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Accumulation of Phosphatidic Acid Increases Vancomycin Resistance in *Escherichia coli*

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In Gram-negative bacteria, lipopolysaccharide (LPS) contributes to the robust permeability barrier of the outer membrane, preventing entry of toxic molecules such as antibiotics. Mutations in *lptD*, the beta-barrel component of the LPS transport and assembly machinery, compromise LPS assembly and result in increased antibiotic sensitivity. Here, we report rare vancomycin-resistant suppressors that improve barrier function of a subset of *lptD* mutations. We find that all seven suppressors analyzed mapped to the essential gene *cdsA*, which is responsible for the conversion of phosphatidic acid to CDP-diacylglycerol in phospholipid biosynthesis. These *cdsA* mutations cause a partial loss of function and, as expected, accumulate phosphatidic acid. We show that this suppression is not confined to mutations that cause defects in outer membrane biogenesis but rather that these *cdsA* mutations confer a general increase in vancomycin resistance, even in a wild-type cell. We use genetics and quadrupole time of flight (Q-TOF) liquid chromatography-mass spectrometry (LC-MS) to show that accumulation of phosphatidic acid by means other than *cdsA* mutations also increases resistance to vancomycin. We suggest that increased levels of phosphatidic acid change the physical properties of the outer membrane to impede entry of vancomycin into the periplasm, hindering access to its target, an intermediate required for the synthesis of the peptidoglycan cell wall.

The outer membrane (OM) of Gram-negative bacteria serves as a robust permeability barrier (1). Composed of lipopolysaccharide (LPS) molecules in the outer leaflet and glycerophospholipids (GPLs) in the inner leaflet, the OM protects the cell from harmful agents, such as detergents and antibiotics (1–3). LPS is synthesized at the inner membrane (IM), transported across the aqueous periplasm, and finally assembled into the outer leaflet of the OM (4). The Lpt proteins are responsible for this transport and assembly (5). Glycerophospholipids (GPLs) are synthesized at the IM, but little is known about their transport across the periplasm and assembly into the OM (6). The coordination of LPS and GPL assembly is important to maintain an effective OM permeability barrier (1).

Because of the high density of LPS in the outer leaflet, the OM carries a net negative charge (7). The human innate immune system takes advantage of this property of the OM by producing cationic antimicrobial peptides (CAMPs) (8). Positively charged CAMPs associate with the negatively charged cell surface and insert into the OM, killing the bacterial cell (8). Bacteria have systems in place, such as the PhoPQ virulence system in *Salmonella enterica* serovar Typhimurium, that modify lipid A and the PL content to decrease the negative charge of the OM, thereby decreasing the affinity of CAMPs for the OM (9–11). This increases bacterial resistance to CAMPs and enables evasion of the immune system.

Like LPS, GPLs can contribute to the electric charge of the membrane. *Escherichia coli* membranes are composed of three major GPLs: phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL). The relative abundances of each of these GPL species are similar in the IM and OM (PE, 75%; PG, 20%; CL, 5%) (12). PE is a zwitterionic GPL with a neutral charge, while PG and CL are acidic GPLs that carry negative charges (6). Although these GPLs have different chemical properties due to their head groups, they are synthesized from the same precursor molecule, phosphatidic acid (PA) (6, 12). The conversion of PA to CDP-diacylglycerol is the last step before commitment to either the zwitterionic GPL pathway or the acidic GPL pathway (6, 12).

In this work, we focus on *E. coli* mutants defective in LPS assembly that exhibit increased sensitivity to antibiotics. Mutations that compromise the function of LptD, the beta-barrel component of the Lpt machinery, are often sensitive to vancomycin. In order to act on its cell wall target, vancomycin must traverse the OM. Once in the periplasm, vancomycin binds to the two terminal D-alanine residues on the cell wall precursor lipid II, thus preventing polymerization of the peptidoglycan cell wall (13, 14). *lptD* mutants exhibit increased sensitivity to vancomycin because they allow for more efficient entry of the drug into the periplasm as a result of a compromised OM barrier.

We report here the isolation and characterization of vancomycin-resistant suppressors of a subset of *lptD* mutations. These suppressors map to the essential gene *cdsA*. Vancomycin-resistant suppression is not specific to the *lptD* mutants; the *cdsA* mutations actually increase vancomycin resistance even in an otherwise wild-type cell. These *cdsA* mutations cause a recessive, partial loss of function, and they accumulate PA like other *cdsA* mutants previously described (15, 16). We suggest that increased PA alters the OM and causes increased resistance to vancomycin.

Received 20 May 2014 **Accepted** 16 June 2014
Published ahead of print 23 June 2014
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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JB.01876-14.

doi:10.1128/JB.01876-14

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference and/or source
Strains		
MC4100	F$^-$ *araD139* Δ*(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR thi*	17
NR754	MC4100 Ara$^+$	18
HC334	NR754 Δ*lptD* pET2342::*lptD*$_{I54E}$	21, 48, 49; this study
HC338	NR754 Δ*lptD* pET2342::*lptD*$_{V52E-I54E}$	21, 48, 49; this study
HC340	NR754 Δ*lptD* pET2342::*lptD*	21, 48, 49; this study
HC342	NR754 *cdsA7 dgt*::Tn*10*	This study
HC351	HC334 Δ*clsA*::*kan*	34; this study
HC386	HC334 *cdsA7 dgt*::Tn*10*	This study
HC509	NR754 Δ*lptD* pET2342::*lptD*$_{\Delta D57\Delta K60}$	21, 48, 49; this study
HC510	HC334 *pgsA444 zed-3069*::Tn*10*	39, 50, 51; this study
HC530	HC509 *cdsA7 dgt*::Tn*10*	This study
HC571	NR754 Δ*clsA*::*kan*	34; this study
HC575	NR754 *pgsA444 zed-3069*::Tn*10*	39, 50, 51; this study
HC630	HC509 *pgsA444 zed-3069*::Tn*10*	39, 50, 51; this study
HC960	HC334 *cdsA9 dgt*::Tn*10*	This study
HC961	HC509 *cdsA9 dgt*::Tn*10*	This study
HC962	NR754 *cdsA9 dgt*::Tn*10*	This study
HC1045	NR754 pASKA::*plsC*	35; this study
HC1198	JAS193 *cdsA7 dgt*::Tn*10*	This study
HC1199	JAS193 *cdsA9 dgt*::Tn*10*	This study
HC1240	HC342 pASKA::*cdsA*	35; this study
HC1241	HC962 pASKA::*cdsA*	35; this study
JAS193	MC4100 Δ*bamE*::*kan*	J. Schwalm, unpublished data
Plasmids		
pET2342::*lptD*	*lptD* cloned into pET2342	48
pASKA::*cdsA*	IPTG inducible, Cam^r	35
pASKA::*plsC*	IPTG inducible, Cam^r	35

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and chemicals. All strains used in the work are isogenic derivatives of NR754, an *araD*$^+$ revertant of MC4100 (17, 18). Strains were constructed by generalized P1 transduction or transformation (19). Suppressor mutations were mapped using pools of random Tn*10* insertions as described previously (20). If necessary, *kan* cassettes were excised using the Flp recombinase (21). Strains and plasmids used in this work are listed in Table 1. All cultures were grown in Luria-Bertani (LB) broth or agar at 37°C. For mass spectrometry (MS), high-pressure liquid chromatography (HPLC)-grade solvents (water, methanol, and isopropanol), acetic acid, and ammonium acetate were obtained from Fisher Scientific (Pittsburgh, PA).

Antibiotic sensitivity assays. Efficiency of plating assays (EOPs) or disk diffusion assays were used to determine antibiotic sensitivity. For EOPs, strains were grown overnight, serially diluted, and then replica plated on LB agar or LB agar supplemented with vancomycin (70 mg/liter, 120 mg/liter, 140 mg/liter, 150 mg/liter, or 200 mg/liter), 625 mg/liter bacitracin, 1 mg/liter gentamicin, or 10 mg/liter rifampin. For disk diffusion assays, the disks contained the following amounts of antibiotics: 1.25 mg ampicillin, 320 μg cetyltrimethylammonium bromide, 125 μg erythromycin, 30 μg moxalactam, 0.1 μg norfloxacin, 260 μg paraquat, 300 units polymyxin B, or 1 mg vancomycin. Isopropyl-β-D-1-thiogalactopyranoside (IPTG; 0.1 mM) was present in the LB agar if indicated.

Growth rate determination. Overnight cultures were diluted 1:100 in 100 μl of LB medium in 96-well plates. One hundred microliters of mineral oil was overlaid to prevent evaporation. Optical density at 600 nm (OD_{600}) was measured every 20 min with intermittent orbital shaking at 37°C. These 4-hour OD_{600} growth curves were used to determine the growth rate. OD_{600} was plotted against time on a logarithmic scale, and linear regression analysis was used to determine the growth rate of each culture. For each strain, three technical replicates of three biological replicates were averaged and the mean of the averages was calculated and reported with standard error of the mean.

Cell size measurements. Overnight cultures were diluted 1:100 and grown to an OD_{600} of approximately 0.4. Cells were suspended on a 1% agarose pad and imaged using a 100× 1.4-numerical-aperture (NA) objective on a Nikon 90i microscope equipped with a QImaging Rolera XR camera and NIS Elements software. Cell dimensions were quantified with MicrobeTracker version 0.936 (22), using MATLAB version R2013a. Approximately 100 cells were examined for each of three independent experiments. *P* values were calculated using Student's *t* test.

Bligh Dyer lipid extraction. Lipids were extracted using an acidic Bligh Dyer method as previously described (23, 24). One-hundred-milliliter cultures were grown to an OD_{600} of 1.5, and then the cells were harvested by centrifugation. Cell pellets were washed once with phosphate-buffered saline (PBS) and then resuspended in 1 ml of 0.1 N HCl. Methanol (2.5 ml) and chloroform (1.25 ml) were added to form a single-phase solution (chloroform-methanol-0.1 N HCl [1:2:0.8 {vol/vol/vol}]) and incubated for 30 min at room temperature with sporadic vortexing. Then, 0.1 N HCl (1.25 ml) and chloroform (1.25 ml) were added to form a two-phase solution (chloroform-methanol-0.1 N HCl [2:2:1.8 {vol/vol/vol}]). These solutions were then centrifuged at 3,000 × *g* for 25 min at room temperature. The lower phase was recovered and dried under nitrogen stream.

Mass spectrometry. Samples were reconstituted into 500 μl 1:1:0.3 methanol-chloroform-water. Mass spectrometric analyses were performed on a 6550 Accurate-Mass quadrupole time of flight (Q-TOF) mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA), equipped with an electrospray ionization (ESI) source operated in both negative- and positive-ion mode. Liquid chromatography (LC) was performed using a 1290 Infinity HPLC system (Agilent Technologies, Inc., Santa Clara, CA) coupled to the mass spectrometer, which scans from *m/z* 200 to 1,000 at 2-GHz extended dynamic range. Nitrogen was used as drying gas, nebulizer gas, and collision gas. Mass spectrometer operation conditions were as follows: capillary voltage, 4,000 V; fragmentor voltage, 225 V; nebulizer gas, 35 lb/in^2; drying gas, 13 liter/min; gas temperature, 275°C. The instrument control, data acquisition, and data analysis were performed by the Agilent Mass Hunter software (Agilent Technologies; version B.05.00), which also controlled the chromatography system.

The LC parameters were as follows: column, Agilent Poroshell-120 EC-C_{18} (2.7 μm, 2.1 by 150 mm); autosampler temperature, 5°C; injection volume, 10 μl; column temperature, 60°C; and solvent flow rate, 150 μl/min. Solvent A is 1 mM ammonium acetate plus 0.2% acetic acid in 90:10 water-methanol, and solvent B is 1 mM ammonium acetate plus 0.2% acetic acid in 2:98 methanol–2-propanol. The gradient was 0 min, 25% B; 2 min, 25% B; 4 min, 65% B; 16 min, 100% B; 20 min, 100% B; 21 min, 25% B; 22 min, 25% B. Postrun time was 6 min.

RESULTS

Vancomycin-resistant suppressors of *lptD* mutations map to *cdsA*. *Escherichia coli lpt* mutants that exhibit defects in transport and assembly of LPS often exhibit sensitivity to detergents and antibiotics (18, 25–31). *lptD*$_{I54E}$, *lptD*$_{V52E-I54E}$, and *lptD*$_{\Delta D57\Delta K60}$ harbor the denoted mutations that alter the soluble N terminus of the OM beta-barrel protein LptD and confer sensitivity to vanco-

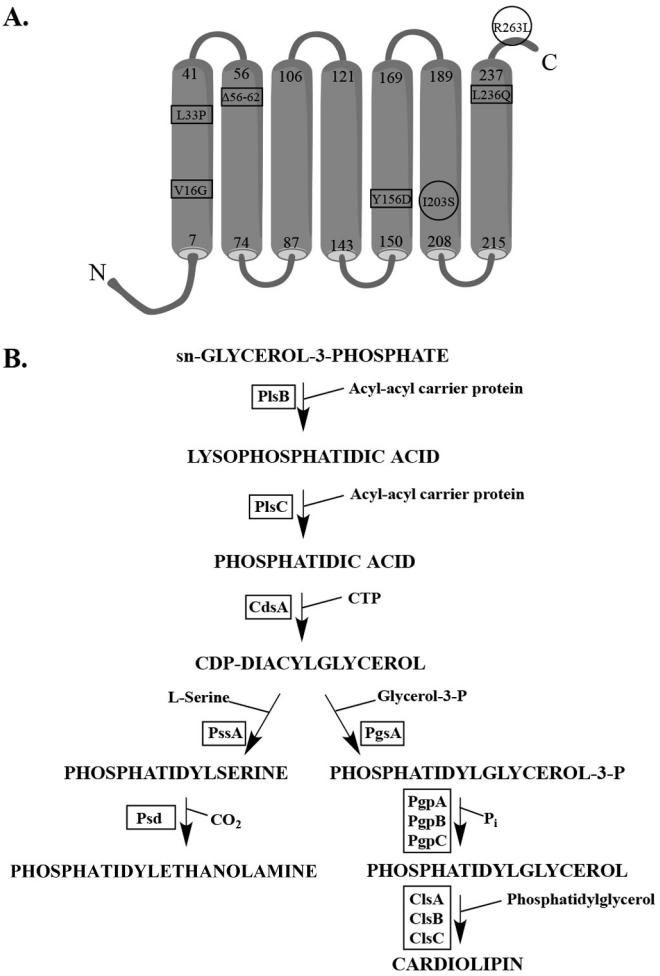

FIG 1 Vancomycin-resistant *lptD* suppressors map to *cdsA*, the gene responsible for the conversion of phosphatidic acid to CDP-diacylglycerol. (A) The inner membrane CdsA protein is predicted to have seven transmembrane domains (33). Vancomycin-resistant *cdsA* suppressors mapped mainly within these domains. Mutations V16G, L33P, Δ56–62, Y156D, and L236Q were isolated in an $lptD_{V52E\text{-}I54E}$ background. I203S was isolated in an $lptD_{I54E}$ background, and R263L was isolated in an $lptD_{\Delta D57\Delta K60}$ background. The mutations enclosed in circles, I203S (*cdsA7*) and R263L (*cdsA9*), are characterized further in this study. (B) Biosynthetic pathway for major phospholipids in *Escherichia coli*. Protein names are in boxes, and they are next to the arrow that signifies the step at which they act.

mycin to differing degrees. Spontaneously arising vancomycin-resistant suppressors were isolated on LB medium supplemented with 120 mg/liter vancomycin.

With strains carrying each of these mutations, survivors arose at a frequency of 10^{-8}, and all suppressors analyzed mapped to an essential gene, *cdsA*. CdsA is an inner membrane protein responsible for the conversion of PA to CDP-diacylglycerol in GPL biosynthesis (6, 15, 32). Figure 1A shows the location of the different *cdsA* mutations on a predicted CdsA structure (33). Most mutations map to the transmembrane helices, and some may affect protein stability in the membrane. Amino acid changes I203S (*cdsA7*) and R263L (*cdsA9*) were chosen as representative examples for this study because they are located in different parts of the protein, and unlike some of the other *cdsA* suppressors isolated, they do not exhibit any growth defects.

Because *cdsA* is an essential gene (16, 34), the suppressors cannot be null mutations. We used complementation analysis to determine whether the *cdsA7* and *cdsA9* mutations were dominant or recessive. The *cdsA* clone from the "A Complete Set of *E. coli* K-12 ORF Archive" (ASKA) collection was introduced into the *cdsA7* and *cdsA9* backgrounds (35). Basal-level (uninduced) expression of a wild-type copy of *cdsA* on the ASKA plasmid decreased vancomycin resistance back to wild-type levels (see Fig. S1 in the supplemental material). Because *cdsA7* and *cdsA9* are recessive, we conclude that both are partial loss-of-function mutations.

The *cdsA* mutations do not alter cell size. Previously, it has been shown that mutations that confer LPS transport defects can be suppressed by mutations that alter fatty acid biosynthesis (36), and we wanted to determine if our suppressors acted in a similar manner. *fabH* and *accD* mutations can suppress certain *lpt* mutations by reducing cell size and growth rate, which in turn decrease the rate of growth of the cell envelope (36). The *cdsA7* and *cdsA9* mutants did not exhibit a decrease in cell volume (see Fig. S2 in the supplemental material), and their growth rates did not differ significantly from that of the wild type (see Table S1). We conclude from these results that these *cdsA* mutations do not suppress *lptD* mutations by decreasing the rate of growth of the cell envelope.

Partial loss-of-function *cdsA* mutants generally increase vancomycin resistance of *E. coli* strains. Because these *cdsA* suppressors were isolated in different *lptD* mutant backgrounds, we asked whether this mode of suppression was specific to each *lptD* mutant. For simplicity, we chose to analyze two of the *cdsA* mutants (*cdsA7* and *cdsA9*) in two of the *lptD* mutant backgrounds ($lptD_{I54E}$ and $lptD_{\Delta D57\Delta K60}$). *cdsA7* and *cdsA9* are both able to cross-suppress $lptD_{I54E}$ and $lptD_{\Delta D57\Delta K60}$, regardless of which *lptD* background they were isolated in (Fig. 2). This demonstrates that these *cdsA* suppressors are not allele specific but rather increase vancomycin resistance in a more generalized manner.

Mutants in the beta-barrel assembly machinery (Bam) increase sensitivity to vancomycin due to impaired assembly of OM proteins (37, 38). The Δ*bamE*::*kan* allele is a null mutation that removes BamE, one of the lipoproteins in the Bam complex. Strains carrying the Δ*bamE*::*kan* allele exhibit mild OM protein assembly defects and display increased sensitivity to vancomycin (37). When *cdsA7* and *cdsA9* were introduced into a Δ*bamE*::*kan* strain, vancomycin resistance increased (Fig. 3A). This suggests that *cdsA7* and *cdsA9* increase vancomycin resistance in a general manner in any strain with a compromised OM.

To test whether *cdsA7* and *cdsA9* increase resistance to vancomycin in wild-type strains, we evaluated these mutants in an otherwise wild-type background. On LB plates supplemented with 200 mg/liter vancomycin, *cdsA7* and *cdsA9* mutants were able to survive better than the wild type (Fig. 3B). This suggests that the *cdsA7* and *cdsA9* strains are altered in a way that protects the cell from vancomycin. Previously isolated *cdsA* mutants showed increased resistance to erythromycin in the strain background tested, but the effect of the mutations on sensitivity to other antibiotics was not reported (16). Using disk diffusion assays, we have checked the *cdsA* mutants against a battery of other antibiotics. We see a very slight increase in resistance to erythromycin but not to any other drug tested (norfloxacin, paraquat, cetyltrimethylammonium bromide, bacitracin, and polymyxin B). Strikingly, however, we do see that the *cdsA* mutations cause a clear increase in sensitivity to ampicillin, moxalactam, gentamicin, and rifampin.

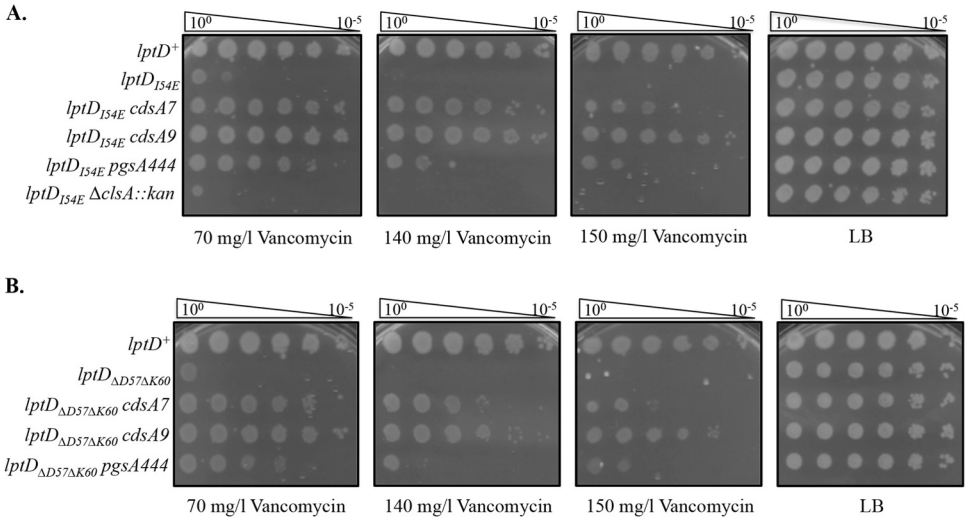

FIG 2 Partial loss-of-function *cdsA* mutants and *pgsA444* suppress the vancomycin sensitivity of *lptD* mutants. (A) Efficiency of plating assay (EOP) on LB agar plates supplemented with 70 mg/liter, 140 mg/liter, or 150 mg/liter vancomycin and grown at 37°C overnight. Suppression is shown for $lptD_{I54E}$. Note that the chromosomal *lptD* allele is Δ*lptD*, while the *lptD* allele listed for each EOP is present in pET2342::*lptD*, which has a leaky T7 promoter. T7 RNA polymerase is not present in these strains. (B) EOPs as described for panel A. Suppression is shown for $lptD_{\Delta D57\Delta K60}$.

Changes in phospholipid content confer vancomycin resistance. The enzymes and intermediates involved in GPL biosynthesis were identified in the lab of Eugene Kennedy (Fig. 1B). Strains harboring partial loss-of-function mutations in *cdsA* have been shown to accumulate PA (15, 16). We would also expect a concomitant decrease in CDP-diacylglycerol levels in *cdsA* mutants, which would result in a decrease in acidic GPL biosynthesis, since CDP-diacylglycerol has a higher affinity for PssA than for PgsA (15, 16).

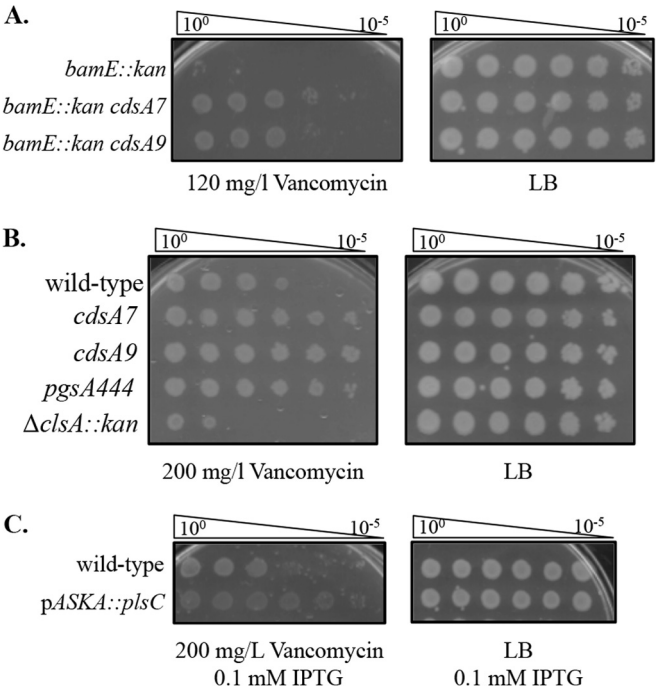

FIG 3 *cdsA* mutants, *pgsA444*, and *plsC* overexpression increase resistance to vancomycin. (A) Partial loss-of-function *cdsA* mutants suppress the vancomycin sensitivity of other cell envelope mutants. EOPs of strains supplemented with 120 mg/liter vancomycin. (B) Partial loss-of-function *cdsA* mutants and *pgsA444* increase vancomycin resistance in wild-type cells. EOPs of strains supplemented with 200 mg/liter vancomycin. (C) Overproduction of PlsC increases vancomycin resistance in wild-type cells. EOPs of strains supplemented with 200 mg/liter vancomycin and 0.1 mM IPTG.

We hypothesized that a change in the phospholipid content in the OM could result in this increased resistance to vancomycin, and we wondered whether it was due to the accumulation of PA or the decrease in acidic GPLs. To distinguish between these two possibilities, we introduced *pgsA444*, a partially inactivating mutation in the phosphatidylglycerophosphate synthetase (39), as well as Δ*clsA*::*kan* from the Keio collection (34) into our *lptD* mutant strains (note that we were unable to construct an $lptD_{\Delta D57\Delta K60}$ Δ*clsA*::*kan* double mutant, so these data are not shown). ClsA is responsible for a significant portion of CL produced in the cell, and *cls* mutants have been shown to have lower levels of CL (24, 40). We found that *pgsA444* partially suppresses the vancomycin sensitivity of the *lptD* mutants, while Δ*clsA*::*kan* does not (Fig. 2). This suggests that the increase in negatively charged PA species in the membrane and not a decrease in acidic GPLs was responsible for the increase in vancomycin resistance. It is likely that *pgsA444* only partially suppresses the vancomycin sensitivity of these *lptD* mutants because it does not accumulate as much PA as the *cdsA* mutants. We also discovered that *pgsA444* confers increased vancomycin resistance in an otherwise wild-type background (Fig. 3B). The Δ*clsA*::*kan* allele does not confer this increased resistance and in fact renders cells more sensitive to vancomycin.

***cdsA* mutants accumulate PA.** To confirm our genetic results, we investigated the GPL content of strains harboring *cdsA7*, *cdsA9*, and the *pgsA444* mutations. GPLs were extracted from whole cells using an acidic Bligh Dyer method (23, 24). Lipids were then separated and quantified by Agilent quadrupole time of flight (Q-TOF) liquid chromatography-mass spectrometry (LC-MS). Distinct PA species were summed, and each sum was normalized to total PS content for each strain. PS levels did not change signifi-

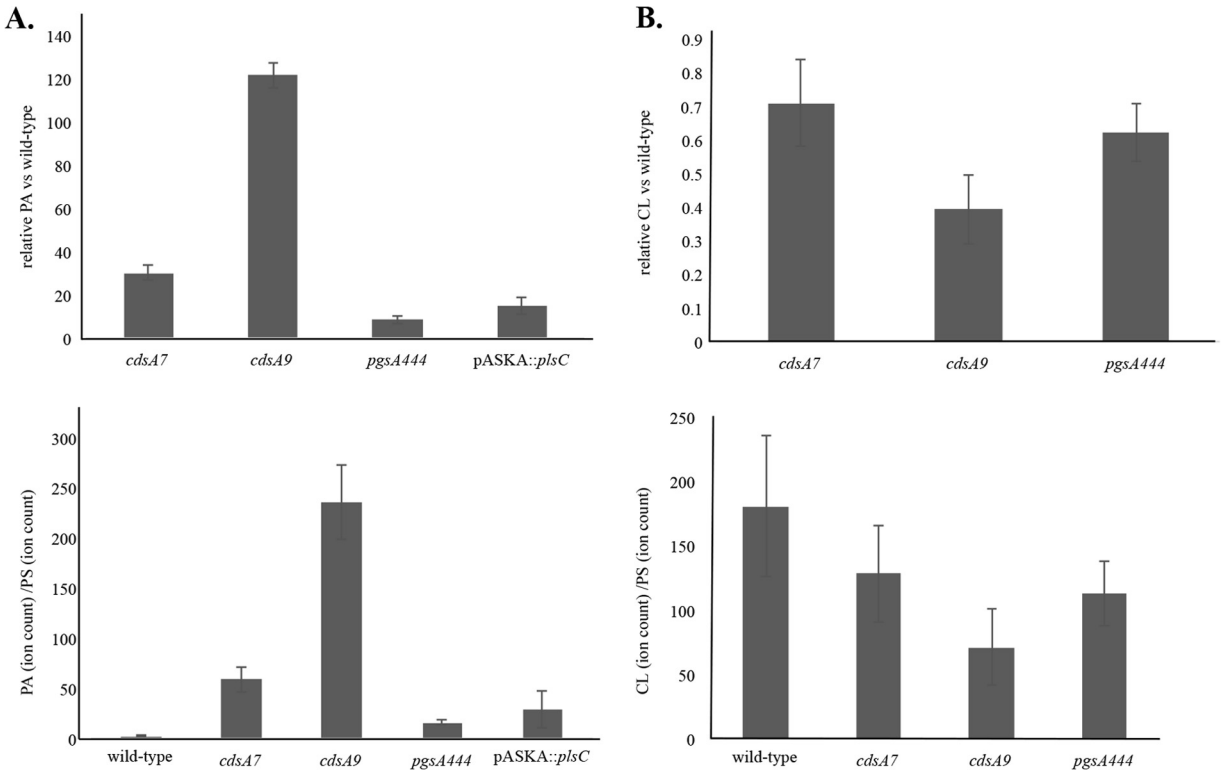

FIG 4 PA accumulates in *cdsA* mutants and *pgsA444* and when *plsC* is overexpressed. PA was quantified by Q-TOF LC-MS. The average fold differences of all PA species ± standard errors from three experiments are shown (top). The changes of PA/PS are reported ± standard errors from three experiments (bottom). (B) CL levels decrease in *cdsA* mutants and *pgsA444*. CL was quantified by Q-TOF LC-MS. The average fold differences of all CL species ± standard errors from three experiments are shown (top). The changes of CL/PS are reported ± standard errors from three experiments (bottom).

cantly in all strains tested. We found that PA accumulates in *cdsA7*, *cdsA9*, and *pgsA444* strains (Fig. 4A). We hypothesize that the *cdsA9* mutation compromises CdsA function more severely than does *cdsA7* because PA levels are much higher in the *cdsA9* mutant. This correlates with the suppression seen with the *lptD* mutants (Fig. 2); at higher vancomycin concentrations, *cdsA9* suppresses vancomycin sensitivity better than does *cdsA7*, suggesting that greater PA accumulation results in better suppression. The *pgsA444* mutant accumulates less PA than either the *cdsA7* or *cdsA9* mutant does, and this correlates with the fact that *pgsA444* does not suppress vancomycin sensitivity as well (Fig. 2). The *cdsA7*, *cdsA9*, and *pgsA444* mutants all show decreased CL levels compared to the wild type (Fig. 4B), most likely because PA is accumulating at the expense of CL.

Overexpression of *plsC* increases PA and increases vancomycin resistance. We have shown that loss-of-function mutations in the phospholipid biosynthesis pathway that cause accumulation of PA increase vancomycin resistance. We wondered whether overproduction of the protein directly upstream of PA synthesis, PlsC, which should cause the accumulation of PA, would also increase vancomycin resistance. We used the *plsC* ASKA clone to overexpress *plsC* in an otherwise wild-type background (35). When *plsC* expression is induced with 0.1 mM IPTG, an increase in vancomycin resistance is observed (Fig. 3C). Q-TOF LC-MS confirms that this level of induction of *plsC* expression results in an accumulation of PA (Fig. 4A). These results confirm that accumulation of PA increases vancomycin resistance in *E. coli*.

DISCUSSION

We show here that OM permeability defects associated with *lptD* mutants can be suppressed by partial loss-of-function mutations in the essential gene, *cdsA*. The results of this selection for increased vancomycin resistance were striking: suppression was rare and the seven suppressors analyzed altered seven different codons in a single essential gene. These *cdsA* mutations increase vancomycin resistance in *lptD* mutants, *bam* mutants, and even in an otherwise wild-type strain. However, they do not markedly increase resistance to any other antibiotic tested. Thus, these suppressors are not gene or pathway specific; rather, they are drug specific. Using classic genetic methods and Q-TOF LC-MS, we show that increasing PA in three different ways (using the *cdsA* suppressors, the *pgsA444* mutation, and overexpression of *plsC*) increases vancomycin resistance in *E. coli*. We conclude that vancomycin resistance increases as a consequence of PA accumulation.

It is difficult to speculate how the accumulation of PA results in increased vancomycin resistance primarily because we do not know how vancomycin enters the periplasm to bind its target, lipid II (13, 14). When the *lpt* system is compromised, LPS transport and assembly slow, allowing for PLs to flip into the outer leaflet of the OM (20). These PLs self-associate to form rafts of PL bilayer in the OM that allow for diffusion of hydrophobic molecules into the periplasm (41). It is not obvious that a large, hydrophilic molecule like vancomycin would be able to cross the membrane in this way. Instead, it is possible that vancomycin enters the

cell through "cracks" in the bilayer at disordered LPS-PL junctions (41). This predicts that an *lpt* mutant would be more vancomycin sensitive due to the increase in LPS-PL junctions, i.e., "cracks" in the membrane.

Ganong et al. have shown that *cdsA* mutants accumulate PA in both the IM and the OM (15). While it is true that there is roughly four times as much PA in the IM as in the OM, PA concentrations in the OM are more than 10-fold higher in their *cdsA* mutant than in the wild type (15). Because vancomycin needs only to traverse the OM to act on its target in the periplasm, it seems unlikely that PA accumulation in the IM is contributing to vancomycin resistance. Rather, we suggest that PA accumulation in the OM impedes efficient entry of vancomycin into the periplasm by reducing the size or altering the properties of the "cracks." Since PA accumulation also increases vancomycin resistance in an otherwise wild-type cell as well, we must propose the presence of a small amount of PLs in the outer leaflet that produce "cracks" in the OM of wild-type strains.

It is possible that the accumulation of PA in the OM reduces the size of "cracks" by increasing LPS assembly, by increasing the activity of the Lpt system, or by decreasing the amount of PLs in the outer leaflet by increasing the activity of PldA or the Mla transport system (20). However, since PA is really an intermediate in PL biosynthesis, and since it is present normally at low levels, we think that this is unlikely.

We propose that the accumulation of PA increases vancomycin resistance by altering the biophysical properties of the OM. PA lacks a head group and therefore increases membrane curvature, and this could affect the size or properties of the "cracks" (42, 43). In addition to increased membrane curvature, PA increases the negative charge of the OM, and this may increase vancomycin association with the OM (43). While this may seem counterintuitive, other studies have shown that CL microdomains interact with CAMPs and prevent them from interacting with their targets, thereby increasing resistance (44, 45).

Changes in GPL composition have been previously shown to alter resistance and susceptibility to CAMPs and antibiotics (9, 46). In *Staphylococcus aureus*, *fmtC* and *lysC* mutations, which reduce lysyl-PG, increase resistance to moenomycin and vancomycin (46). The increased net negative charge of these mutant membranes may increase interaction with these drugs and prevent them from interacting with their targets. In *Salmonella* Typhimurium, the PhoPQ system responds to acidic pH and CAMPs by increasing levels of cardiolipin and palmitoylated acyl-PG (9). This increase in these GPLs correlates with an increase in resistance to CAMPs that helps evade the host immune system (9). There is speculation that this resistance may be due to increased membrane curvature as well as CAMP association with CL microdomains, thereby occluding CAMP interactions with their targets (9, 44, 45).

The mode of resistance shown here is inherently different from that regulated by the PhoPQ system. When this system is turned on, palmitoylation of PG increases, which results in a reduction in polarity and an increase in the hydrophobicity and saturation of the membrane (9, 10). In this work, we show an overall increase in PA, regardless of the chain length or saturation state (see Fig. S3 in the supplemental material). As a result of the accumulation of PA, we also show a reduction in CL levels. This also contrasts with the increase in CL levels observed in *Salmonella* when the PhoPQ system is activated (9). Accumulation of PA is yet another way in which the OM can be modified to increase resistance to antibiotics.

The emergence of multidrug-resistant bacteria is a serious, life-threatening problem (47). Owing to the barrier properties of the OM, this problem is especially acute for Gram-negative pathogens. Many antibiotics that work well for infections caused by Gram-positive bacteria fail when used against Gram-negative bacteria because of the effectiveness of the OM barrier, and we do not know how to modify these agents so that they can reach their targets. A better understanding of the permeability properties of the OM and how they can be modified should help facilitate the design of new antibacterials.

ACKNOWLEDGMENTS

This material is based upon work supported by the National Science Foundation Graduate Research Fellowship Program under grant no. DGE1148900 (H.A.S.). This work was also supported by the National Institute of General Medical Sciences grant GM34821 (T.J.S.) and NIH/NIGMS grant P50 GM071508 (S.Z.).

Any opinions, findings, and conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the National Science Foundation.

We thank the Silhavy lab, present and past, for helpful discussion and comments. We also thank Mark Rose and Zemer Gitai for helpful discussion and critical analysis. We are grateful to Thomas Bartlett for help with imaging and cell size analysis as well as Jaclyn Schwalm for strains.

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