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Replacement of Lipopolysaccharide with Free Lipid A Molecules in *Escherichia coli* Mutants Lacking All Core Sugars†

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ABSTRACT: *Escherichia coli* mutants deficient in 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo) biosynthesis are conditionally lethal, but their phenotypes are bypassed by certain suppressor mutations or by overexpression of MsbA, the inner membrane flippase for core−lipid A. These strains grow on broth with the tetraacylated precursor lipid IV_A replacing lipopolysaccharide [Meredith, T. C., et al. (2006) *ACS Chem. Biol. 1*, 33−42]. Deletion of *kdtA*, which encodes the Kdo transferase, is possible under these conditions. We now show that lipid IV_A reaches the outer surface of the outer membrane in these strains, as judged by its accessibility to the lipase PagL. On the assumption that MsbA is optimized to transport penta- or hexaacylated lipid A, we overexpressed the lauroyl- or the myristoyltransferase of lipid A biosynthesis, encoded by *lpxL* and *lpxM*, respectively, and demonstrated that *kdtA* deletion mutants were also viable in this setting. Although *E. coli* LpxL is stimulated by the presence of the Kdo disaccharide in its acceptor substrate, LpxL does slowly acylate lipid IV_A. Overexpression of LpxL from a plasmid suppressed the lethality of *kdtA* deletions on nutrient broth at 30 or 37 °C without the need for MsbA overproduction. These strains accumulated penta- and hexaacylated free lipid A containing a secondary laurate chain or a laurate and a myristate chain, respectively. Deletion of *kdtA* in strains overexpressing LpxM accumulated pentaacylated lipid A with a secondary myristate moiety. None of the strains lacking *kdtA* grew in the presence of bile salts at any temperature or on nutrient broth at 42 °C. Our findings show that the main function of Kdo is to provide the right substrates for the acyltransferases LpxL and LpxM, resulting in the synthesis of penta- and hexaacylated lipid A, which is optimal for the MsbA flippase.

Mutants of *Escherichia coli* or *Salmonella* defective in the biosynthesis of ADP-heptose or in the transfer of heptose to the inner 2-keto-3-deoxy-D-*manno*-octulosonic acid (Kdo)[1] residue of nascent lipopolysaccharide (LPS) accumulate Kdo_2−lipid A (Figure 1), which is transported to their outer membranes (*1*−*3*). The Kdo_2−lipid A substructure of LPS (*4*) is sufficient to support bacterial growth on nutrient broth at 30 or 42 °C, albeit with alterations in outer membrane permeability (*5*). Kdo_2−lipid A is the most conserved portion of LPS in all Gram-negative bacteria (*2*, *3*) and is a potent agonist against the Toll-like receptor 4 (TLR4)/MD2 complex of the innate immune system (*6*−*8*), which detects LPS as foreign and triggers inflammation.

E. coli or *Salmonella* mutants defective in Kdo biosynthesis, or lacking Kdo-transferase, are not viable on nutrient broth, and conditional mutants accumulate lipid IV_A (Figure 1) in their inner membranes (*9*−*14*). However, Kdo biosynthesis genes can be deleted in the presence of certain point mutations in MsbA (*15*, *16*), an essential ABC transporter that normally functions as the inner membrane flippase for nascent LPS (Figure 2) (*17*−*20*), or in the nonessential inner membrane of protein YhdJ (*15*), the function of which is unknown. Alternatively, Kdo biosynthesis genes can be deleted if MsbA is overexpressed (*16*), and the gene encoding Kdo transferase can be deleted if cells are grown very slowly on minimal medium at 21 °C (*21*). In all of these situations, cells replace their LPS with the precursor lipid IV_A (Figure 1), which accumulates to high levels (*16*, *21*).

Following several covalent modifications of core−lipid A and attachment of O-antigen on the outer surface of the inner membrane (*2*, *3*), nascent LPS is transported to the outer surface of the outer membrane by the Lpt proteins (Figure 2) (*22*−*27*). The inner membrane proteins LptB, LptF, and LptG, in conjunction with the accessory protein LptC, constitute a second essential ABC transporter (*26*), apparently required for the ejection of LPS from the outer surface of the inner membrane (Figure 2). The soluble periplasmic protein LptA may bind to lipid A directly (*28*) and deliver it to the LptD/E complex, or it may function to appose the inner and outer membranes (*27*, *29*) so that the LptD and LptE proteins can incorporate nascent LPS into the outer surface of the outer membrane (Figure 2). The biochemical mechanisms by which these transport proteins accomplish their tasks have not been elucidated.

Enzymatic modifications to the lipid A moiety of LPS occur on the outer surface of the inner membrane or in the outer membrane (*3*). For instance, in polymyxin-resistant mutants, phosphoethanolamine (pEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) units are attached to the phosphate groups of lipid A on the outer surface of the inner membrane (*18*, *30*). After

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[1]Abbreviations: L-Ara4N, 4-amino-4-deoxy-L-arabinose; ESI/MS, electrospray ionization mass spectrometry; IPTG, isopropyl β-D-thiogalactoside; Kdo, 2-keto-3-deoxy-D-*manno*-octulosonic acid; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; pEtN, phosphoethanolamine; PG, phosphatidylglycerol; TLR4, Toll-like receptor 4.

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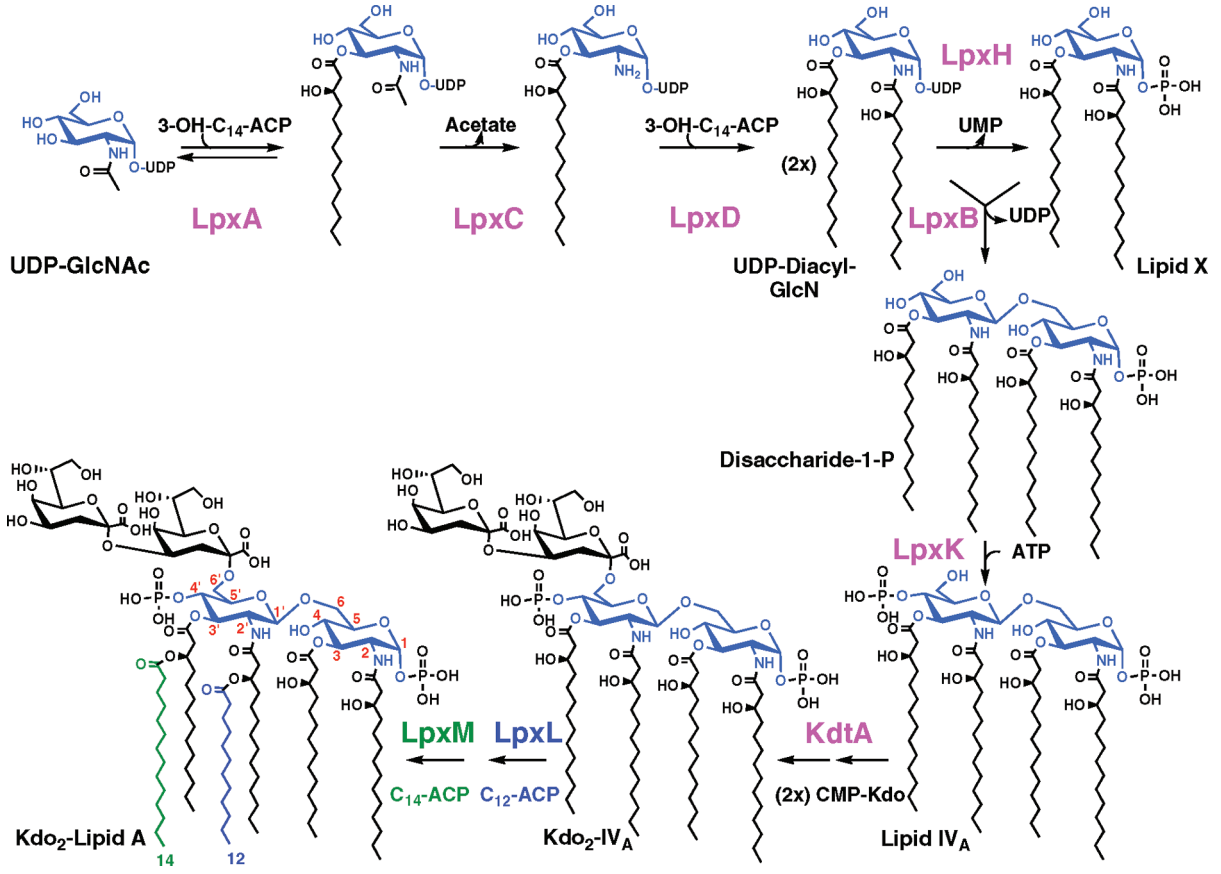

FIGURE 1: Biosynthesis of lipid A in wild-type *E. coli* K-12. The first three enzymes are cytoplasmic, whereas the others are peripheral or integral inner membrane proteins that face the cytoplasm (*2*, *3*). LpxL is stimulated ~1000-fold in vitro by the presence of the Kdo disaccharide in its substrate, but its residual activity with lipid IV_A is significant (*38*). The Kdo dependency of LpxM has not been studied in vitro. Mutants of *E. coli* or *Salmonella* defective in Kdo biosynthesis or transfer accumulate mainly tetraacylated lipid IV_A and some of its modified derivatives (not shown) (*11*, *12*). No free hexaacylated lipid A accumulates in Kdo-deficient mutants of cells grown at 30 °C or above.

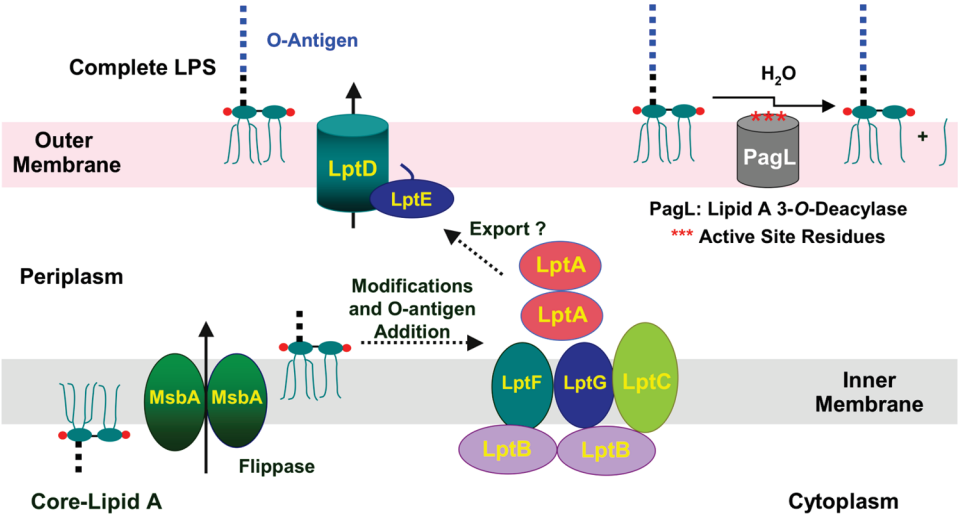

FIGURE 2: Proteins involved in the export of nascent LPS to the outer membrane. The main steps of core−lipid A biosynthesis occur on the inner surface of the inner membrane, whereupon the essential ABC transporter MsbA flips the nascent LPS to the outer surface of the inner membrane (*2*, *3*). In wild-type strains (though not *E. coli* K-12), O-antigen is polymerized and transferred to the nascent LPS on the outer surface of the inner membrane (*2*, *3*). The downstream ABC transporter, consisting of the LptA, LptB, LptC, LptG, and LptF proteins, is thought to deliver LPS to the outer membrane LptD/E complex, which is responsible for the proper integration of LPS into the outer surface of the outer membrane (*22*−*27*). Modification of lipid A with pEtN or L-Ara4N units in polymyxin-resistant mutants occurs on the outer surface of the inner membrane (not shown), whereas PagL-catalyzed removal of the 3-*O*-acyl chain or PagP-catalyzed addition of palmitate (not shown) occurs in the outer surface of the outer membrane (*3*).

incorporation into the outer surface of the outer membrane, the lipase PagL from *Salmonella* (Figure 2) can remove the (*R*)-3-hydroxyacyl chain from the 3-position of lipid A (*31*, *32*). Alternatively, the outer membrane acyltransferase PagP (not shown) can add a secondary palmitate chain to the (*R*)-3-hydroxymyristate unit at the 2-position of lipid A (*33*−*35*). These

Table 1: Bacterial Strains and Plasmids

	relevant genotype	source or ref
E. coli strains		
W3110	wild type, F^-, λ^-	*E. coli* Genetic Stock Center, Yale University
DY330	W3110 △*lacU169 gal490 λcI857* △ (*cro-bioA*)	*44*
XL1-Blue	△*mcrABC recA1 endA1 gyrA96 relA1 supE44 thi-1 lac*	Stratagene
WBB06	W3110 *mtl*, (Δ*waaC-waaF*)::*tet6*, heptose deficient	*1*
MST100[a]	DY330 Pm^R	*55*
CMR100	DY330 (*kdtA::kan*) pWMsbA	this work
CMR101	DY330 (*kdtA::kan*) pWLpxL	this work
CMR102	DY330 (*kdtA::kan*) pWLpxM	this work
CMR103	DY330 (*kdtA::kan*) pLptCAB2	this work
CMR200	MST100 (*kdtA::kan*) pWMsbA	this work
CMR300	W3110 (*kdtA::kan*) pWMsbA	this work
CMR301	W3110 (*kdtA::kan*) pWLpxL	this work
CMR302	W3110 (*kdtA::kan*) pWLpxM	this work
plasmids		
pWSK29	low copy vector, *lac* promoter, Amp^R	*36*
pWSK130	low copy vector, *lac* promoter, Kan^R	*36*
pACYC184	medium copy vector, Tet^R, Cam^R	New England Biolabs
pBAD33	medium copy vector, Cam^R	*62*
pBAD-pagL	pBAD33 harboring *pagL*	*27*
pWTD1	pET28b harboring *msbA*	*19*
pWTD2	pET23a(+) harboring *msbA*	this work
pACYC-KdtA	pACYC184 harboring *kdtA*	this work
pWMsbA	pWSK29 harboring *msbA*	this work
pWLpxL	pWSK29 harboring *lpxL*	*38*[b]
pMsbB	pET21a(+) harboring *lpxM*	*39*
pWLpxM	pWSK29 harboring *lpxM*	this work
pLptCAB2	pWSK29 harboring *lptC*, *lptA*, *lptB*	*27*

[a] Pm^R: polymyxin resistant. [b] Same as pWSK-LpxL (*38*).

modification enzymes are useful as reporters for the trafficking of lipid A from the inside of the cell to the outer leaflet of the outer membrane.

We now report several new *E. coli* constructs in which the *kdtA* gene (Figure 1) can be deleted. Overexpression of MsbA from the plasmid pWSK29 (*36*) permits the deletion of *kdtA* in *E. coli* cells grown on nutrient broth at 30 or 37 °C, accompanied by the accumulation of lipid IV_A, but these strains do not grow at 42 °C or on MacConkey agar (*37*), which contains bile salts. The *kdtA* gene can also be deleted when LpxL or LpxM (*38*−*41*) (Figure 1) is overexpressed from pWSK29. When LpxL is overexpressed in strains lacking *kdtA*, large amounts of penta- and hexaacylated free lipid A species are produced. The pentaacylated species contains laurate as its secondary acyl chain, whereas the hexaacylated material contains laurate and myristate, as does the lipid A moiety of wild-type LPS (*3*). When LpxM is overexpressed in strains lacking *kdtA*, pentaacylated free lipid A bearing a secondary myristate chain accumulates. These results support the view that penta- and hexaacylated lipid A are better substrates for the MsbA flippase than is the tetraacylated precursor lipid IV_A (*17*, *20*); consequently, overexpression of MsbA is not necessary to support growth in these constructs. Finally, *kdtA* can also be deleted in cells overexpressing LptA, LptB, and LptC (Figure 2), but these cells only grow slowly at 30 °C.

EXPERIMENTAL PROCEDURES

Materials. Chloroform, methanol, and silica gel 60 (0.25 mm) thin-layer chromatography plates were from EMD Chemicals Inc. (Gibbstown, NJ). Tryptone, yeast extract, and agar were from Becton, Dickinson and Co. (Franklin Lakes, NJ). Isopropyl 1-thio-β-D-galactopyranoside (IPTG) was from Invitrogen Corp. (Carlsbad, CA). $^{32}P_i$ (10 μCi/μL) was from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA). All other chemicals were of reagent grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

Bacterial Strains. Bacterial strains used in this study are described in Table 1. They are all derivatives of *E. coli* K-12, which makes a complete LPS core but no O-antigen (*2*). Typically, bacteria were grown in LB medium, a nutrient broth that contains 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter (*37*), and tested for viability on MacConkey medium (*37*). When required for the selection of plasmids, cells were grown in the presence of 100 μg/mL ampicillin, 25 μg/mL chloramphenicol, 12.5 μg/mL tetracycline, and/or 20 μg/mL kanamycin.

Molecular Biology Applications. Protocols for handling of DNA samples and preparing *E. coli* for electroporation were those of Sambrook and Russell (*42*). Transformation-competent cells of *E. coli* were prepared by the method of Inoue et al. (*43*). Plasmids were isolated from cell cultures using the QIAprep miniprep kit or extracted from agarose gel with the QIAquick gel extraction kit (Qiagen, Valencia, CA). Genomic DNA was isolated using the protocol for bacterial cultures in the Easy-DNA kit (Invitrogen, Carlsbad, CA). T4 DNA ligase (Invitrogen), restriction endonucleases (New England Biolabs, Ipswich, MA), and shrimp alkaline phosphatase (USB, Cleveland, OH) were used according to the manufacturers' instructions. Double-stranded DNA sequencing was performed with an ABI Prism 377 instrument at the Duke University DNA Analysis Facility. Primers were purchased from MWG-Biotech (Huntsville, AL).

Construction of MsbA, LpxM, and KdtA Expression Vectors. The *msbA* gene was excised from pWTD1 (*19*) by

Table 2: Oligonucleotide Primers Used in This Work

name	sequence
KdtAKOFOR	5′-CATAGAATCCCCAGCACATCCATAAGTCAGCTATTTACT*AAGGAGATATAATGAGCCATATTCAACGGGAA*-3′ [a]
KdtAKOREV	5′-GAAAGTACCCGGATAAATCGCCCGTTTTTGCATAACAACC*TTAGAAAAACTCATCGAGCAT*-3′ [a]
KdtACFor	5′-CCAAACTGAAGCTATTTAAGTC-3′
KdtACRev	5′-GTGATCGAACATCTGCGTCGTG-3′
KdtAXbaI	5′-GCGCGC*TCTAGA*AAGGAGATATAATGCTCGAATTGCTTTACACC-3′ [b]
KdtABamHI	5′-GCGCGC*GGATCC*TCAATGCGTTTTCGGTGGCAG-3′ [b]

[a]The letters in italics are part of the *kan* gene plus the ribosome binding site. The *kdtA* flanking genomic sequence is not italicized. [b]The underlined letters are the recognition sites for the restriction enzymes.

digesting the plasmid with *Nde*I and *Bam*HI. The *msbA* gene was then ligated into the corresponding restriction sites of pET23a(+) to create pWTD2. Next, the *Xba*I/*Bam*HI-digested fragment, consisting of the *msbA* gene as well as the pET23a(+)-derived ribosome binding site, was ligated to the corresponding sites of pWSK29, a lactose-inducible, low-copy expression vector (*36*). This plasmid, designated pWMsbA, was transformed into competent cells of *E. coli* WBB06, DY330, W3110, or MST100 (Table 1).

The *lpxM* gene was excised from pMsbB (*39*) using the *Xba*I and *Hin*dIII sites. The *Xba*I/*Hin*dIII-digested fragment, consisting of the *lpxM* gene as well as the pET21a(+)-derived ribosome binding site, was ligated to the corresponding sites of pWSK29. This plasmid, designated pWLpxM, was transformed into competent cells of *E. coli* DY330 or W3110.

The *kdtA* gene of *E. coli* W3110 was cloned into pACYC184. The coding region for *kdtA* was amplified by PCR from *E. coli* W3110 genomic DNA using the primers KdtAXbaI and KdtA-BamHI (Table 2). The PCR reaction was carried out using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The PCR product and the vector were digested with both *Xba*I and *Bam*HI, ligated together, and transformed into XL1-Blue cells (Table 1) for propagation of the desired plasmid. This plasmid, designated pACYC-KdtA, was transformed into CMR100 (see below).

Construction of kdtA Deletions in E. coli DY330 Derivatives. PCR was used to construct a linear piece of DNA containing the kanamycin resistance cassette (*kan*), flanked on the 5′ end by a ribosome binding site plus 39 bp of chromosomal DNA upstream of *kdtA* and flanked on the 3′ end by 40 bp of chromosomal DNA located downstream of *kdtA*. The sequences of the forward (KdtAKOFOR) and reverse (KdtAKOREV) primers used to construct this PCR product are presented in Table 2. The kanamycin resistance gene (Tn*903*) from plasmid pWSK130 (*36*) served as the template. The PCR reaction was carried out using *Pfu* DNA polymerase (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The PCR product was resolved on a 1% agarose gel and purified with the QIAquick gel kit. The product was electroporated into DY330/pWMsbA, DY330/pWLpxL, DY330/pWLpxM, DY330/pLptCAB, and MST100/pWMsbA (Table 1), which had been grown at 30 °C in the presence of 100 μg/mL ampicillin and 1 mM IPTG until the A_{600} reached ≈0.4. Just prior to electroporation, each culture was shifted to 42 °C for 15 min to activate the λ-RED genes (*44*), and the cells were washed twice with 30 mL of ice-cold distilled water. The cells were resuspended in cold distilled water and then electroporated with 100−250 ng of the PCR product. After growth for 2 h at 30 °C in LB medium with 100 μg/mL ampicillin and 1 mM IPTG, the cells were plated on LB agar, containing kanamycin, ampicillin, and IPTG. The plates were incubated at 30 °C, and the resulting colonies were repurified on LB−kanamycin, ampicillin, and IPTG plates at 30 °C. Colonies of all the strains remained viable for only 1−2 days on LB agar plates. Liquid cultures lost viability in stationary phase, and therefore they were only grown to A_{600} = 0.3−0.4, except as indicated. All of the strains remained viable at −80 °C in glycerol stocks. The *kdtA*::*kan* replacement on the chromosome of DY330 harboring the various covering plasmids was verified by PCR using external primers KdtACFor and KdtACRev (Table 2). The PCR products were resolved on a 1% agarose gel, purified with the QIAquick gel extraction kit, and sequenced to confirm the replacement. The *kdtA::kan* derivatives of DY330/pWMsbA, DY330/pWLpxL, DY330/pWLpxM, DY330/pLptCAB2, and MST100/pWMsbA were designated as CMR100, CMR101, CMR102, CMR103, and CMR200, respectively.

Construction of kdtA Deletions in E. coli W3110. DY330 is inherently temperature-sensitive (*44*); thus, it was necessary to transduce the *kdtA::kan* marker into W3110 harboring the various pWSK29-derived multicopy suppressor constructs (Table 1) in order to evaluate the viability of the *kdtA::kan* strains at higher temperatures. To accomplish this, we first transformed CMR100 with pACYC-KdtA to restore LPS, which is needed for $P1_{vir}$ infection. Next, a $P1_{vir}$ bacteriophage lysate of donor strain CMR100/pACYC-KdtA was made (*37*) and used to transduce stationary cells of W3110/pWMsbA, W3110/pWLpxL, and W3110/pWLpxM, which had been grown at 30 °C in the presence of 100 μg/mL ampicillin and 1 mM IPTG. The transduction mixtures were plated onto LB agar, containing 20 μg/mL kanamycin, 100 μg/mL ampicillin, 1 mM IPTG, and 4 mM citrate. Colonies were repurified with the same selection, and the *kdtA*::*kan* replacements on the chromosome of each of the constructs were verified by PCR using external primers KdtACFor and KdtACRev (Table 2). The PCR products were resolved on a 1% agarose gel, purified with the QIAquick gel extraction kit, and sequenced to confirm the replacement. The *kdtA::kan* derivatives of W3110/pWMsbA, W3110/pWLpxL, and W3110/pWLpxM were designated as CMR300, CMR301, and CMR302, respectively. As in the DY330 background, colonies on LB agar remained viable for 1 or 2 days, and in liquid culture the strains lost viability in stationary phase.

Growth Phenotypes of kdtA Deletion Mutants. Strains were grown overnight on LB broth containing 1 mM IPTG and 100 μg/mL ampicillin at 30 °C and used to inoculate 50 mL of prewarmed LB broth containing 1 mM IPTG and 100 μg/mL ampicillin to A_{600} = 0.02. Growth was allowed to continue with shaking at 220 rpm. Whenever the A_{600} reached 0.3−0.4, the cultures were diluted 10-fold into 50 mL of prewarmed LB broth containing 1 mM IPTG and 100 μg/mL ampicillin in order to keep the cells in mid-log phase.

Lipid Isolation from kdtA Deletion Mutants. Typically, strains were grown from single colonies to $A_{600} = 0.3$ on LB broth containing 1 mM IPTG and 100 μg/mL ampicillin at 30 °C and used to inoculate 50 mL of LB broth containing 1 mM IPTG and 100 μg/mL ampicillin to $A_{600} = 0.02$. Growth was allowed to continue with shaking at 220 rpm. When the A_{600} reached 1.0, the cultures were harvested by centrifuging at 4000*g* for 20 min. Cells were then washed once with 30 mL of phosphate-buffered saline (PBS) (*45*). The pellet was then resuspended in 8 mL of PBS. Chloroform (10 mL) and methanol (20 mL) were added to make a one-phase Bligh−Dyer system (*46*). The mixture was incubated at room temperature for 1 h with occasional mixing and then centrifuged at 4000*g* for 20 min. The supernatant was converted to a two-phase Bligh and Dyer mixture by the addition of chloroform (10 mL) and PBS (10 mL). The mixture was vortexed and centrifuged at 4000*g* for 20 min. The lower phase was removed, and the upper water−methanol phase was extracted a second time by the addition of preequilibrated lower phase. The lower phases were pooled and dried by rotary evaporation. The lipids were stored at −80 °C.

Electrospray Ionization Mass Spectrometry of Lipids. Spectra were acquired on an ABI QSTAR XL quadropole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Toronto, Canada) equipped with an electrospray ionization (ESI) source. Spectra were acquired in the negative ion mode and typically were the accumulation of 60 scans collected from *m*/*z* 200 to *m*/*z* 2000. Typically, one-third of the total lipids extracted from a 50 mL culture were dissolved in 200 μL of chloroform−methanol (2:1 v/v), supplemented with 1% piperidine, and immediately infused into the ion source at 5−10 μL/min. The negative ion ESI was carried out at −4200 V. Data acquisition and analysis were performed using the Analyst QS software (ABI/MDS-Sciex, Toronto, Canada).

RESULTS

Meredith et al. first reported that *E. coli* mutants defective in Kdo biosynthesis, which are conditionally lethal in a wild-type background, were rendered viable by point mutations in either MsbA (Figure 2) or YhdJ, a nonessential, inner-membrane protein of unknown function (*15*, *16*). Their constructs lack LPS but still synthesize the precursor lipid IV_A (Figure 1) and transport it to the outer membrane (*16*). Meredith et al. did not determine the orientation of the lipid IV_A within the outer membrane.

Phenotypes of kdtA Deletions in Cells Overexpressing msbA. We constructed a new strain of *E. coli* in which the *kdtA* gene, encoding Kdo transferase (Figure 1), could be deleted without loss of viability. The MsbA-expressing plasmid pWMsbA was introduced into DY330 (Table 1), a strain that makes the λ-red recombinase to permit homologous recombination between exogenous linear DNA molecules and their corresponding chromosomal sequences (*44*). In this setting it was possible to obtain kanamycin-resistant recombinants of DY330 on nutrient broth at 30 °C. The *kan* cassette in DY330 *kdtA::kan*(pWMsbA) replaced the entire *kdtA* gene; the strain was designated CMR100. When lipids of CMR100 were analyzed by ESI/MS in the negative ion mode, peaks consistent with accumulation of lipid IV_A were observed (Supporting Information Figure 1).

The presence of the heat-inducible λ-red recombinase in DY330 renders this strain temperature-sensitive for growth (*44*). To evaluate the growth phenotype of *kdtA* deletion mutations in the absence of the λ-red recombinase, the *kan* cassette of DY330 *kdtA::kan*(pWMsbA) was transferred into *E. coli* W3110-(pWMsbA) by $P1_{vir}$ transduction and selection for kanamycin-resistant transductants on LB broth at 30 °C. W3110 *kdtA::kan*(pWMsbA), renamed CMR300 (Table 1), grew normally on broth at 30 and 37 °C (Figure 3A) but stopped growing after ~2 h at 42 °C (Figure 3D). CMR300 failed to grow at any temperature on MacConkey agar (not shown), possibly because of the presence of bile salts in this medium. CMR300 remained very sensitive to the selective LpxC inhibitor CHIR-090 (*47*) (data not shown); apparently, the lipid IV_A that replaces LPS in CMR300 is still essential for some aspect of cell physiology or outer membrane biogenesis. All *kdtA* deletion mutants lose viability if A_{600} exceeds 1 (data not shown).

ESI/MS and TLC of Lipids from W3110(pWMsbA) and CMR300. Like DY330 *kdtA::kan*(pWMsbA), CMR300 cells accumulate high levels of lipid IV_A when grown at 30 °C on LB broth, representing between 15% and 20% of the total chloroform-soluble material, as quantified by $^{32}P_i$ labeling (see below). ESI/MS of the total cellular lipids in the negative ion mode showed new peaks interpreted as the $[M - 2H]^{2-}$ and $[M - 3H + Na]^{2-}$ ions of lipid IV_A at *m*/*z* 701.448 and *m*/*z* 712.419 in CMR300 but not in W3110(pWMsbA) (panels B and A of Figure 4, respectively). These values are in reasonable agreement with the predicted *m*/*z* of 701.420 and 712.411 for the $[M - 2H]^{2-}$ and the $[M - 3H + Na]^{2-}$ ions of lipid IV_A, which ionizes predominantly as a dianion during ESI/MS. The slight discrepancy between the observed and predicted values for the $[M - 2H]^{2-}$ ion may reflect the presence of overlapping minor glycerophospholipids in this region of the spectrum (Figure 4B). The overall distribution of phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) molecular species was very similar in both strains (Figure 4). The accumulation of lipid IV_A in CMR300 versus W3110(pWMsbA) was confirmed by TLC and charring with 10% sulfuric acid in ethanol (Figure 4, insets).

Presence of Lipid IV_A on the Outer Surface of the Outer Membrane in CMR300. In order to demonstrate that lipid IV_A in CMR300 is transported to the outer surface of the outer membrane, a second compatible hybrid plasmid (pBAD-PagL) (*27*) was transformed into CMR300 (Table 1). In parallel, the pBAD vector without insert was introduced into CMR300. The resulting constructs were grown at 37 °C in LB broth and induced with 0.2% L-arabinose when A_{600} reached 0.3 (Figure 5A). Concomitantly, the cells were labeled with $^{32}P_i$ and grown for a additional 3 h with occasional 10-fold back-dilution when A_{600} reached 1.0 to maintain exponential growth. As shown in Figure 5B, about two-thirds of the lipid IV_A was deacylated when PagL was present, supporting the view that much of the lipid IV_A reaches the outer surface of the outer membrane in CMR300. The loss of one of the four acyl chains of lipid IV_A (*31*, *32*) does not further compromise the viability of CMR300 under these conditions.

Phosphoethanolamine Addition to Lipid IV_A in Polymyxin-Resistant DY330. In polymyxin-resistant mutants of *E. coli*, the lipid A moiety of LPS is modified with pEtN and L-Ara4N units (*3*), which reduce its affinity for polymyxin and other cationic antimicrobial peptides. The pEtN and L-Ara4N moieties are added to lipid A on the outer surface of the inner membrane (*18*). In the presence of the Kdo disaccharide, pEtN goes mainly to position 1, whereas L-Ara4N is targeted to position 4′ (*48*). In temperature-sensitive *Salmonella* mutants defective in Kdo biosynthesis, pEtN is incorporated exclusively at

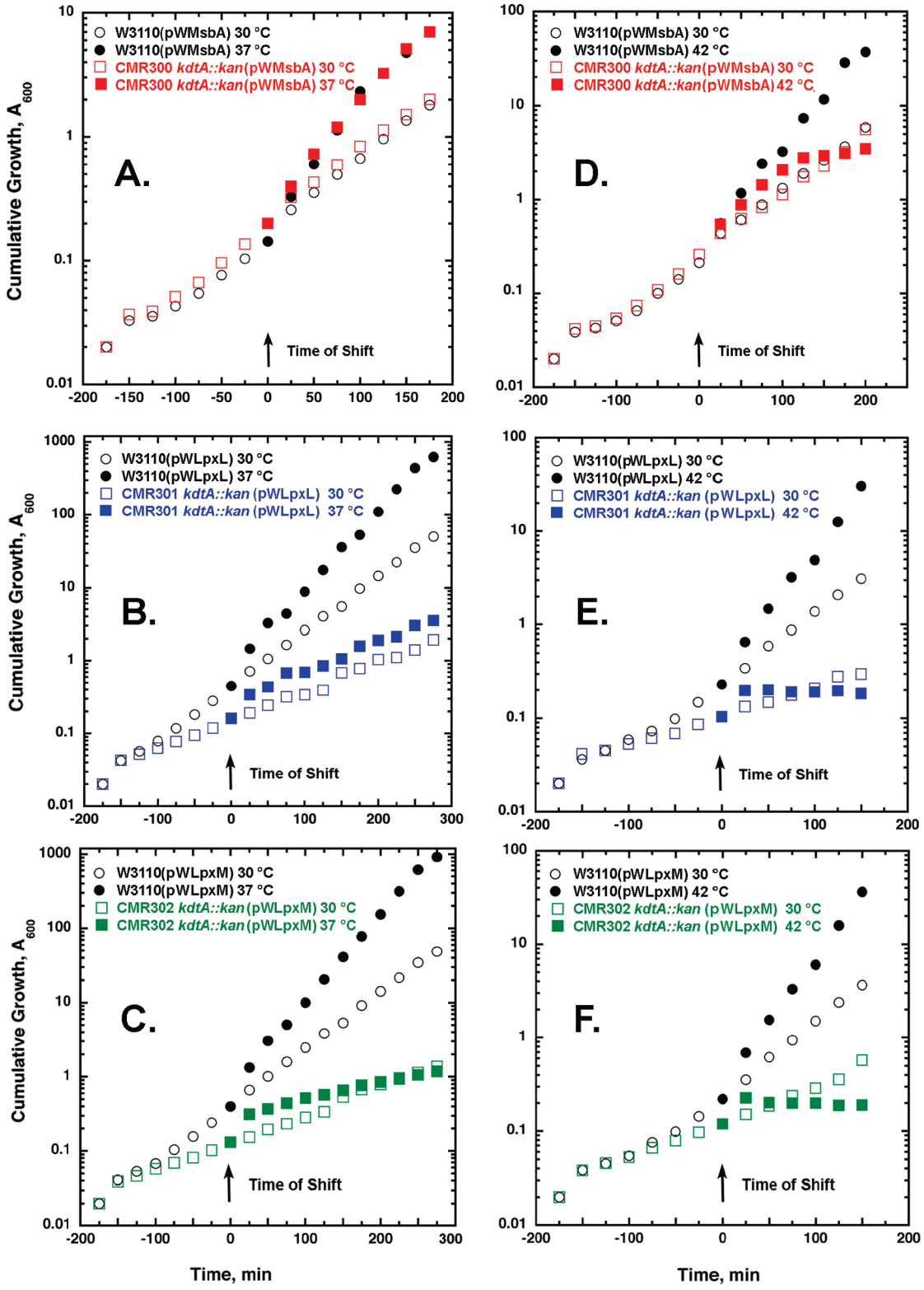

FIGURE 3: Temperature-sensitive growth of CMR300, CMR301, and CMR302 on LB broth. Cells were grown on LB broth with 100 μg/mL ampicillin and 1 mM IPTG from A_{600} 0.02 to 0.2 at 30 °C; then half of each culture was shifted to 37 or 42 °C, as indicated. Cultures were diluted 10-fold whenever A_{600} reached 0.3−0.4. The cumulative A_{600} was corrected for the dilutions. Panels A, B, and C show the temperature shifts of CMR300, CMR301, and CMR302, respectively, from 30 to 37 °C. CMR300 nearly matches the W3110(pWMsbA) control at both temperatures. Reduced growth rates, albeit with good viability, were observed for CMR301 and CMR302 at both 30 and 37 °C versus their matched vector control strains. Panels D, E, and F show the temperature shifts of exponentially growing CMR300, CMR301, and CMR302, respectively, from 30 to 42 °C in LB broth, demonstrating the rapid growth cessation of all three *kdtA* deletion constructs.

position 4′ and a small amount of L-Ara4N is added to position 1 (*12*, *48*, *49*).

To demonstrate that lipid IV_A reached the outer surface of the inner membrane (Figure 2), we deleted *kdtA* in MST100(pWMsbA), a polymyxin-resistant derivative of DY330 (Table 1). In the *kdtA* deletion strain of MST100 (designated CMR200 in Table 1), relatively little lipid IV_A accumulated, but a new compound migrating more slowly was observed (Figure 6, insets). ESI/MS in the

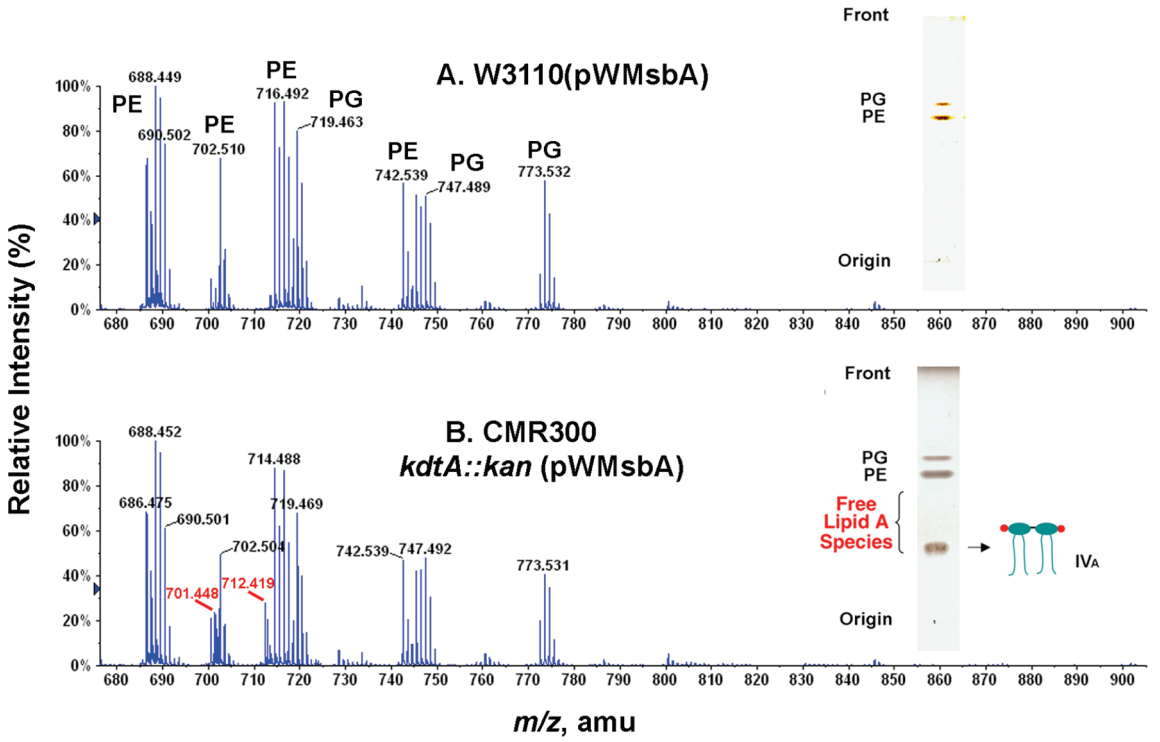

FIGURE 4: ESI/MS and TLC demonstrating accumulation of lipid IV_A in CMR300. Exponentially growing cells in 50 mL of LB broth supplemented with 1 mM IPTG and 100 μg/mL ampicillin were harvested in late log phase. The lipids were extracted with a two-phase neutral Bligh−Dyer system (*46*), redissolved in chloroform/methanol/piperidine (2:1:0.03 v/v/v), and immediately analyzed in the negative ion mode by direct infusion ESI/MS, using an ABI QSTAR XL quadrupole time-of-flight mass spectrometer. Panel A: Major glycerophospholipid ions of the control strain W3110(pWMsbA) between *m/z* 680 and 900 consist mainly of molecular species of PE and PG, as indicated. Panel B: The *kdtA* deletion mutant CMR300 contains similar glycerophospholipids but accumulates additional peaks (red), which are interpreted as the $[M - 2H]^{2-}$ and $[M - 3H + Na]^{2-}$ ions of lipid IV_A. The accumulation of lipid IV_A was confirmed by TLC (insets).

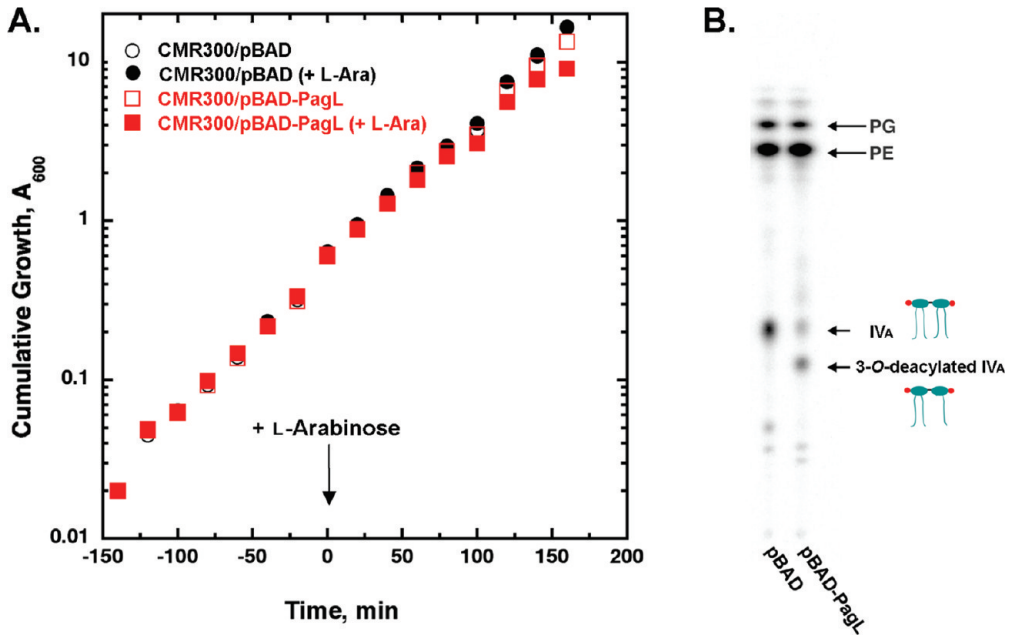

FIGURE 5: Efficient transport of lipid IV_A to the outer surface of the outer membrane in CMR300. Panel A: Cultures of CMR300 harboring either pBAD33 or pBAD-pagL were grown at 37 °C in the presence of 100 μg/mL ampicillin and 25 μg/mL chloramphenicol from an initial A_{600} of 0.02. When the A_{600} reached 0.3, 0.2% L-arabinose and 5 μCi/mL $^{32}P_i$ were added, and growth was allowed to continue for an additional 3 h with the cultures back-diluted as needed. The cells were harvested when the cumulative A_{600} reached 10 and washed once with PBS. Panel B: A single-phase neutral Bligh−Dyer mixture (*46*) was used to extract the lipids, followed by conversion to two phases and drying down of the lower phase that contains the lipids, as described previously (*17*, *48*). The lipids were dissolved in chloroform/methanol (2:1 v/v) and spotted onto a silica gel 60 TLC plate. The plate was developed with a chloroform:pyridine:88% formic acid:water (50:50:16:5 v/v/v/v) system and then analyzed with a PhosphorImager.

negative ion mode of the lipids from CMR200 revealed new peaks at *m/z* 762.931 and *m/z* 882.052, interpreted as the $[M - 2H]^{2-}$ ions of lipid III_A (i.e., lipid IV_A derivatized with one pEtN moiety) (*12*) and lipid III_B (i.e., lipid IV_A derivatized with one pEtN and one palmitoyl

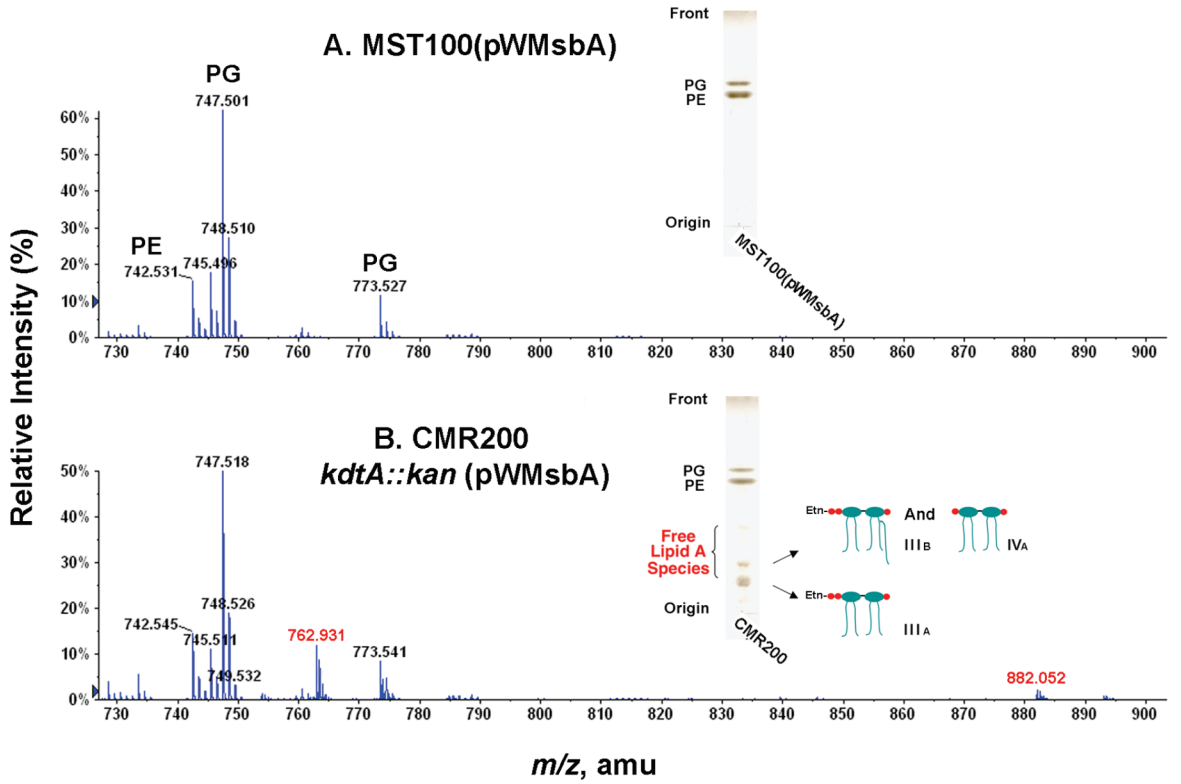

FIGURE 6: Phosphoethanolamine-modified lipid IV_A in CMR200, a PmrA-constitutive *kdtA* deletion mutant. Cells were grown at 30 °C, and lipids were extracted as in Figure 4. Panel A: ESI/MS in the negative ion mode of the major lipid species in the control strain MST100(pWMsbA). Panel B: Accumulation of the pEtN-modified lipids III_A and III_B (*11*, *12*) in CMR200, indicated by the red numbers. The accumulation of lipids III_A ($[M - 2H]^{2-}$ at *m*/*z* 762.931) and III_B ($[M - 2H]^{2-}$ at *m*/*z* 882.052) was confirmed by TLC (insets).

moiety) (*12*), respectively (Figure 6B). The predicted $[M - 2H]^{2-}$ ions for these species are *m*/*z* 762.923 and *m*/*z* 882.088, in reasonable agreement with the observed values. The proposed attachment of the pEtN moiety to the 4′-phosphate group (Figure 6B, inset) was confirmed by ^{31}P NMR analysis (not shown) (*12*). The presence of some lipid III_B (Figure 6B) provides additional confirmation that lipid III_A reaches the outer surface of the outer membrane in this construct. The palmitate moiety of III_B is incorporated by the outer membrane acyltransferase PagP (*33*, *34*). No peaks corresponding to L-Ara4N-modified lipid IV_A were observed in CMR200 (Figure 6B), presumably reflecting the requirement for the Kdo moiety and/or the secondary myristoyl chain of lipid A for L-Ara4N transferase activity (*49*, *50*). CMR200 is polymyxin sensitive when compared to MST100, consistent with the loss of the L-Ara4N modification (data not shown).

Viability of kdtA Deletions in Cells Overexpressing lpxL or lpxM. Overexpression of the *msbA* gene suppresses the temperature-sensitive phenotype of deletions in *lpxL*(*htrB*), which encodes in the lauroyltransferase of lipid A biosynthesis (Figure 1) (*17*, *51*, *52*). LpxL-deficient strains accumulate LPS containing a tetraacylated lipid A moiety at 42 °C (*17*, *39*). The requirement for MsbA overexpression to permit growth of *lpxL* mutants at 42 °C suggests that tetraacylated lipid A is a relatively poor substrate for MsbA (*39*).

LpxL activity is stimulated ~1000-fold by the presence of the Kdo disaccharide in its acceptor substrate (Figure 1), but the residual activity is significant (*38*). To determine whether or not hexaacylated lipid A by itself is the preferred substrate for MsbA, we constructed W3110 *kdtA::kan*(pWLpxL), renamed CMR301, which grew more slowly on LB broth at 30 and 37 °C than did CMR300 (Figure 3B) but formed single colonies on LB agar (not shown). Like CMR300, CMR301 failed to grow at 42 °C (Figure 3D) on LB agar or on MacConkey agar at any temperature.

The same strategy for LpxL was used to delete the *kdtA* gene of W3110 in the presence of pWLpxM, which directs the overexpression of the myristoyltransferase of lipid A biosynthesis (Figure 1) (*40*). The growth phenotypes of W3110 *kdtA::kan*-(pWLpxM), renamed CMR302 (Figures 3C and 3F), were very similar to those of CMR301 (Figure 3B,E). No growth of CMR302 was observed at 42 °C on LB broth or LB agar or on MacConkey agar at any temperature.

Penta- and Hexaacylated Free Lipid A in CMR301 and CMR302. TLC and ESI/MS were used to evaluate the lipid compositions of CMR301 and CMR302 grown at 30 °C on LB broth with 1 mM IPTG and 100 μg/mL ampicillin. The control strain W3110(pWLpxL) contained no free lipid A, and its major PE and PG molecular species (Figure 7A) were essentially the same as those in W3110(pWMsbA) (Figure 4A) or W3110-(pWLpxM) (not shown). Both CMR301 *kdtA::kan*(pWLpxL) and CMR302 *kdtA::kan*(pWLpxM) accumulated several free lipid A species, two of which migrated faster than lipid IV_A (Figure 7B,C, insets). ESI/MS in the negative ion mode showed a major new peak in the lipids of CM301 at *m*/*z* 792.522. This is interpreted as the $[M - 2H]^{2-}$ ion of a pentaacylated, free lipid A species, bearing a secondary laurate chain at the 2′-position (calculated *m*/*z* of 792.503), consistent with the selectivity of LpxL (Figure 8) (*38*, *39*). The additional peak at *m*/*z* 897.638 (Figure 7B) is interpreted as the $[M - 2H]^{2-}$ ion of a hexaacylated, free lipid A species, bearing one secondary laurate and one secondary myristate chain (calculated *m*/*z* of 897.602), as in the lipid A moiety of wild-type *E. coli* LPS (*3*). The small peak at

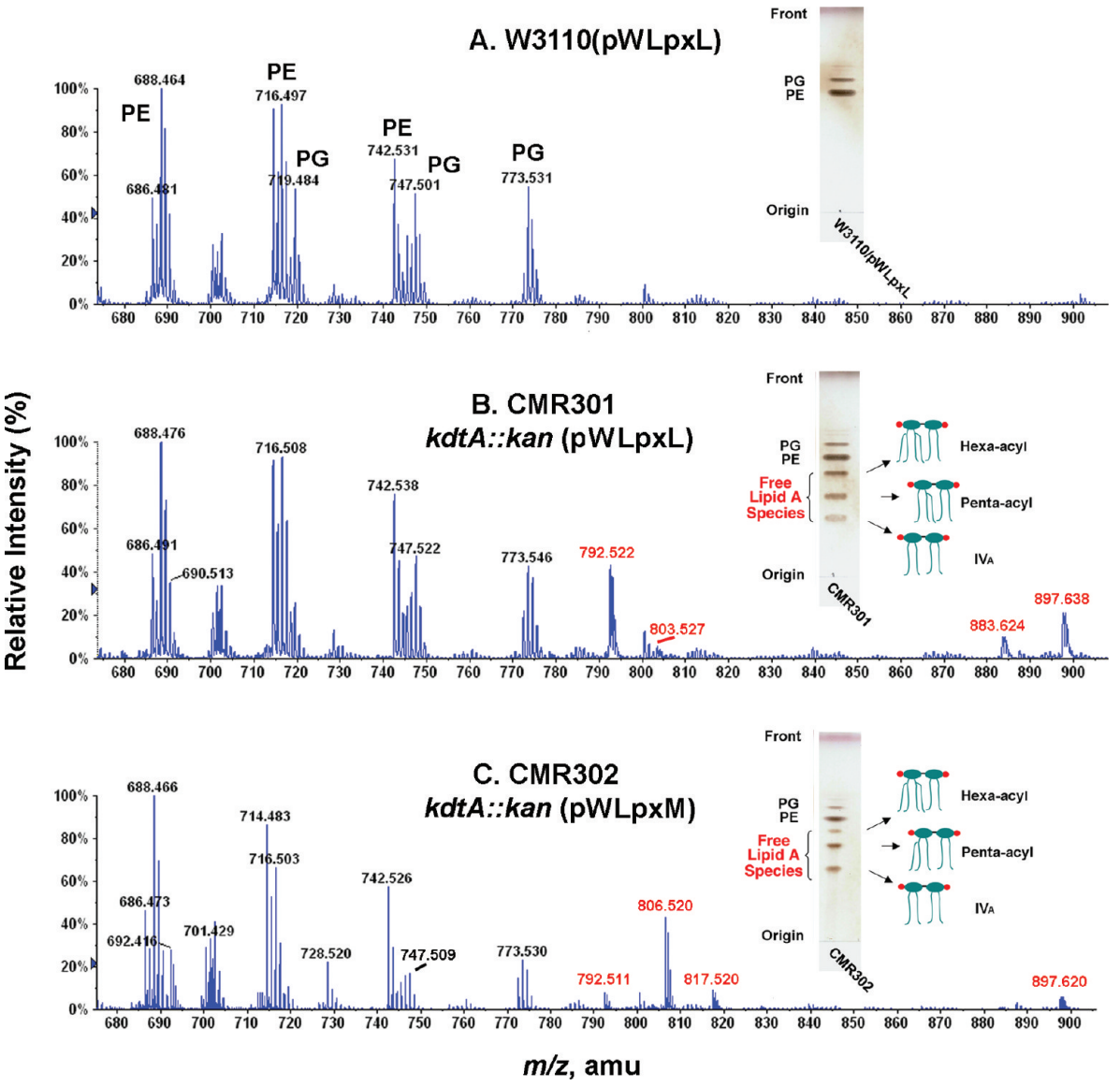

FIGURE 7: Penta- and hexaacylated free lipid A molecules in a *kdtA* deletion mutants overexpressing LpxL or LpxM. Exponentially growing cells in 50 mL of LB broth with 100 μg/mL ampicillin and 1 mM IPTG at 30 °C were harvested in late log phase. The lipids were extracted and analyzed by direct infusion ESI/MS in the negative ion mode, using an ABI QSTAR XL quadruplole time-of-flight mass spectrometer, as in Figure 4. Panel A: Major glycerophospholipid ions of the control strain W3110(pWLpxL) between *m*/*z* 680 and 900 consist mainly of molecular species of PE and PG, as indicated. Panel B: The *kdtA* deletion mutant CMR301, which overexpresses LpxL, contains similar glycerophospholipids but accumulates additional peaks (red), which are interpreted as the $[M - 2H]^{2-}$ and $[M - 3H + Na]^{2-}$ ions of pentaacylated and hexaacylated free lipid A (see also Figure 8), in addition to some lipid IV_A (not labeled). The pentaacylated lipid A contains laurate as its secondary acyl chain but no myristate. A significant amount of hexaacylated lipid A with a wild-type $[M - 2H]^{2-}$ is also generated, suggesting that LpxM may not be as Kdo-dependent as LpxL. Panel C: The *kdtA* deletion mutant CMR302, which overexpresses LpxM, accumulates a different pentaacylated lipid A than does CMR301 (see Figure 8). In this case the predominant secondary acyl chain is myristate, although some laurate is also present because of the relative lack of selectivity of LpxM (*40*). The accumulation of these free lipid A species was confirmed by TLC (insets).

m/*z* 803.527 represents the monosodium adduct $[M - 3H + Na]^{2-}$ of the species at *m*/*z* 792.522 (Figure 7B), whereas the peak at *m*/*z* 883.624 (Figure 7B) likely arises from a hexaacylated lipid A in which both secondary acyl chains are laurate because of the lack of absolute selectivity of LpxM (*4*, *40*). The dependency of LpxM on the Kdo domain has not been studied, but the results of Figure 7B suggest that it functions fairly well in its absence. The proposed pathway for the formation of these species in CMR301 is shown in the upper part of Figure 8.

In the ESI/MS analysis of the CMR302 lipids, a prominent new peak is seen at *m*/*z* 806.520 (Figure 7C), which is not detectable in CMR301 (Figure 7B). This species is consistent with the $[M - 2H]^{2-}$ ion of a pentaacylated lipid A, bearing a secondary myristate chain (calculated *m*/*z* of 806.519). The myristate chain is presumably attached at the 3′-position, assuming the usual regioselectivity of LpxM (lower half of Figure 8). The small peak at *m*/*z* 817.520 is interpreted the $[M - 3H + Na]^{2-}$ adduct ion of the same pentaacylated species. The small peak at *m*/*z* 792.511 likely reflects the relaxed selectivity of LpxM for myristate versus laurate (Figure 7C) (*40*), which also accounts for the minor hexaacylated species at *m*/*z* 883.624 in CMR301 (Figure 7B). The small peak at *m*/*z* 897.620 in CMR302 is interpreted as the $[M - 2H]^{2-}$ ion of a hexaacylated lipid A, bearing one secondary laurate and one secondary myristate chain, perhaps arising as shown in the lower half of Figure 8 with LpxL functioning after LpxM.

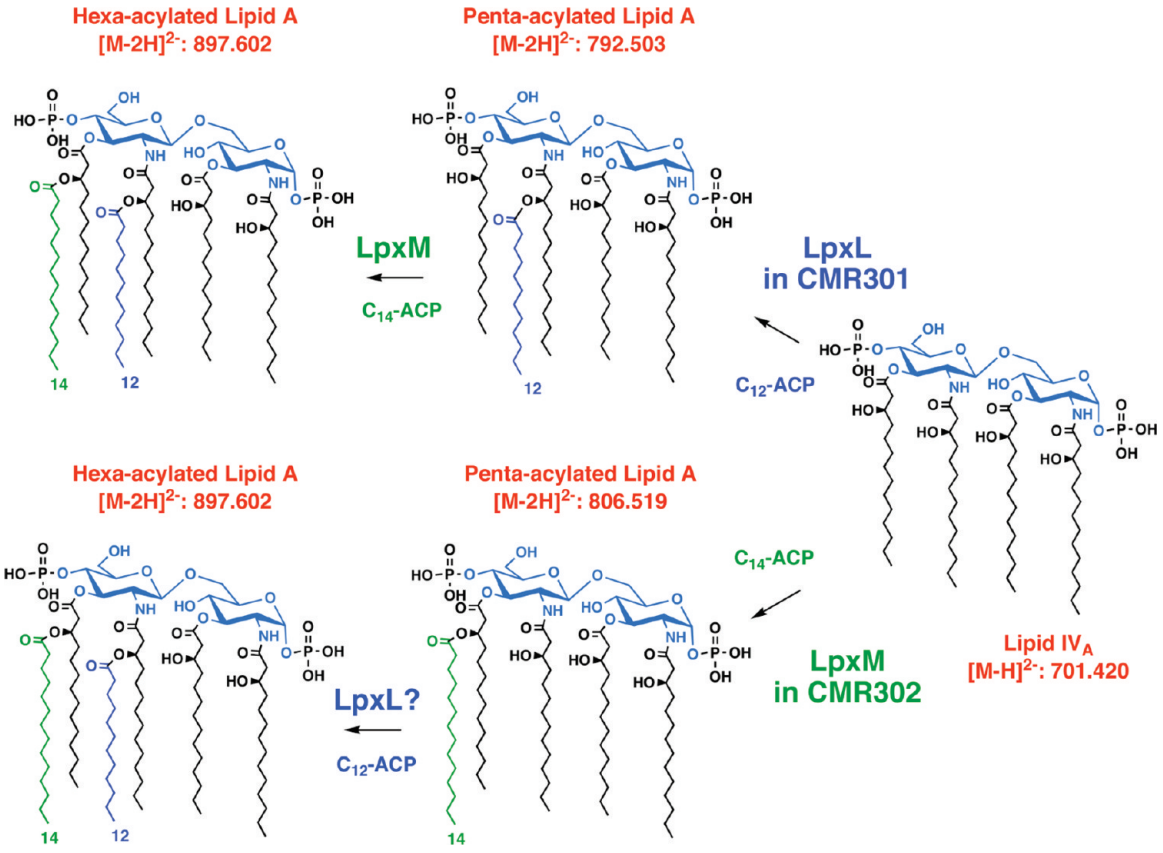

FIGURE 8: Origin of free lipid A molecules in *kdtA* deletion mutants overexpressing LpxL or LpxM. High levels of LpxL or LpxM bypass the need for the Kdo disaccharide in the acceptor substrate. Consequently, CMR301 and CMR302 are able to synthesize significant quantities of penta- and hexaacylated lipid A, which is exported at a sufficient rate by wild-type levels of MsbA to support cell growth. The locations of the secondary acyl chains are proposed based on their normal locations in wild-type lipid A (*3*) and have not been validated by independent methods.

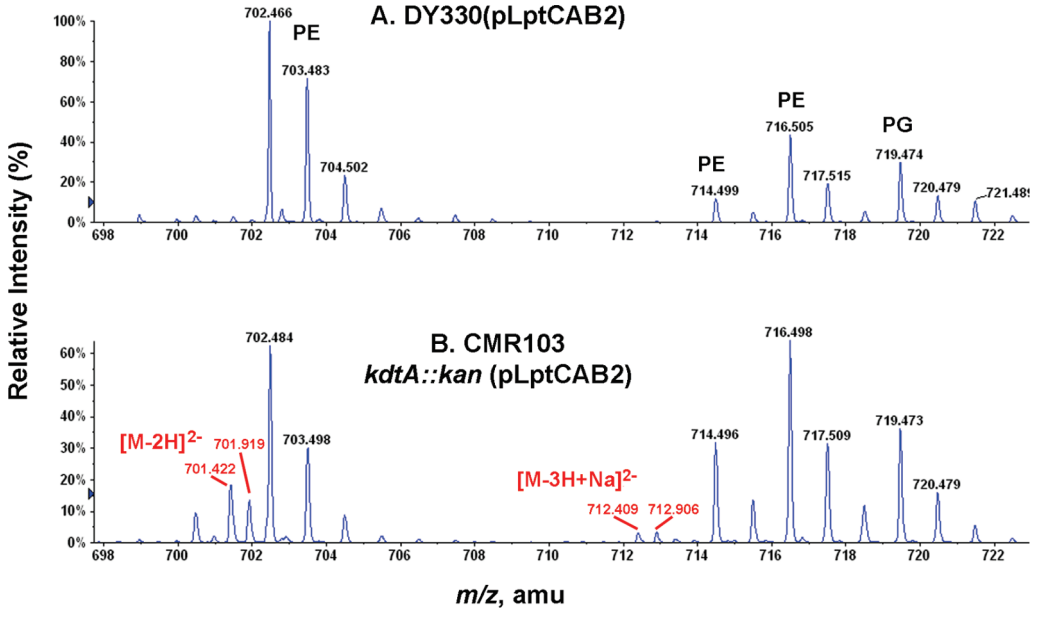

FIGURE 9: Accumulation of lipid IV_A in CMR103 (*kdtA::kan*) overexpressing LptC, LptA, and LptB. Cells were grown at 30 °C on LB broth, and lipids were extracted as in Figure 4. Panel A: ESI/MS in the negative ion mode of the major lipid species in the control strain DY330(pLptCAB2). Panel B: Accumulation of lipid IV_A in CMR103, as indicated by the red numbers for its $[M - 2H]^{2-}$ and $[M - 3H + Na]^{2-}$ ions.

Taken together, our results demonstrate that chromosomal levels of wild-type MsbA are sufficient to support *E. coli* growth in the absence of Kdo transfer to lipid IV_A, provided that an alternative mechanism is available for the generation of sufficient amounts of penta- and hexaacylated lipid A (Figure 1 versus Figure 8). The primary function of Kdo in *E. coli* therefore appears to be the enhancement of LpxL and possibly LpxM activity with the generation of the optimal penta- and hexaacylated lipid A substrates for the flippase, MsbA (Figure 2).

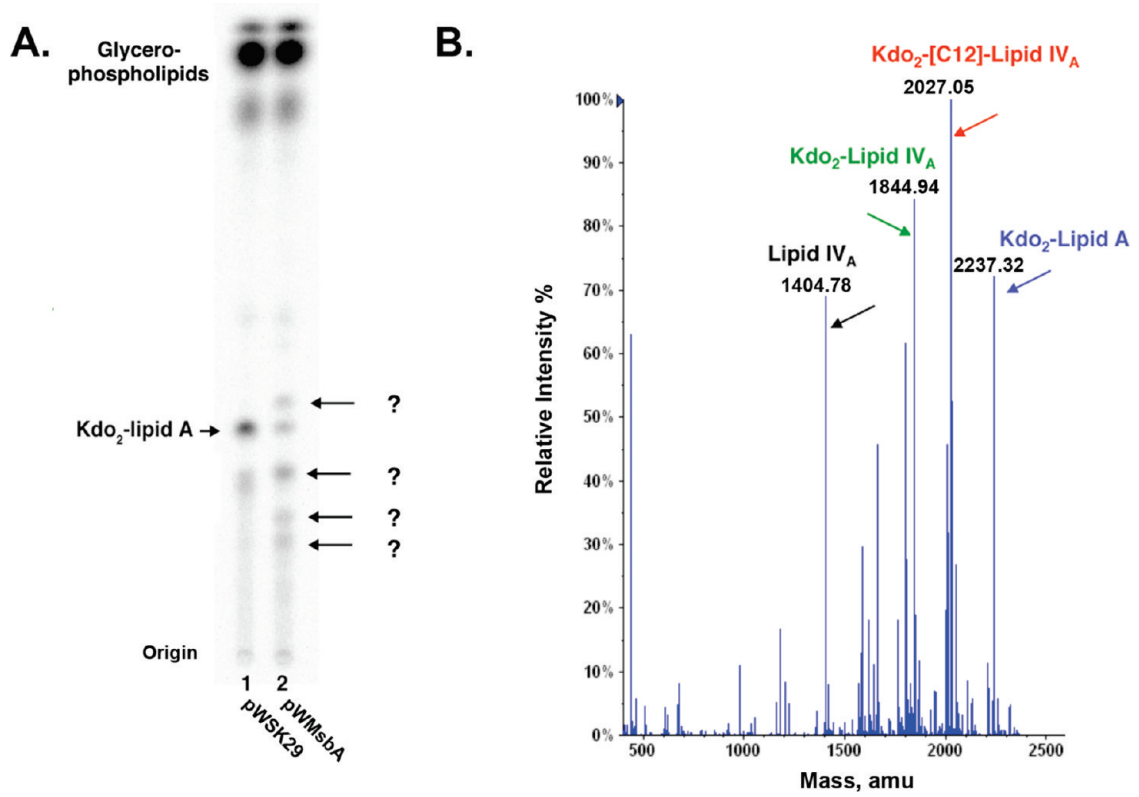

FIGURE 10: Accumulation of Kdo_2–lipid A precursors in a heptose-deficient mutant overexpressing MsbA. Strain WBB06 harboring pWMsbA was grown in the presence of $^{32}P_i$ to late log phase on LB medium at 30 °C. Labeled lipids were extracted and analyzed by TLC (*17*) as in Figure 5. Panel A: Accumulation of additional lipids in WBB06/pWMsbA versus its vector control. Panel B: The lipids from WBB06/pWMsbA were fractionated by DEAE-cellulose chromatography (*17*, *48*) and analyzed by ESI/MS in the negative ion mode. The deconvoluted spectrum of the lipids contained in the 480 mM ammonium acetate fraction (*17*, *48*) revealed the measured exact masses of the peaks arising from Kdo_2–lipid A and its precursors bearing four or five acyl chains.

DISCUSSION

Mutants lacking the lauroyltransferase of lipid A biosynthesis, encoded by *lpxL* (Figure 1), do not grow on nutrient broth at 42 °C (*41*, *53*) and concurrently accumulate LPS bearing a tetraacylated lipid A moiety (*39*). Overexpression of the essential ABC transporter MsbA (*51*) suppresses this phenotype, permitting growth with tetraacylated LPS (*17*). These findings led to the proposal that MsbA is the inner membrane flippase for nascent LPS (Figure 2) and that MsbA might preferentially transport hexaacylated lipid A species (*17*, *20*). Subsequent genetic, biochemical, and structural studies have supported this hypothesis (*18*, *19*, *39*, *54*). However, in vitro assays showing that purified MsbA can flip hexaacylated LPS across a lipid bilayer have not yet appeared.

Mutants defective in Kdo biosynthesis because of a mutation in D-arabinose-5-phosphate isomerase (*kdsD*/*gutQ*) require the addition of D-arabinose 5-phosphate to the growth medium to support growth and LPS assembly (*16*). Meredith et al. also discovered that certain point mutations in MsbA or in the inner membrane protein YhdJ bypass this arabinose 5-phosphate auxotrophy, resulting in strains that can grow with lipid IV_A as their sole LPS substructure (*15*, *16*). The physiological function of YhdJ is unknown, but it is not normally required for growth (*15*). Presumably, certain point mutations in MsbA and YhdJ (*15*) somehow enhance the export of lipid IV_A, which otherwise tends to accumulate in the inner membrane of mutants defective in Kdo biosynthesis or Kdo transfer to lipid IV_A. Very recently, Klein et al. have shown that Kdo-transferase can be deleted in a wild-type background if the cells are grown very slowly on a minimal medium at 21 °C or below with lipid IV_A as the only remaining LPS substructure (*21*).

We have now discovered several additional multicopy suppressors that restore the growth of *E. coli* on nutrient broth when the *kdtA* gene is deleted. As suggested by the work of Meredith et al. (*16*), MsbA overexpression from a multicopy plasmid permits the deletion of the *kdtA* gene in *E. coli* cells at 30 or 37 °C with the accumulation of lipid IV_A (Figures 3 and 4). Furthermore, this lipid IV_A reaches the outer surface of the outer membrane (Figure 2), as judged by its accessibility to cleavage by the outer membrane lipase PagL (Figure 5), the crystal structure of which is known (*31*). When *kdtA* is deleted in the polymyxin-resistant strain MST100 (*55*), the lipid IV_A is modified with a pEtN unit (Figure 6), demonstrating that it may transiently reside on the outer surface of the inner membrane (*18*). In the absence of the Kdo disaccharide, the pEtN unit is attached to the 4′-phosphate group of lipid IV_A (Figure 6) (*12*). The additional modification with palmitate by PagP demonstrates the further movement of the pEtN-modified product to the outer leaflet of the outer membrane (*35*). The absence of L-Ara4N-modified lipid IV_A in this setting is explained by the specificity of the *E. coli* aminoarabinose transferase ArnT for hexaacylated lipid A substrates (*50*) (not shown).

Given that MsbA may be optimized to transport hexaacylated lipid A, we reasoned that overexpression of LpxL or LpxM (Figure 1) might also permit the deletion of *kdtA* under these conditions. Wild-type LpxL greatly prefers Kdo_2–lipid IV_A as its acceptor substrate, but it does have measurable activity with lipid IV_A in vitro (*38*). Accordingly, the overexpression of LpxL from

a multicopy plasmid might enable the formation of penta- and/or hexaacylated lipid A in the absence of KdtA (Figure 8). The preference of LpxM for the Kdo disaccharide has not been analyzed in vitro. Although LpxM prefers pentaacylated acceptor substrates containing a secondary laurate chain (*40*), it does function slowly when overexpressed in cells lacking LpxL (*39*).

Strains CMR301 and CMR302, which overexpress LpxL or LpxM, respectively, can grow when their *kdtA* gene is deleted (Figure 3), albeit more slowly than CMR300, the matched MsbA overexpressing construct (Table 1). As anticipated from the in vitro selectivity of LpxL and LpxM, CMR301 and CMR302 accumulate penta- and hexaacylated free lipid A and relatively little lipid IV_A (Figure 7). The pentaacylated lipid A in CMR301 contains a secondary laurate chain, as judged by the ESI/MS analysis (Figure 7B,C), whereas the pentaacylated lipid A of CMR302 mainly contains a secondary myristate unit. CMR301 also accumulates hexaacylated material (Figure 7B) with the molecular weight expected for wild-type lipid A (*3*, *4*). Taken together, these phenotypes demonstrate that MsbA prefers penta- or hexaacylated substrates to tetraacylated molecules in the absence of core sugars. A primary function of the Kdo disaccharide in *E. coli* therefore appears to be the production of the optimal substrate for LpxL. Chromosomal levels of LpxM show significant activity with pentaacylated free lipid A generated by LpxL in the absence of the Kdo disaccharide (Figures 7B and 8).

The idea that MsbA preferentially transports hexaacylated lipid A gains further support from the effects of overexpressing *msbA* in the heptose-deficient mutant WBB06. This well-characterized strain synthesizes mostly hexaacylated Kdo_2−lipid A when grown on LB broth (*4*). However, when pWMsbA is introduced into WBB06, the pattern of lipid A species becomes more complex, and the levels of Kdo_2−lipid A are reduced (Figure 10A). Analysis by ESI/MS demonstrates the accumulation of the precursors lipid IV_A, Kdo_2−lipid IV_A, and Kdo_2-(lauroyl)−lipid IV_A, as in the deconvoluted negative ion mode spectrum (Figure 10B). When MsbA flippase function is enhanced by overexpression in WBB06, we suggest that the rate of lipid IV_A export may become comparable to the rates of Kdo and secondary acyl chain addition to lipid IV_A, consistent with the ability of *msbA* overexpression to suppress the lethality of *kdtA* deletions. In the wild-type W3110, which generates a complete core, overproduction of MsbA does not have a dramatic effect on the acyl chain composition of the lipid A moiety (not shown). The reasons for the different responses of WBB06 and W3110 to MsbA overexpression are unclear and require further investigation.

Overexpression of MsbA does not enable the deletion of *lpxK* (Figure 2) (data not shown), suggesting that MsbA has a strong preference for substrates with two monophosphate groups. Expression of the *Francisella* lipid A 4′-phosphatase (*56*), the active site of which resides on the outer surface of the inner membrane, is toxic in CMR300, suggesting that the 4′-phosphate group may have additional functions in *E. coli* following the export of lipid IV_A by MsbA.

The first six enzymes of the lipid A pathway are excellent targets for new antibiotic development, as illustrated by CHIR-090 (*47*, *57*), a potent, selective inhibitor of LpxC (Figure 2). Kdo-deficient strains like CMR300 remain sensitive to killing by CHIR-090 under all conditions examined to date, demonstrating that CMR300 cannot grow without lipid IV_A. Nevertheless, it may yet be possible to delete the *E. coli* lipid A pathway entirely, as in the case of *Neisseria meningitidis* (*58*, *59*), by introducing additional, as yet unidentified suppressor mutations. However, most other Gram-negative bacteria are like *E. coli* in that lipid A is essential for growth.

The recent discovery of the Lpt transport proteins (Figure 2) has provided the first molecular insights into the trafficking of nascent LPS from the outer surface of the inner membrane to the outer membrane (*22*−*27*). Interestingly, concurrent overexpression of LptA, LptB, and LptC (Figure 2), which are encoded in an operon (*22*), partially suppresses the lethality of *kdtA* deletion at 30 °C with accumulation of lipid IV_A (Figure 9). These constructs grew very slowly compared to the others described above (not shown). The partial suppression of *kdtA* deletions by LptABC overexpression suggests that rapid removal of lipid IV_A from the outer surface of the inner membrane might increase the efficiency of MsbA (Figure 2), perhaps by mass action. Overexpression of the entire Lpt complex (Figure 2) might improve MsbA efficiency even further.

The ability of *E. coli* cells overexpressing LpxL or LpxM to grow with free lipid A replacing their LPS resembles the normal situation in *Francisella novicida*, in which most of the lipid A is not covalently attached to LPS (*60*, *61*). In fact, *kdtA* deletion in *Francisella* does not inhibit cell growth (J. Zhao and C. R. H. Raetz, in preparation). One of the two LpxL orthologues present in *F. novicida* may be Kdo-independent (D. A. Six, W. Chen, and C. R. H. Raetz, in preparation).

The growth phenotypes associated with *kdtA* deletions in CMR300, CMR301, and CMR302 (Figure 3) should facilitate the selection of second-site suppressor mutations that can grow without LPS at 42 °C or in the presence of bile salts. This genetic approach might reveal the presence of additional protein components required for lipid A trafficking or for the proper assembly of a functional outer membrane. If the strategy of replacing LPS with free lipid A is applicable to pathogens, like *Salmonella* or *Shigella*, it may be possible to attenuate these organisms and to use them for the development of novel vaccines.

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SUPPORTING INFORMATION AVAILABLE

The negative ion ESI/MS spectrum of the lipids of strain CMR100 (Table 1), demonstrating the accumulation of lipid IV_A when compared to a wild-type control. This material is available free of charge via the Internet at http://pubs.acs.org.

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