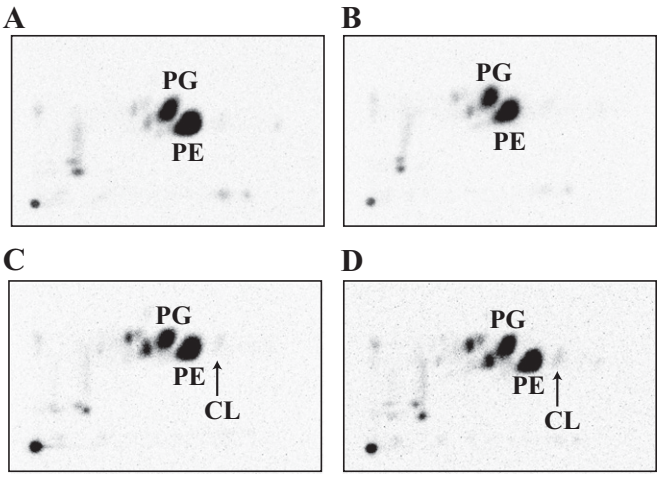

Fig. S7. Presence of CL in midlog cells grown at elevated NaCl levels. BTK10 (Δ*clsA*) was grown in LB medium containing either 5g/L (*A* and *B*) or 10 g/L (*C* and *D*) of NaCl to midlog phase (A_{600} of 0.5–0.7). Cultures were supplement with [^{32}P]PO_4 either during overnight growth and after dilution to A_{600} of 0.05 to initiate log growth (*B* and *D*) or only after dilution of the overnight culture (*A* and *C*) to monitor only phospholipids synthesized during logarithmic growth. Quantification of phospholipid content after TLC as in Fig. 3A showed PE and PG to be 73% and 26%, respectively, in all panels, with CL detectible only in *C* and *D* at 0.6% of the three major phospholipids.

Table S1. Strains of *Escherichia coli* K12 and plasmids used in this work

	Relevant genotype	Source
Strain		
W3110	Wild type	*E.coli* Genetic Stock Center, Yale University
JW1241	BW25113 Δ*clsA*::*Kan*^{R}	(1)
JW0772	BW25113 Δ*clsB*::*Kan*^{R}	(1)
JW5150	BW25113 Δ*clsC*::*Kan*^{R}	(1)
JW1032	BW25113 Δ*ymdB*::*Kan*^{R}	(1)
BKT10	W3110 Δ*clsA*::*Kan*^{R}	This work
BKT10a	W3110 Δ*clsA;* derived from BKT10	This work
BKT11	W3110 Δ*clsA,* Δ*clsB*::*Kan*^{R}; derived from BKT10a	This work
BKT11a	W3110 Δ*clsA,* Δ*clsB*; derived from BKT11	This work
BKT12	W3110 Δ*clsA,* Δ*clsB,* Δ*clsC*::*Kan*^{R}*;* derived from BKT11a	This work
BKT13	W3110 Δ*clsB*::*Kan*^{R}	This work
BKT14	W3110 Δ*clsC*::*Kan*^{R}	This work
BKT14a	W3110 Δ*clsC*; derived from BKT14	
BKT15	W3110 Δ*clsA*, Δ*clsC*::*Kan*^{R}*;* derived from BKT10a	This work
BKT16	W3110 Δ*clsB*::*Kan*^{R}*,* Δ*clsC*; derived from BKT14a	This work
DY330	W3110 ΔlacU169 gal490 λcI857 Δ(cro-bioA)	(2)
BKT19	DY330 Δ*clsC,* Δ*ymdB*::*Kan*^{R}; derived from DY330	This work
BKT20	W3110 Δ*clsC,* Δ*ymdB*::*Kan*^{R}	This work
BKT21	W3110 Δ*clsA,* Δ*clsB,* Δ*ymdB*::*Kan*^{R}	This work
BKT22	W3110 Δ*clsA,* Δ*clsB,* Δ*clsC,* Δ*ymdB*::*Kan*^{R}	This work
UE54	MG1655 *lpp2* Δ*ara714 rcsF*::mini*Tn10*$_{cam}$ Δ*pgsA*::FRT-*Kan*-FRT	(3)
BKT25	MG1655 *lpp2* Δ*ara714 rcsF*::mini*Tn10*$_{cam}$ Δ*pgsA*	This work
BKT29	BKT25 Δ*clsA*, Δ*clsB*, Δ*clsC,* Δ*ymdB*::*Kan*^{R}	This work
Plasmid		
pCP20	FLP recombinase expression; AmpR CamR; temperature-sensitive replicon	(4)
pBAD30	Low copy number expression plasmid	(5)
pBAD-A	clsA inserted between XbaI and SphI of pBAD30	This work
pBAD-B	clsB inserted between XbaI and HindIII of pBAD30	This work
pBAD-C	clsC inserted between XbaI and HindIII of pBAD30	This work
pBAD-YC	ymdB-clsC inserted between XbaI and HindIII of pBAD30	This work
pWSK29	Low copy number expression plasmid	(6)
pWSK29-Y	ymdB inserted between XbaI and HindIII of pWSK29	This work
pBAD-YC H130A	clsC with H130A substitution, ymdB wild type	This work
pBAD-YC H369A	clsC with H369A substitution, ymdB wild type	This work

1. Baba T, et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* 2:2006–0008.
2. Yu D, et al. (2000) An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc Natl Acad Sci USA* 97:5978–5983.
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Table S2. Phospholipid composition as a function of growth phase and strain genetic background

Strain	GP	PA	PG	PE	CL	X
Wild type (W3110)	M	2.1	12	78	5.7	1.7
	M*		6.4	78	16	
	S	1.5	15	70	11	1.7
Δ*clsA* (BKT10)	M	1.5	20	77	"0.6"	1.4
	M*		20	78	2.2	
	S	1.6	22	68	6.8	1.6
Δ*clsB* (BKT13)	M	1.4	12	80	5.5	1.5
	M*		7.2	81	12	
	S	3.1	17	67	12	1.2
Δ*clsC* (BKT14)	M	1.5	12	81	4.4	1.3
	M*		5.0	80	15	
	S	2.6	18	68	11	1.3
Δ*clsAB* (BKT11)	M	1.4	19	77	"0.7"	1.5
	M*		19	78	2.6	
	S	1.8	23	67	6.8	1.3
Δ*clsAC* (BKT15)	M	1.3	19	78	"0.3"	1.5
	M*		21	79	0.3	
	S	2.1	25	69	2.2	1.5
Δ*clsBC* (BKT16)	M	0.8	13	81	4.5	1.2
	M*		6.4	82	12	
	S	2.2	16	68	12	1.5
Δ*clsABC* (BKT12)	M	1.4	18	79	"0.4"	1.2
	M*		23	77	ND	
	S	2.5	26	70	ND	1.8

Strain W3110 (wild type) and strains with the indicated *cls* gene deletions were grown in LB medium containing 5 g/L of NaCl and [^{32}P]PO_4 to either midlog phase (M) or overnight to stationary phase (S). The percent of the indicated phospholipids from Fig. 4 *A* and *B* (M and S rows, respectively) was determined as described in the *Materials and Methods*. Lipid composition was analyzed by 2D TLC and only the major lipids quantified. CL, cardiolipin; GP, growth phase; M*, medium supplemented with 0.6 M mannitol and grown to midlog phase; ND, not detected (i.e., signal was at background level of chromatography plate); PA, phosphatidic acid; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; X, sum of other minor lipids; " ", no CL detected by 2D TLC (Fig. S4).