

Escherichia coli K-12 Suppressor-free Mutants Lacking Early Glycosyltransferases and Late Acyltransferases

MINIMAL LIPOPOLYSACCHARIDE STRUCTURE AND INDUCTION OF ENVELOPE STRESS RESPONSE^{*[5]}

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To elucidate the minimal lipopolysaccharide (LPS) structure needed for the viability of *Escherichia coli*, suppressor-free strains lacking either the 3-deoxy-D-manno-oct-2-ulosonic acid transferase *waaA* gene or derivatives of the heptosyltransferase I *waaC* deletion with lack of one or all late acyltransferases (*lpxL/M/P*) and/or various outer membrane biogenesis factors were constructed. $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ and *waaA* mutants exhibited highly attenuated growth, whereas simultaneous deletion of *waaC* and *surA* was lethal. Analyses of LPS of suppressor-free *waaA* mutants grown at 21 °C, besides showing accumulation of free lipid IV_A precursor, also revealed the presence of its pentaacylated and hexaacylated derivatives, indicating *in vivo* late acylation can occur without Kdo. In contrast, LPS of $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ strains showed primarily Kdo₂-lipid IV_A, indicating that these minimal LPS structures are sufficient to support growth of *E. coli* under slow-growth conditions at 21/23 °C. These lipid IV_A derivatives could be modified biosynthetically by phosphoethanolamine, but not by 4-amino-4-deoxy-L-arabinose, indicating export defects of such minimal LPS. $\Delta waaA$ and $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ exhibited cell-division defects with a decrease in the levels of FtsZ and OMP-folding factor PpiD. These mutations led to strong constitutive additive induction of envelope responsive CpxR/A and σ^E signal transduction pathways. $\Delta(lpxL\ lpxM\ lpxP)$ mutant, with intact *waaC*, synthesized tetraacylated lipid A and constitutively incorporated a third Kdo in growth medium inducing synthesis of *P-EtN* and L-Ara4N. Overexpression of *msbA* restored growth of $\Delta(lpxL\ lpxM\ lpxP)$ under fast-growing conditions, but only partially that of the $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ mutant. This suppression could be alleviated by overexpression of certain mutant *msbA* alleles or the single-copy chromosomal MsbA-498V variant in the vicinity of Walker-box II.

Lipopolysaccharides (LPS)⁴ are the major amphiphilic constituents of the outer leaflet of the outer membrane (OM) of

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1.

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⁴ The abbreviations used are: LPS, lipopolysaccharide; Kdo, 3-deoxy- α -D-manno-oct-2-ulosonic acid; OMP, outer membrane proteins; *P-EtN*, phos-

phoethanolamine; L-Ara4N, 4-amino-4-deoxy-L-arabinose; ESI FT-ICR MS, electrospray ionization Fourier transform-ion cyclotron mass spectrometry; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Gram-negative bacteria, including *Escherichia coli*. LPS share a common architecture composed of a membrane-anchored phosphorylated and acylated $\beta(1\rightarrow6)$ -linked GlcN disaccharide, termed lipid A, to which a carbohydrate moiety of varying size is attached (1, 2). The latter may be divided into a lipid A proximal core oligosaccharide and, in smooth-type bacteria, a distal O-antigen. LPS always contain 3-deoxy- α -D-manno-oct-2-ulosonic acid (Kdo) linked to the lipid A. The physiological importance of the Kdo/lipid A region is reflected by its specific position within the pathway of LPS biosynthesis. In *E. coli* K-12, a bisphosphorylated lipid A precursor molecule with two amide and two ester-bound (*R*)-3-hydroxymyristate residues (lipid IV_A) is synthesized from UDP-GlcNAc, following 6 distinct enzyme reactions (1). This intermediate serves as an acceptor for the Kdo transferase (WaaA), which transfers two Kdo residues from CMP-Kdo to yield an $\alpha(2\rightarrow4)$ -linked Kdo disaccharide-attached $\alpha(2\rightarrow6)$ to the non-reducing GlcN residue of lipid IV_A (3). The latter reaction product, termed Kdo₂-lipid IV_A, comprises a key intermediate of LPS biosynthesis that acts 2-fold as a specific substrate: (i) for glycosyltransferases catalyzing further steps of the core oligosaccharide biosynthesis (4) and (ii) for acyltransferases that complete the lipid A moiety by the transfer of 2 additional fatty acids to the (*R*)-3-hydroxyl groups of both acyl chains, which are directly bound to position 2' and 3' of the non-reducing GlcN residue (1). Three acyltransferases, encoded by paralogous genes, have been described in *E. coli* K-12, which catalyze the latter enzyme reactions using acyl carrier protein-activated fatty acids as co-substrates (5–10). At ambient temperatures, a lauroyl residue is first transferred by LpxL (6) to the OH group of the amide-bound (*R*)-3-hydroxymyristate residue at position 2'. This catalytic step is partially replaced at low temperature (12 °C) by LpxP, which transfers palmitoleate to the same position in ~80% of the LPS molecules (7). The free OH group of the ester-bound (*R*)-3-hydroxymyristate residue at position 3' within both pentaacylated intermediates is then myristoylated by LpxM to give a hexaacylated lipid A moiety (Fig. 3) (5).

Consistent with the essentiality of LPS in *E. coli*, all the genes, whose products are required for committed steps of biosynthesis of lipid IV_A and subsequent transfer of Kdo to it, are essential (1, 2). However, individually neither the subsequent steps of

phoethanolamine; L-Ara4N, 4-amino-4-deoxy-L-arabinose; ESI FT-ICR MS, electrospray ionization Fourier transform-ion cyclotron mass spectrometry; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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addition of the secondary lauroyl and myristoyl residues to the distal glucosamine unit by LpxL and LpxM to synthesize hexaacylated lipid A nor the later glycosylation of hexaacylated Kdo₂-lipid A is essential for viability of bacteria like *E. coli* K-12 under defined growth conditions (8). Although Re mutants that possess LPS with only hexaacylated Kdo₂-lipid A or mutants that synthesize complete LPS core with only lipid IV_A are viable, they are impaired in several growth properties, including constitutive induction of RpoE signal transduction in Re mutants (8, 11–13). A triple null mutant, which lacks all 3 late acyltransferases, is viable but only in slow-growth conditions in accordance with lipid IV_A being a poor substrate of the lipid A transporter MsbA (8). Mutants impaired in the synthesis of Kdo, which synthesize only lipid IV_A lacking any glycosylation, can be constructed, but they require additional suppressor mutations either in *msbA*, or the *yhjD* gene (14, 15). Strains that potentially can only synthesize Kdo₂-lipid IV_A have not been reported up to now. Thus, suppressor-free minimal LPS structures that can support growth of *E. coli* K-12 bacteria known up to now have genetic compositions of $\Delta(lpxL\ lpxM\ lpxP)$ or Re mutants.

We describe the construction and characterization of suppressor-free $\Delta waaA$ and $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ mutants, synthesizing either free lipid IV_A derivatives or Kdo₂-lipid IV_A LPS, respectively. Analyses of lipid A of $\Delta waaA$ also revealed the presence of free penta- and hexaacylated lipid A derivatives, arising due to incorporation of secondary acyl chains. Such suppressor-free strains could be constructed only in slow-growth conditions at lower temperatures. Growth of $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ could be restored by extragenic chromosomal *MsbA*-D498V suppressor mutation or by the overexpression of the *msbA* wild-type gene product. The LPS of $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ and lipid IV_A precursor of $\Delta waaA$ was found to be substituted by *P-EtN*, but not *L-Ara4N*, under LPS-modifying growth conditions. Deletion of late acyltransferases in $\Delta waaC$ or deletion of the *waaA* gene resulted in constitutively elevated levels of periplasmic protease HtrA, due to additive induction of the envelope stress responsive CpxR/A two-component system and σ^E pathway.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—Bacterial strains and plasmids used in this study are described in Table 1. Luria-Bertani (LB) broth, M9, and 121 phosphate-limiting minimal media were prepared as described (16, 17). When necessary, media were supplemented with ampicillin (100 $\mu\text{g ml}^{-1}$), tetracycline (10 $\mu\text{g ml}^{-1}$), kanamycin (50 $\mu\text{g ml}^{-1}$), spectinomycin (50 $\mu\text{g ml}^{-1}$), or chloramphenicol (20 $\mu\text{g ml}^{-1}$).

Generation of Null Mutations and Construction of Their Combinations—Non-polar antibiotic-free deletion mutations of various genes were generated using the λ Red recombinase/FLP-mediated recombination system (18). The coding sequence of each gene was replaced with either the kanamycin (*aph*) or chloramphenicol (*cat*) resistance cassette flanked by FRT recognition sequences using plasmids pKD13 and pKD3 as templates (18), and recombined on the chromosome of BW25113 containing the λ Red recombinase-encoding plasmid pKD46. Gene replacements and their exact chromosomal loca-

tions were verified by PCR. All the initial single gene disruptions were made on minimal M9 medium at 30 °C, except for the *waaA* deletion, which was constructed at 21 °C. Deletion mutations were then transduced into W3110 selecting for antibiotic markers. Multiple null combinations were made through a series of transductions using bacteriophage T4-mediated transductions, followed by the removal of the *aph* or *cat* cassettes. The initial *lpxM lpxP* double null combinations were built using M9 minimal medium and limited growth at 37 °C to facilitate excision of the *aph* cassette and removal of Ts FLP recombinase plasmid pCP20 without causing accumulation of suppressors. The rationale for the first construction of the $\Delta(lpxP\ lpxM)$ strain was that such a genetic knock-out combination does not confer any known growth defects (8). SR7465 $\Delta(lpxM\ lpxP)$ was then used as a recipient to further transduce *waaC::cm*, *waaC::kan*, or *waaCF tet::6*, or *lpxL (aph or cat)* deletion mutations. As a control another set of strains was constructed by replacing *lpxL*- and *lpxP*-coding sequences with *cat* and spectinomycin (*ada*) cassettes and then transferred to the chromosome, following λ Red recombineering. Recombinants were selected on minimal M9 plates with appropriate antibiotics and used as donors to transduce deletion alleles in *lpxM* mutants. This resulted in the construction of *lpxM lpxL::cm lpxP::spec* (SR8277) and similar derivatives. All the transductions were performed in parallel at 30 or 23 °C on minimal M9 medium. Finally, strains lacking all of the late acyltransferase genes with the addition of deletion of *waaC*, SR7870 (*lpxL lpxP lpxM waaCF::tet*) and SR8352 (*lpxL lpxP lpxM waaC::cm*) constructed by transductions, were obtained only at 23 °C on the M9 minimal medium. Control transduction consisted of using *lpxL lpxM lpxP* null but carrying either *waaC* or *msbA* genes on the plasmid as a recipient SR8432 for bringing in the $\Delta waaC::cm$ allele at 23 and 30 °C (Tables 1 and 3). Identical sets of single and multiple deletion strains were constructed in isogenic W3110 (GK1111) and BW25113 strains carrying single-copy promoter fusions to the *rpoHP3* and *cpxP* promoter (Table 1). Disruptions and null allelic combinations involving *eptA*, *basR*, and *eptB* were constructed in the same manner as described above (Table 1). Finally, suppressor-free deletions of the *waaA* gene on chromosomes of W3110 and BW25113 were constructed by substitution of the entire coding sequence with either *aph* or *cat* cassettes. Such recombinants were constructed on the M9 minimal medium at 21 °C and subsequently transduced under the same growth conditions, resulting in SR8614, SR8621, and SR8647 (Table 1). Coding sequences of *lpxL*, *waaC*, and *msbA* genes were PCR amplified and cloned in tightly controlled expression vectors pSE420 (Invitrogen) and pCA24N (19).

LPS Extraction and Growth Analysis—Bacterial cultures were grown with shaking in M9 liquid medium with appropriate antibiotics at 23 °C to early log phase. To compare growth rates, cultures were washed in 10 mM MgSO₄ and resuspended at an optical density A_{600} of 0.01 in LB and M9 minimal medium. Cultures were further incubated at 23, 30, 37, 39, and 43 °C with shaking. The optical density A_{600} was recorded at various times. For LPS extraction, bacterial cultures were grown under permissive growth conditions of 23 or 30 °C either in M9 minimal medium (non-modifying) or in 121 medium

TABLE 1
Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristic	Reference or source
Strains		
W3110	λ^- , <i>IN(rrnD-rrnE)1</i> , <i>rph-1</i>	<i>E. coli</i> Genetic Stock Center, Yale
BW25113	<i>lacI^r rrnB_{T14} ΔlacZ_{-wJ16} hsdR514 ΔaraBAD_{AH33}</i>	18
GK1111	Δ <i>rhaBAD</i> _{LD78}	This study
WBB01	W3110 Δ <i>lac</i>	45
SR7330	JC7623 <i>waaCF</i> tet	This study
SR7465	W3110 <i>lpxP</i> <> <i>aph</i>	This study
SR7751	W3110 <i>lpxP</i> <> <i>frt lpxM</i> <> <i>frt</i>	This study
SR7770	W3110 Δ <i>waaCF</i> tet	This study
SR7774	W3110 <i>lpxM</i> <> <i>cat</i>	This study
SR7781	W3110 <i>lpxM</i> <> <i>frt lpxL</i> <> <i>aph</i>	This study
SR7787	W3110 <i>lpxM</i> <> <i>frt lpxP</i> <> <i>frt lpxL</i> <> <i>aph</i>	This study
SR7790	SR7751 <i>lpxL</i> <> <i>aph</i>	This study
SR7825	SR7751 <i>lpxM</i> <> <i>aph</i>	This study
SR7870	W3110 <i>waaCF</i> tet <i>lpxM</i> <> <i>frt lpxL</i> <> <i>aph</i>	This study
SR7870	W3110 <i>waaCF</i> tet <i>lpxM</i> <> <i>frt lpxP</i> <> <i>frt lpxL</i> <> <i>aph</i>	This study
SR8035	SR7781 Δ <i>waaCF</i> tet	This study
SR8101	W3110 <i>waaC</i> <> <i>aph</i>	This study
SR8163	W3110 <i>lpxL</i> <> <i>cat</i>	This study
SR8233	W3110 <i>lpxP</i> <> <i>cat</i>	This study
SR8258	W3110 <i>waaC</i> <> <i>cat</i>	This study
SR8277	W3110 <i>lpxP</i> <> <i>ada</i>	This study
SR8352	SR7774 <i>lpxP</i> <> <i>ada</i>	This study
SR8129	W3110 <i>lpxM</i> <> <i>frt lpxP</i> <> <i>frt waaC</i> <> <i>aph lpxL</i> <> <i>cat</i>	This study
SR8190	GK1111 ϕ (<i>rpoHP3-lacZ</i>)	This study
SR8165	SR8129 <i>waaC</i> <> <i>aph</i>	This study
SR8159	SR8129 <i>lpxL</i> <> <i>aph</i>	This study
SR8168	SR8129 <i>lpxL</i> <> <i>cat</i>	This study
SR8160	SR8129 <i>lpxM</i> <> <i>aph</i>	This study
SR8162	SR8129 <i>lpxM</i> <> <i>cat</i>	This study
SR8225	SR8129 <i>lpxP</i> <> <i>cat</i>	This study
SR8221	SR8129 <i>waaC</i> <> <i>aph lpxL</i> <> <i>cat</i>	This study
SR8481	SR8129 <i>waaC</i> <> <i>aph lpxM</i> <> <i>cat</i>	This study
SR8510	SR8129 <i>lpxM</i> <> <i>cat lpxL</i> <> <i>aph lpxP</i> <> <i>ada</i>	This study
SR7718	BW25113 ϕ (<i>pcpxP-lacZ</i>)	This study
SR8201	SR7718 <i>waaCF</i> tet	This study
SR8177	SR7718 <i>lpxL</i> <> <i>cat</i>	This study
SR8180	SR7718 <i>lpxM</i> <> <i>cat</i>	This study
SR8212	SR7718 <i>waaCF</i> tet <i>lpxL</i> <> <i>cat</i>	This study
SR8209	SR7718 <i>waaCF</i> tet <i>lpxM</i> <> <i>cat</i>	This study
SR8352	SR8277 <i>waaC</i> <> <i>cat</i>	This study
SR8356	SR7781 <i>waaC</i> (<i>pmsbA</i> ⁺)	This study
SR8433	SR8356 <i>pmsbA</i> (L279V)	This study
SR8437	SR8356 <i>pmsbA</i> (A510P)	This study
SR8478	SR7870 <i>msbA</i> (D498V)	This study
SR8445	SR7781 <i>waaC</i> (<i>psurA</i> ⁺)	This study
SR8469	GK1162 <i>waaC</i> <> <i>cat</i>	This study
SR8432	SR7781 (<i>pmsbA</i> ⁺)	This study
SR8614	BW25113 <i>waaA</i> <> <i>aph</i>	This study
SR8621	SR8129 <i>waaA</i> <> <i>aph</i>	This study
SR8647	SR7718 <i>waaA</i> <> <i>cat</i>	This study
GK1077	W3110 <i>lpxM</i> <> <i>aph</i>	This study
GK1162	W3110 <i>eptB</i> <> <i>aph</i>	This study
GK1275	W3110 <i>lpxL</i> <> <i>aph</i>	This study
GK1395	W3110 <i>eptA</i> <> <i>aph</i>	This study
GK1400	W3110 <i>basR</i> <> <i>aph</i>	This study
GK1735	W3110 <i>eptB</i> <> <i>frt basR</i> <> <i>aph waaC</i> <> <i>cat</i>	This study
GK1753	GK1395 <i>waaC</i> <> <i>cat</i>	This study
Plasmids		
pCP20	Contains temperature-sensitive replicon and a thermally inducible FLP recombinase	<i>E. coli</i> Genetic Stock Center, Yale
pKD3	<i>oriR6K₊ bla</i> (Amp ^R), <i>kan</i> , <i>rgnB</i> (Ter), <i>cat</i>	<i>E. coli</i> Genetic Stock Center, Yale (18)
pKD13	<i>oriR6K₊ bla</i> (Amp ^R), <i>kan</i> , <i>rgnB</i> (Ter)	<i>E. coli</i> Genetic Stock Center, Yale (18)
pKD46	<i>araBp-gam-bet-exo</i> , <i>bla</i> (Amp ^R), <i>repA101</i> (ts) <i>oriR101</i>	<i>E. coli</i> Genetic Stock Center, Yale (18)
pRS415	<i>lacZ</i> YA transcriptional fusion vector Amp ^R	
pSE420	Amp ^R expression vector <i>lacIQ</i>	Invitrogen
pCA24N	Cm ^R expression vector <i>lacIQ</i>	19
pCL1921	<i>ori pSC101 spec</i> ^R	46
pGK1663	pRS415::(<i>eptA-lacZ</i>)	This study
pGK1665	pRS415::(<i>arnB-lacZ</i>)	This study
pSR7409	<i>msbA</i> cm ^R in pCA24N	This study
pSR8262	<i>msbA</i> amp ^R in pSE420	This study
pSR2777	<i>surA</i> amp ^R	13

(LPS-modifying medium) until optical density A_{600} of 0.3 to 0.6 was reached. Cultures (400 ml) were harvested by centrifugation at 7000 \times g for 30 min and dried. LPS was extracted by the

phenol/chloroform/petroleum ether procedure (20) and lyophilized. For LPS analysis, lyophilized material was dispersed in water by sonication and resuspended at a concentration of 2 mg

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ml⁻¹. Lipid IV_A and its derivatives were extracted from 200-ml cultures of $\Delta waaA$ mutants grown either in LB or different types of minimal media at either 30 or 21 °C, following the procedure described for isolation of LPS from deep-rough mutants (21). Glycerophospholipids and the lipid A mixture was resuspended in a chloroform/methanol mixture (4:1, v/v) at a concentration of 2 mg ml⁻¹. For detection of chemotype 1 μ g of purified LPS or a portion of whole cell lysate treated with proteinase K was applied to a 16.5% Tricine gel. Gels were silver stained for LPS analysis. TLC immunostaining for verification of the absence of Kdo but presence of tetraacylated lipid A in $\Delta waaA$, monoclonal antibodies A20 or A6 were used as described (22–24), using compounds 406, 506, and Re LPS as controls.

Mass Spectrometry—Electrospray ionization Fourier transform-ion cyclotron (ESI FT-ICR) mass spectrometry was performed in negative ion mode using an APEX II Bruker Daltonics, equipped with a 7-tesla actively shielded magnet and an Apollo ion source. Samples at a concentration of ~ 10 ng μ l⁻¹ were sprayed at a flow rate of 2 μ l min⁻¹ as described (25). Capillary entrance voltage was set to 3.8 kV, and dry gas temperature to 200 °C. For unspecific fragmentation the DC offset (collision voltage) of the quadrupole interface was set from 5 to 30 V. Under these conditions the labile linkage between lipid A and the core oligosaccharide is cleaved. If not otherwise stated the mass spectra were charge deconvoluted and mass numbers given refer to the monoisotopic masses of the neutral molecules. Mass calibration was done externally by well characterized similar compounds of known structure. Mass accuracy was better than 5 ppm.

Western Blot Analysis—Cultures were grown at either 21 or 23 °C for 24–48 h in 30 ml of M9 minimal medium, harvested by centrifugation at 3000 $\times g$ for 10 min, and resuspended in SDS lysis buffer. Proteins were resolved by 12% SDS-PAGE. After electrophoresis, proteins were blotted to nitrocellulose membrane. HtrA, PpiD, RseB, DnaK, and FtsZ proteins were detected with the respective antibody (26, 27).

β -Galactosidase Assays—The activity of CpxR/A and RpoE pathways in *waaA*, *waaC*, *lpxL*, and *lpxM* mutants, and in derivatives with their null allelic combinations were analyzed in strains carrying either *rpoHP3-lacZ* or *cpxP-lacZ* promoter fusions in a single copy on the chromosome. The construction of *rpoHP3-lacZ* or *cpxP-lacZ* and other RpoE-regulated promoter fusions has been previously described (28). Putative BasS/R-regulated promoter regions of the *eptA-basS-basR* operon, and *arn* operon were amplified by PCR, using primers listed in supplemental Table S1. The amplified PCR products were cloned in pRS415 vector and transferred to chromosome as described previously for other promoter fusions (13, 17). Isogenic bacterial strains, carrying promoter fusions, were grown in M9 medium, at 21, 23, or 30 °C, harvested by centrifugation, and diluted to an A₆₀₀ of ≈ 0.03 in M9 or 121 medium. Cultures were allowed to grow for another 90 min and β -galactosidase activity was measured in Miller units at different growth intervals. At least four independent cultures were assayed for each mutant and the corresponding isogenic parent.

RESULTS

Construction of Chromosomal Deletions of *lpxL*, *lpxM*, *lpxP*, and *waaC* Genes and Their Combinations—To construct and characterize *E. coli* strains that synthesize LPS with only Kdo₂-lipid IV_A, deletions in genes encoding the late acyltransferases, namely lauroyl-, myristoyl-, and palmitoleoyl acyltransferases, and heptosyltransferase I were constructed as described under “Experimental Procedures.” In one case ($\Delta(lpxL\ lpxM\ lpxP)$ strains (SR7781 and SR8277), constructed by a combination of individual deletions of the respective genes at 30 °C on M9 medium, were used to receive *waaCF::tet* or *waaC::cm* using T4-mediated transductions, resulting in SR7870 and SR8352, respectively. Alternatively, $\Delta(lpxM\ lpxP\ waaC)$ (SR7807), synthesizing Kdo₂-lipid A_{penta}, was used as recipient to transduce *lpxL::kan*, resulting in SR7877. Transductions were performed at 23 and 30 °C in the presence or absence of plasmids expressing either the *waaC* or *lpxL* genes. In all cases viable transductants, resulting in $\Delta(waaC\ lpxL\ lpxM\ lpxP)$, were obtained at 23 °C on minimal medium but not on the LB medium, when *waaC* or *lpxL* genes were not expressed from the plasmid. The frequency of transductions at 23 °C was similar in the presence or absence of *waaC/lpxL* plasmids (Table 3). No viable transductants could be obtained at 30 °C even in slow-growth conditions of minimal medium in the absence of the *waaC* plasmid. However, in the presence of *waaC* plasmid, viable transductants were obtained at 23 and 30 °C at the same frequency. Taken together, these results suggest that a viable suppressor-free $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ mutant synthesizing Kdo₂-lipid IV_A can be constructed under slow-growth conditions of minimal medium at 23 °C, but not at or above 30 °C even on minimal medium. However, as described in later sections, $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ could be constructed even on rich medium at both 23 and 30 °C, when extra copies of MsbA were provided in *trans*.

Growth Properties of the Mutant Strains at Different Temperatures—Consistent with the reported Ts phenotype of *rfaD* mutants (12), $\Delta waaC$ mutants exhibited a temperature-sensitive growth phenotype at or above 43 °C. Furthermore, $\Delta(waaC\ lpxM)$ strain exhibited an additive temperature-sensitive phenotype above 39 °C, which is permissive for both $\Delta waaC$ and $\Delta lpxM$ bacteria. Significantly, the growth of $\Delta(waaC\ lpxL)$ on rich medium was severely compromised and supported the colony forming ability only at 23 or 30 °C, but not above such temperatures on minimal medium, with synthetic lethality on rich medium. Furthermore, the addition of $\Delta lpxM$ to such as strain $\Delta(waaC\ lpxL)$ caused even higher growth reduction in the temperature range of 23 to 30 °C (Table 2). The colony forming ability of $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ and growth in liquid culture was restricted strictly to slow-growth conditions, within a narrow temperature range with slower growth rates (Table 2). The growth occurred on minimal medium at 23 °C with inability to form colonies at temperatures at or above 30 °C. These results demonstrate that *E. coli* strains, which synthesize the Kdo₂-lipid IV_A predicted LPS structure, can sustain growth in genetically suppressor-free backgrounds within a limited growth range unless Kdo₂-lipid IV_A is more efficiently transported.

TABLE 2

Growth rate h^{-1} (μ) of the wild-type and $\Delta waaC$ derivatives with and without MsbA overexpression

	M9		LB	
	23 °C	30 °C	23 °C	30 °C
Wild type	0.40	0.52	0.61	0.99
$\Delta waaC$	0.31	0.51	0.60	0.98
$\Delta(waaC\ lpxL)$	0.20	0.18	0.50	0.34
$\Delta(waaC\ lpxM)$	0.22	0.30	0.31	0.47
$\Delta(waaC\ lpxL\ lpxM)$	0.15	0.17	0.26	0.24
$\Delta(waaC\ lpxL\ lpxM\ lpxP)$	0.14	NL ^a	NL	NC, ^b NL
$\Delta(lpxL\ lpxM\ lpxP)$	0.29	0.41	0.43	NC, NL

	LB		
	23 °C	30 °C	37 °C
Wild type	0.60	1.01	1.34
$\Delta(lpxL\ lpxM\ lpxP) + pmsbA^+$	ND ^c	0.65	0.67
$\Delta(waaC\ lpxL\ lpxM\ lpxP) + pmsbA^+$	0.54	0.45	ND

^a NL, non-linear growth and hence data not used to calculate growth rate.^b NC, inability to support colony forming ability.^c ND, not determined.

The waaA Gene Is Dispensable at or below 30 °C—Because viable $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ strains capable of synthesizing only Kdo₂-lipid IV_A could be constructed under slow-growth conditions, we wondered if under the same growth conditions, a suppressor-free chromosomal deletion in the otherwise essential *waaA* gene can be constructed. This allowed us to test if Kdo attachment to lipid IV_A is absolutely required for viability of *E. coli*. Strains with viable non-polar deletions in the *waaA* gene were obtained on minimal medium at 21–23 °C in W3110 and BW25113 genetic backgrounds (SR8614, SR8621, and SR8647). Such strains grew even on LB medium up to 30 °C, although with a smaller colony size than the parental wild-type (Fig. 11C), unlike $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ strains, which did not form colonies on rich LB medium (Table 3). Thus, under slow-growth conditions and at low temperatures the *waaA* gene is dispensable for *E. coli* viability, although such strains grew poorly. These should be to date the first viable suppressor-free deletions in the *waaA* gene in *E. coli* K-12. However, $\Delta waaA$ mutants exhibited severe membrane defects, resulting in the inability to form colonies on MacConkey agar, extremely sensitive to antibiotics, detergents, and chelating agents like EDTA (data not shown). $\Delta waaA$ were also found to have cell division defects and lower amounts of the cell division protein FtsZ (Fig. 11F). To rule out any accumulation of suppressors under slow-growth conditions (30 °C or below) besides transductions, genomic DNA of 3 independent $\Delta waaA$ strains was prepared and found to have no mutations either in the *msbA* or *yhjD* genes, the two potential genes in which suppressors could arise. Furthermore, subsequent analysis of LPS of $\Delta waaA$ mutants confirmed the presence of free lipid IV_A precursors and its derivatives, lacking any Kdo and further glycosylation (see below). This was also verified by TLC immuno-overlay, using the Kdo-specific monoclonal antibody A20 (23) (data not shown).

$\Delta(waaC\ lpxL\ lpxM\ lpxP)$ Mutants Exhibit Defects in Cell Division—Because $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ exhibited a slow-growth phenotype as compared with the parental strain even under permissive growth conditions, we examined cellular morphology. $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ mutant bacteria formed short filaments with ~2–3-fold longer dimension than the nor-

TABLE 3

Colony forming ability of $\Delta waaC$ derivatives in transductional combinations with OMP/LPS-specific factors and suppression by MsbA variants

	Transductional efficiency			
	M9		LB	
	30 °C	37 °C	30 °C	37 °C
$\Delta(lpxM\ lpxP) + pmsbA^+ + \Delta lpxL$	++ ^a	++	++	++
$\Delta(lpxL\ lpxM\ lpxP) + pmsbA^+ + \Delta waaC$	++	+ ^b	++	– ^g
$\Delta(lpxL\ lpxM\ lpxP) + pmsbA\ L279V + \Delta waaC$	++	++	++	++
$\Delta(lpxL\ lpxM\ lpxP) + pmsbA\ A510P + \Delta waaC$	++	++	++	++
$\Delta(lpxL\ lpxM\ lpxP)\ msbA^c\ D498V + \Delta waaC$	++	++	++	++
$\Delta(lpxL\ lpxM\ lpxP) + \Delta surA$	++	ND ^d	ND	ND
$\Delta skp + \Delta waaC$	++	++	++	++
$\Delta fkpA + \Delta waaC$	++	++	++	++
$\Delta ppiD + \Delta waaC$	++	++	++	++
<i>surA</i> ::kan + <i>waaC</i> ::cm	FC ^e	FC	FC	FC
<i>surA</i> ::cm + <i>waaC</i> ::kan	FC	FC	FC	FC
<i>surA</i> ::kan + <i>psurA</i> ⁺ + <i>waaC</i> ::cm	++	++	++	++
<i>htrA</i> ::tet + <i>waaC</i> ::cm	++	++	++	++
<i>htrA</i> ::tet + <i>waaC</i> ::kan	++	+	++	+
$\Delta hfq + \Delta waaC$	++	++	++	++
$\Delta rybB + \Delta waaC$	++	++	++	++
$\Delta yfgL + \Delta waaC$	++	++	++	+

	M9		LB	
	21 °C	23 °C	30 °C	37 °C
$\Delta(lpxL\ lpxM\ lpxP) + \Delta waaC$	++	++	–	–
Wild type + $\Delta waaA$	++	++	+ ^f	–

^a ++, ≥500 colonies.^b +, 100–500 colonies, but small in size.^c Chromosomal MsbA D498V mutation.^d ND, not determined.^e FC, few colonies <10.^f Small colony size.^g –, inability to support colony forming ability.

mal cell shape of the isogenic wild-type strain (Fig. 1A). This could be further attributed to impairment of cell division machinery, with an apparent 50% decrease in the levels of FtsZ in $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ with only Kdo₂-lipid IV_A LPS (Fig. 1C). Thus, the cell division defects in $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ can be ascribed to limiting amounts of the key cell division protein FtsZ.

msbA Overexpression Suppresses $\Delta(lpxL\ lpxM\ lpxP)$ Triple Mutants but Only a Partial Rescue for $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ —Because the growth of *lpxL* mutants, which predominantly contain tetraacylated LPS, can be suppressed by overexpression of the *msbA* gene product (29), we tested if the MsbA in high dosage could suppress conditional lethality of $\Delta(lpxL\ lpxM\ lpxP)$ and $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ strains. Introduction of plasmid, bearing the *msbA* gene with the inducible promoter, in $\Delta(lpxL\ lpxM\ lpxP)$ restored their growth and colony forming ability in the presence of inducer (isopropyl β -D-thiogalactopyranoside) even at 37 °C on rich medium (faster growing conditions), where $\Delta(lpxL\ lpxM\ lpxP)$ mutants are incapable of growth (Tables 2 and 3).

To verify if *msbA* overexpression would also restore growth in $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ mutants, we first transduced at 23 °C the *waaC* null allele in strain SR7781 (*lpxL\ lpxM\ lpxP*) carrying the *msbA* gene on a plasmid. Such transductants were then tested for their ability to grow at different temperatures. $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ with *msbA* overexpression grew nearly to wild-type levels at 23 and 30 °C both on rich and minimal medium (Tables 2 and 3). However, a similar complementation could not be observed at 37 °C, indicating partial suppression.

Tetraacylated LPS of *E. coli* K-12

Mutations in the *msbA* Gene That Elevate *Kdo*₂-Lipid IV_A Mutants—Because mild overexpression of the *msbA* gene allowed growth of $\Delta(waaC lpxL lpxM lpxP)$ mutants at 30 °C, we looked for suppressor mutations under the same conditions

that could rescue growth at 37 °C on LB medium. Temperature-resistant survivors at 37 °C of SR7870, carrying the *msbA* on plasmids, were obtained at a frequency of $\sim 10^{-4}$. Plasmid DNA was prepared from individual temperature-resistant clones and used to confirm if the suppressor mutation was in the coding region of the plasmid-encoded *msbA* gene and their ability to breed true upon retransformation. Three independent clones identified changes in the *msbA*-coding sequence at residues L279V, D498V, and A510P, respectively. Among these, the D498V clone was found to suppress SR7870 and SR7781, even without the presence of inducer, thus at the low level of MsbA expression. Thus, we constructed a chromosomal *msbA*-498Val mutation in SR7870 (Table 3), which supported the colony forming ability up to 37 °C in LB and minimal media, leading us to conclude that this mutant allele can suppress the $\Delta(waaC lpxL lpxP lpxM)$ mutant at the chromosomal copy.

Analysis of LPS Composition of $\Delta waaC$ and Its Derivatives in Minimal Medium with and without Induction of LPS Modifications—The chemotype of the LPS of *waaC* mutant and its derivatives deleted for genes encoding the late acyltransferases such as lauroyl, myristoyl, and palmitoleoyl acyltransferase was determined by mass spectrometry. LPS was extracted from bacterial cultures grown at permissive temperatures, either in the lipid A non-modifying M9 medium or 121 medium, which we found induces lipid A and core modifications that are commonly observed in polymyxin-resistant mutants. In *E. coli*, it is known that lipid A modifications by *P-EtN* and *L-Ara4N* are under control of the inducible two-component system BasS/R (30, 31). We chose minimal medium, because it is permissive for growth of all these mutants, including $\Delta waaA$ and $\Delta(waaC lpxL lpxM lpxP)$, and provides suppressor-free conditions.

To establish the molecular basis of lipid A modifications in 121 medium, *basS/R*-regulated *eptA-lacZ* and *arnB-lacZ* promoter fusions were constructed and assayed for transcriptional activity. Shift of cultures, carrying either *arnB-lacZ* or *eptA-lacZ* promoter fusions, from LB or M9 medium to 121 medium

resulted in 20–90-fold induction of β -galactosidase activity (Fig. 2A). This induction in 121 medium was attributed to the cumulative effect of phosphate limitation and the simultaneous presence of non-toxic amounts (20 μ M each) of Fe³⁺ and Zn²⁺ ions (Fig. 2B). When 121 medium was supplemented with 2 mM K₂HPO₄, the transcriptional activity of the *eptA-lacZ* was reduced nearly 6-fold (Fig. 2B). However, when Fe³⁺ and Zn²⁺ were omitted from 121 medium, the induction of *eptA-lacZ* fusion was drastically reduced, even in the phosphate-limiting conditions (Fig. 2B). Because the *eptA* gene is transcribed as an *eptA-basS-basR* operon, induction of the *eptA* pro-

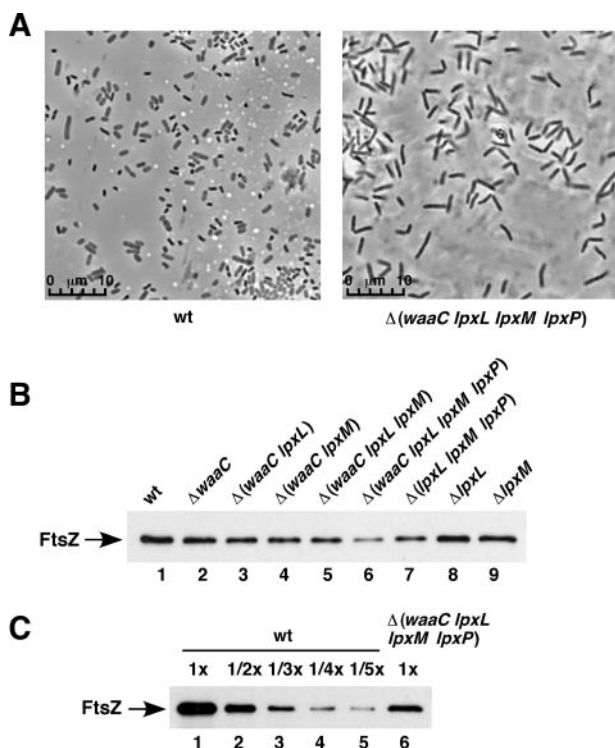


FIGURE 1. Cell division defects of $\Delta waaC$ and its derivatives. A, cultures of wild-type (*wt*) and $\Delta(waaC lpxL lpxM lpxP)$ were grown in M9 minimal medium at 23 °C to early log phase and samples were fixed and visualized by confocal microscopy. B, aliquots of cultures grown as described above at 23 °C in M9 medium of *waaC* and its derivatives with mutations in *lpxL* or *lpxM*, or triple null $\Delta(lpxL lpxM lpxP)$ were harvested by centrifugation. Equivalent amounts of cells were lysed in SDS-sample buffer and proteins were resolved on 12% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and probed with antisera raised against FtsZ. C, lysed samples of wild-type prepared as described above were diluted as indicated, and applied on 12% SDS-PAGE along with the undiluted sample from $\Delta(waaC lpxL lpxM lpxP)$ and analyzed for FtsZ levels.

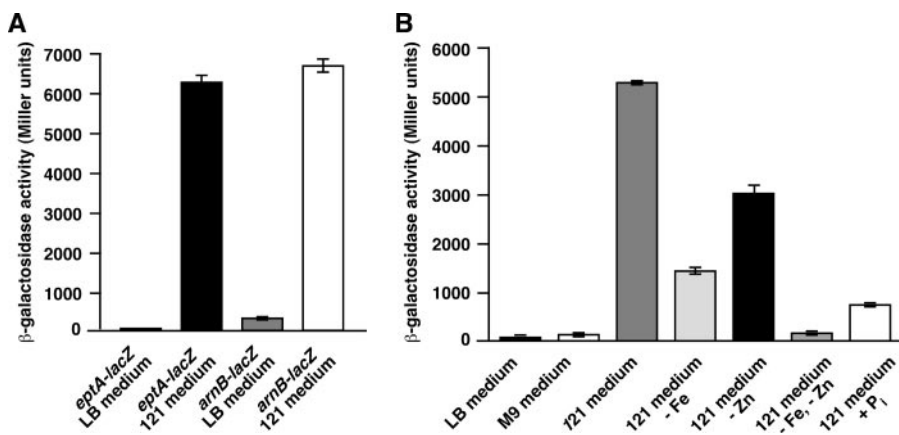


FIGURE 2. Induction of transcription of *eptA* and *arnB* promoters in 121 medium. Cultures of *E. coli* strain GK1111 carrying *eptA-lacZ* and *arnB-lacZ* promoter fusions were grown to early log phase in LB medium at 37 °C, washed, and adjusted to OD of 0.02 at 600 nm in LB, M9, or 121 medium. Aliquots of samples were drawn at different intervals and analyzed for β -galactosidase activity. Data of one representative set are presented (A). Cultures of GK1111 carrying *eptA-lacZ* promoter fusion were analyzed for β -galactosidase activity in LB, M9, 121 medium, or 121 with or without supplementation of various components as indicated for the various bars (B). Error bars represent S.E. of four independent measurements.

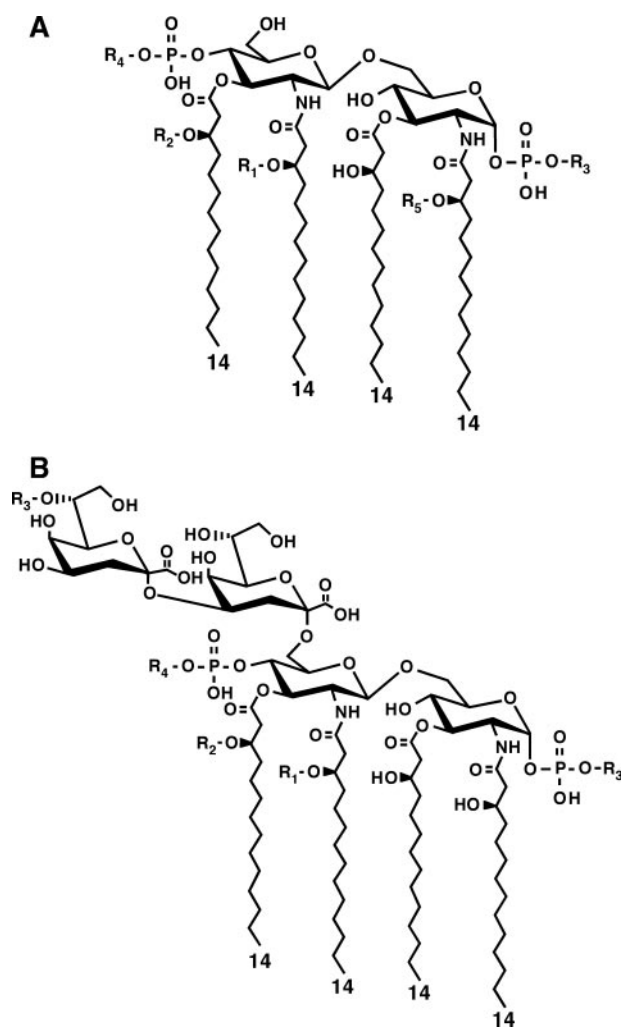


FIGURE 3. Chemical structure of tetraacylated lipid IV_A precursor (A) and Kdo₂-lipid IV_A (B). R1 represents C12:0 or C16:1; R2, C14:0; R3 and R4 are under LPS-modifying conditions *P-EtN* and *L-Ara4N*, respectively, and R5, C16:0.

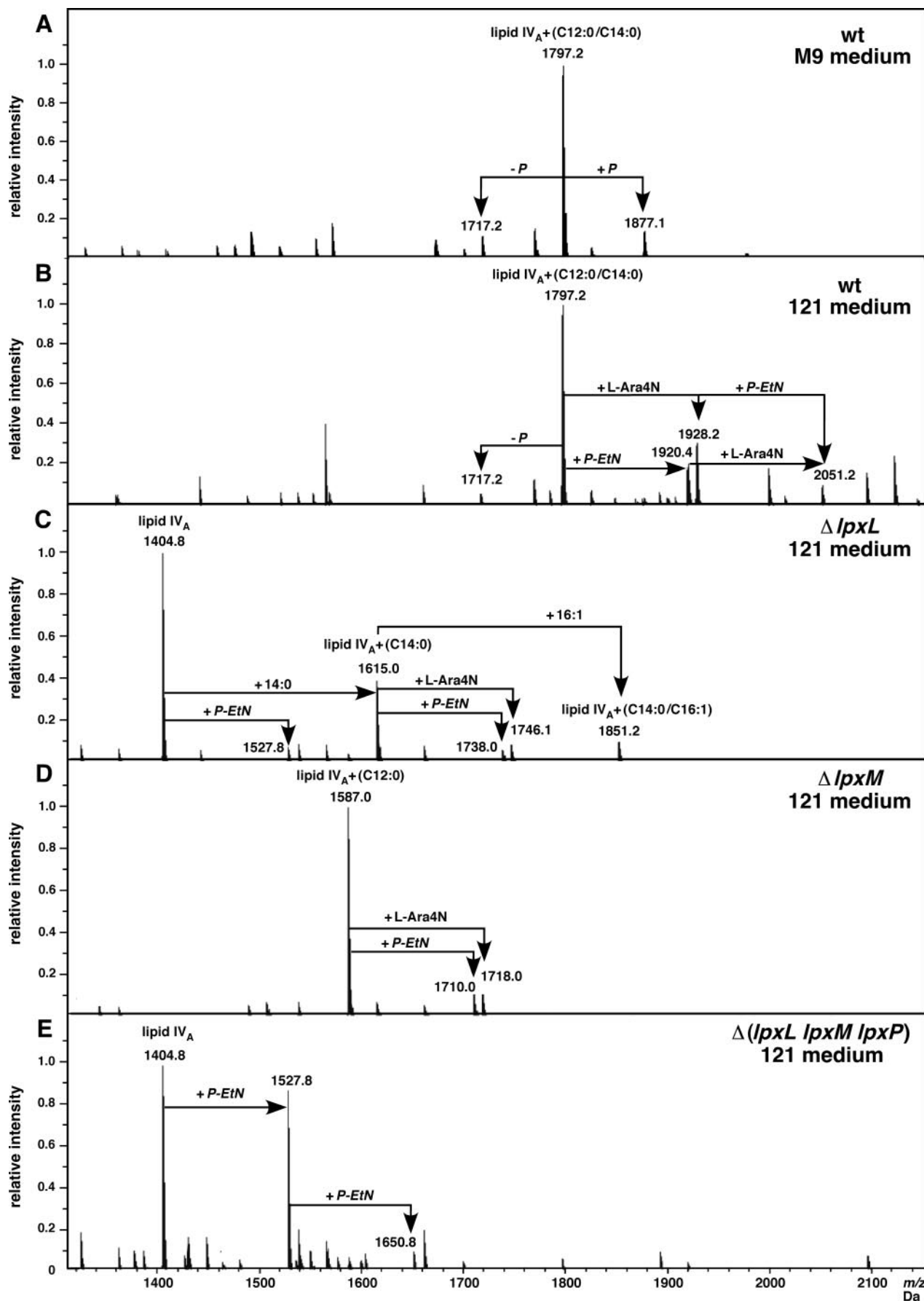
moter should activate all members of the BasS/R two-component system. Thus, using 121 medium growth conditions allowed us to monitor efficiency of LPS translocation, given some of the modifications are supposed to occur after translocation on the periplasmic side and serve as good markers for lipid A translocation (32, 33). This was important for analyses of the LPS of $\Delta waaA$ and $\Delta(waaC lpxL lpxM lpxP)$ mutant strains, synthesizing free lipid IV_A precursor and Kdo₂-lipid IV_A, respectively, given tetraacylated LPS as being a poor substrate for the LPS transporter MsbA (33).

The Lipid A Modifications of Wild-type and Isogenic $\Delta lpxL$, $\Delta lpxM$, and $\Delta(lpxL lpxM lpxP)$ Mutants in 121 Medium—All LPS isolates were analyzed in their native, non-derivatized form by high resolution ESI FT-ICR MS under soft ionization conditions to obtain the intact molecular species and after unspecific fragmentation, generating Y-fragment ions comprising the heterogeneous lipid A part structures. The lipid A part of LPS of the wild-type *E. coli* K-12 grown in M9 revealed mass peaks of 1796.2 Da corresponding to hexaacylated lipid A and another peak with 1877.1 Da, which can be attributed to the substitution by 1-pyrophosphate (Figs. 3 and 4A). However, the lipid A part

of LPS of wild-type bacteria grown in 121 medium, in addition to the presence of the hexaacylated 1,4'-bisphosphorylated species (1796.2 Da), also contained molecular species with *P-EtN* (1920.4 Da), *L-Ara4N* (1928.2 Da), and both substitutions (2051.2 Da) (Fig. 4B). Overall, these data confirm that the lipid A part of LPS from the wild-type bacteria grown in 121 medium contains *P-EtN* and *L-Ara4N*, due to the induction of *basS/R*-induced transcription of genes whose products mediate such additions.

Deletion of the *lpxM* gene, encoding myristoyltransferase, resulted in the synthesis of the pentaacylated lipid A species, characterized by a peak at 1587.0 Da. However, two additional peaks with 1710.0 and 1718.0 Da (Fig. 4D) were observed corresponding to the addition of *P-EtN* or *L-Ara4N* to the pentaacylated lipid A, respectively. Deletion of the *lpxL* gene, encoding lauroyltransferase, revealed tetra-, penta-, and hexaacylated lipid A (peaks with 1404.8, 1615.0, and 1851.2 Da, respectively) (Fig. 4C). The mass peak corresponding to hexaacylated lipid A can arise due to the addition of one secondary myristate and one secondary pantoic acid. Tetraacylated lipid A was also found to be modified by *P-EtN*, giving rise to a peak at 1527.8 Da. Similarly, pentaacylated lipid A (with mass of 1615.0 Da) was also modified by *P-EtN* and *L-Ara4N* (Fig. 4D). Thus, the pentaacylated lipid A species in $\Delta lpxL$, as well as in $\Delta lpxM$, seem to be modified in 121 medium growth conditions. However, under the same growth conditions tetraacylated lipid A was not found to be modified by *L-Ara4N* (Fig. 4, C and E). The tetraacylated lipid A in $\Delta(lpxL lpxM lpxP)$ was found to be modified with 1 or 2 *P-EtN* residues as revealed by mass peaks at 1527.8 and 1650.8 Da, respectively (Fig. 4E). However, no species containing *L-Ara4N* could be detected. These results help explain the poor translocational ability of tetraacylated LPS export and hence limited access to modification systems, like *L-Ara4N* addition, which operate upon translocation across the inner membrane.

Kdo-independent Acylation of Lipid IV_A Precursor in $\Delta waaA$ by Late Acyltransferases—Lipid A composition of $\Delta waaA$ mutants grown at either 21 or 30 °C in different growth medium was determined from the extracts containing a mixture of glycerophospholipids and lipid A. These growth conditions are permissive for suppressor-free $\Delta waaA$ without requiring extra copies of the *msbA* gene. Due to the isolation procedure, phospholipids were not removed and are also present in the spectra. For example, the peak at *m/z* 1404.0 could be either a phospholipid dimer or a cardiolipin (Fig. 5). The non-deconvoluted mass spectrum of LPS from $\Delta waaA$ grown at 30 °C in LB medium revealed an ion peak ($M - H^+$)⁻ at *m/z* 1403.8 Da, corresponding to the structure of tetraacylated 1,4'-bisphosphorylated lipid IV_A precursor (Figs. 3 and 5A). Under these growth conditions no detectable penta- or hexaacylated derivatives of lipid IV_A were observed. However, analysis of LPS/lipid A, extracted from $\Delta waaA$ mutants grown at 21 °C, revealed a more complex composition of free lipid A. In addition to the ion peak, corresponding to tetraacylated lipid IV_A, ion peaks corresponding to penta- and hexaacylated derivatives were also present, despite the lack of Kdo transferase. Thus, lipid A extracted from $\Delta waaA$, grown in LB medium at 21 °C, also revealed ion peak at *m/z* 1640.1 Da, corresponding to the



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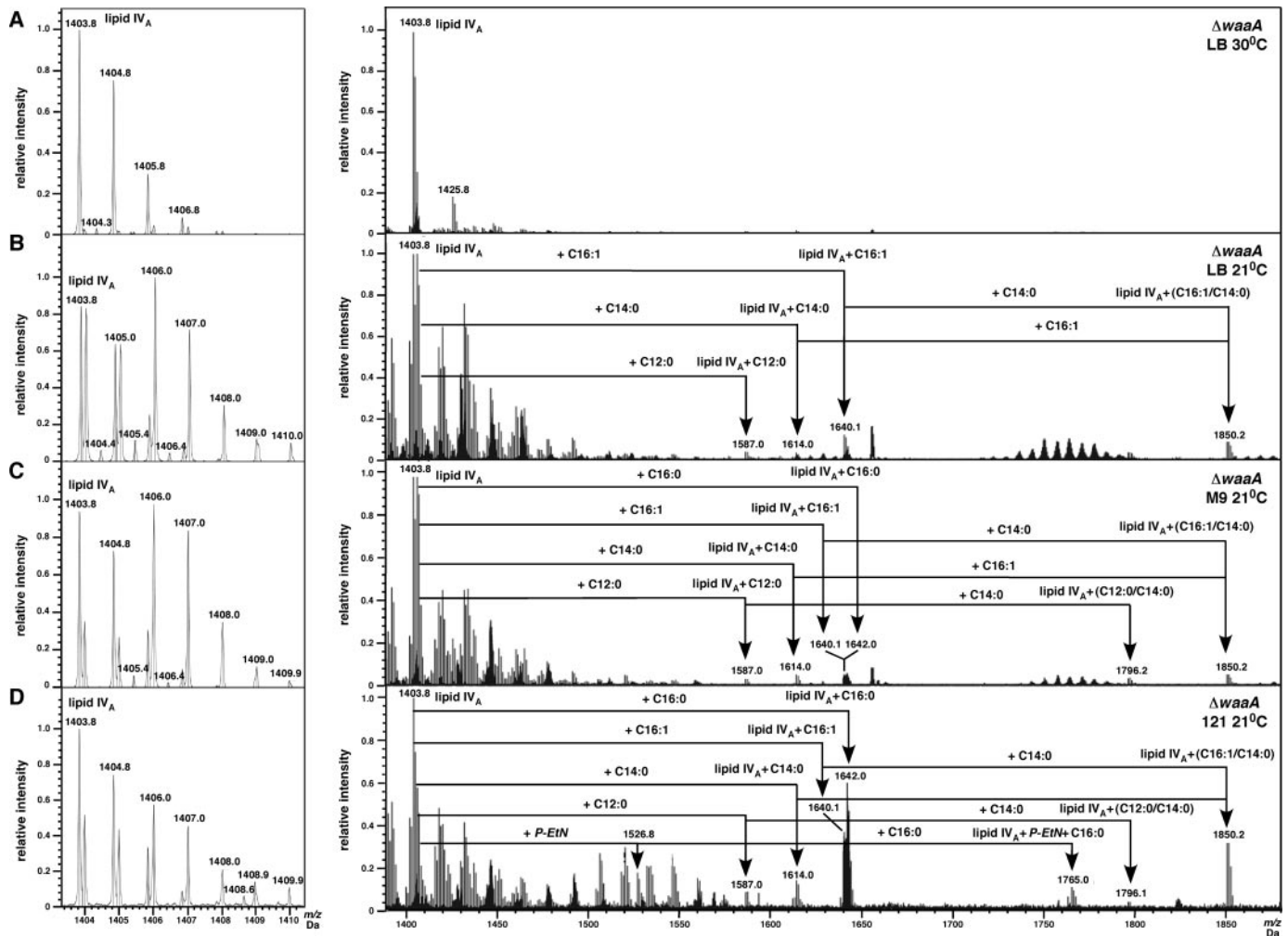


FIGURE 5. ESI FT-ICR mass spectra in negative ion mode of LPS isolated from *waaA* deletion grown in LB medium at 30 °C (A) and 21 °C in LB medium (B), in M9 medium (C), and 121 medium (D). The left inset shows in detailed part of the spectra containing tetraacylated lipid IV_A precursor. Mass numbers correspond to (M - H⁺)⁻ ions. Addition of lauroyl, myristoyl, palmitoleate, palmitoyl, and *P-EtN* moieties to lipid IV_A precursor and their derivatives are drawn schematically. These spectra also contain intensive signals for phospholipids. The ion peak at *m/z* 1404.0 in the inset could correspond to either phospholipid dimmer or a cardiolipin.

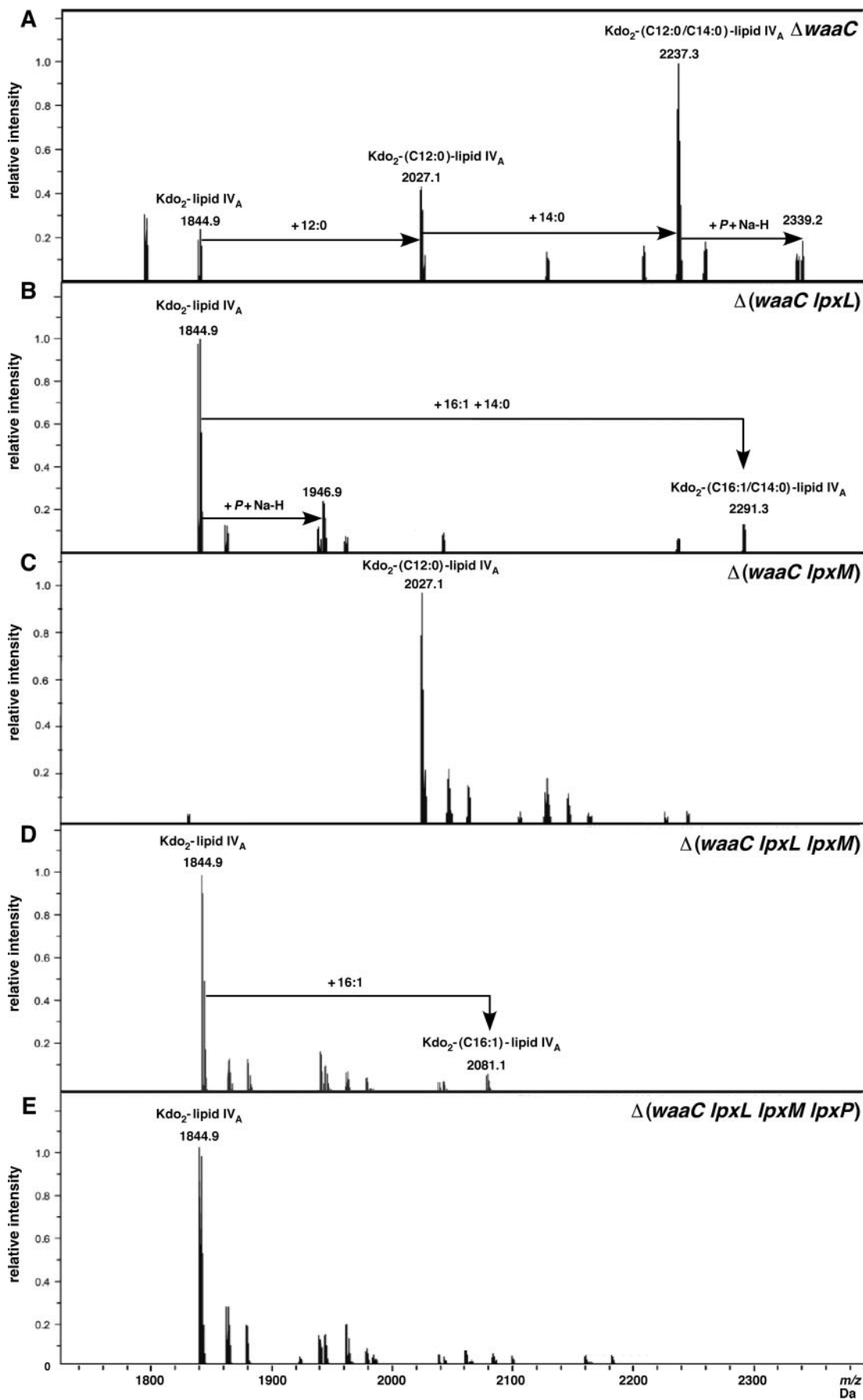
predicted incorporation of the C16:1 secondary palmitoleate (Fig. 5B). Furthermore, ion peaks at *m/z* 1614.0 and 1850.2 Da indicated an addition of myristate to lipid IV_A precursor and conversion of lipid IV_A + C16:1, to the hexaacylated derivative of lipid IV_A C16:1 + C14:0, respectively.

Analyses of lipid A extracted from $\Delta waaA$, grown at 21 °C in M9 or 121 medium, revealed the presence of four different ion peaks, corresponding to the synthesis of distinct pentaacylated lipid IV_A derivatives, besides lipid IV_A precursor (Fig. 5, C and D). Out of these, ion peaks at *m/z* 1640.1 and 1642.0 Da correspond to the addition of palmitoleate and the palmitoyl moiety to lipid IV_A precursor. The other two ion peaks at *m/z* 1587.0 and 1615.0 Da represent the characteristic incorporation of secondary laurate and myristate groups to (*R*)-3-hydroxymyr-

istate chains located at the 2' and 3' positions of the distal glucosamine, respectively (Figs. 3 and 5).

The presence of palmitoyl-modified species in $\Delta waaA$, represented by ion peaks at *m/z* 1642.0 Da (Fig. 5, C and D), indicates that the lipid IV_A precursor, synthesized at 21 °C in the minimal medium, is translocated to the outer membrane, because it requires the activity of the outer membrane PagP enzyme. Furthermore, the lipid IV_A precursor was found to be modified by *P-EtN*, corresponding to ion peaks at *m/z* 1526.8 Da (lipid IV_A + *P-EtN*) and 1765.0 Da (lipid IV_A + *P-EtN* + C16:0) (Fig. 5D) in $\Delta waaA$ grown in 121 medium. Like the hexaacylated lipid A represented by the ion peak at *m/z* 1850.2 Da, with the predicted composition of lipid IV_A + C16:1 + C14:0, observed in LB grown $\Delta waaA$ at 21 °C, also accumulated

FIGURE 4. Charge deconvoluted ESI FT-ICR mass spectra in negative ion mode depicting lipid A modifications of LPS from wild-type (wt) or its derivatives with deletions of late acyltransferases genes grown at 30 °C. Part of the negative ion mass spectra of the native LPS after unspecific fragmentation leading to cleavage of the labile lipid A-Kdo linkage are presented. Spectra of lipid A section from LPS extracted from wild-type grown in M9 (A) or 121 medium (B) and its derivatives carrying deletions of $\Delta lpxL$ (C), $\Delta lpxM$ (D), and $\Delta(lpxL\ lpxM\ lpxP)$ (E) all grown in 121 minimal medium. Only the mass peaks corresponding to the lipid A part are marked and substitutions with *P-EtN* and/or L-Ara4N are indicated.



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in 121 medium growth conditions (Fig. 5D). The Kdo-independent incorporation of some secondary laurate group in minimal medium at 21 °C also resulted in synthesis of the typical hexaacylated lipid A (ion peak at m/z 1796.1 Da) with the predicted composition of lipid IV_A + C16:1 + C14:0, due to further addition of the myristate acyl chain (Fig. 5, C and D). Thus, at low temperatures in slow-growth conditions *in vivo* lipid IV_A can be used as the substrate for late acyltransferases independent of Kdo attachment. Of these, interesting is the incorporation of the LpxP-dependent C16:1 palmitoleate moiety, whose synthesis and incorporation is favored at low temperatures. This can be ascribed to a significant burst in the activity of RpoE in $\Delta waaA$, resulting in elevated RpoE-dependent *lpxP* transcription (Figs. 5 and 11).

Mass Spectrometry of Intact LPS from $\Delta waaC$ and Its Isogenic Derivatives Lacking the Late Acyltransferases Grown in Non-modifying or Modifying Minimal Medium—The composition of LPS of $\Delta waaC$ in either phosphate-rich M9 medium (LPS non-modifying) or 121 medium (LPS modifying conditions) revealed the common mass peak of 2237.3 Da. This is in agreement with the composition of Kdo₂-lipid A_{hexa} 1,4'-bisphosphate (Figs. 6 and 7A). The other common peak at 2027.1 Da could be interpreted as Kdo₂-lauroyl-lipid IV_A. However, LPS of $\Delta waaC$ from 121 medium also revealed mass peaks of 2368.4 (addition of L-Ara4N) and 2491.4 Da (addition of L-Ara4N and *P-EtN*) (Fig. 7A). Modification with only *P-EtN* on Kdo₂-lipid A_{hexa} or Kdo₂-lipid A_{penta} was also revealed with peaks at 2360.3 and 2150.1 Da, respectively (Fig. 7A). Besides the differences in the presence of the *P-EtN* and L-Ara4N species on Kdo₂-lipid A_{hexa} and Kdo₂-lipid A_{penta}, the species corresponding to Kdo₂-lipid IV_A was conspicuously missing in the LPS of $\Delta waaC$ mutants from 121 medium as compared with its presence in the M9 medium.

LPS extracted from $\Delta(waaC\ lpxL)$, grown in either M9 or 121, revealed a common mass peak of 1844.9 Da, characteristic of Kdo₂-lipid IV_A (Figs. 6B and 7B). Consistent with the known substitution of palmitoleate (*cis*Δ⁹C16:1) to the same position of the missing laurate (C12:0) in *lpxL* mutants at or below 30 °C, a compound of 2291.3 Da corresponded to hexaacylated LPS, representing Kdo₂-lipid IV_A C16:1, C14:0 was observed. Such a hexaacylated compound was totally absent in $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ (Figs. 6E and 7E). The LPS of $\Delta(waaC\ lpxL)$ grown in 121 medium also revealed peaks at 2055.1 and 2081.2 Da, corresponding to Kdo₂-lipid IV_A containing an additional myristate or palmitoleate, respectively. Interestingly, species representing modification by *P-EtN* on Kdo₂-lipid IV_A or Kdo₂-lipid IV_A C16:1, C14:0 forms in $\Delta(waaC\ lpxL)$ LPS were also observed with peaks at 1967.9 and 2414.3 Da, respectively (Fig. 7B). Thus, such structures seemed to be accessible and act as substrates for *P-EtN* modification.

LPS of $\Delta(waaC\ lpxM)$ revealed a common peak at 2027.1 Da, both under M9 and 121 growth conditions (Figs. 6C and 7C), corresponding to the expected structure of the Kdo₂-lauroyl-

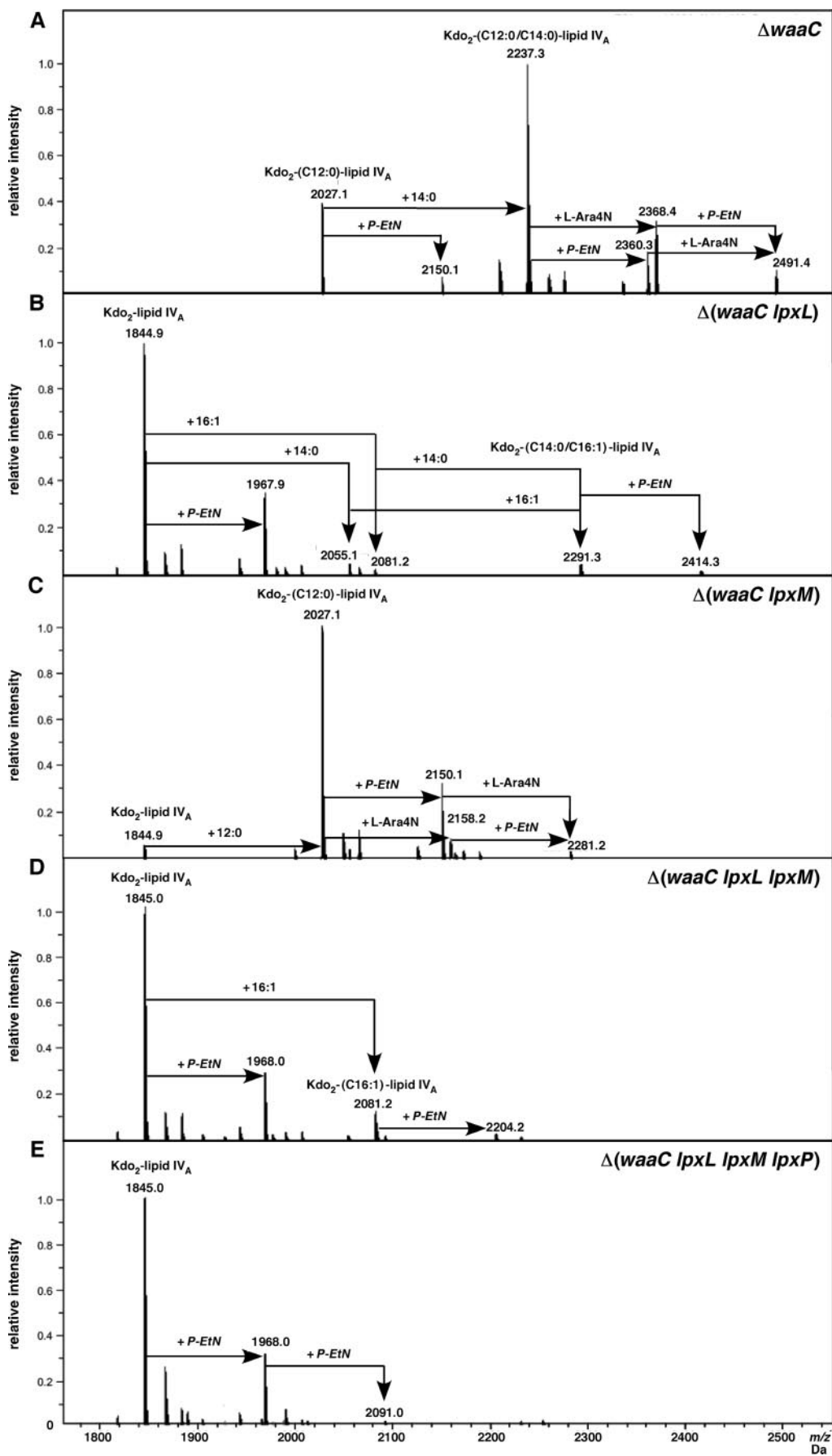
lipid IV_A. However, two additional peaks at 2150.1 and 2281.2 Da correspond to Kdo₂-lauroyl-lipid IV_A with the addition of *P-EtN* and *P-EtN*+L-Ara4N, respectively, were detected in LPS from $\Delta(waaC\ lpxM)$ grown in 121 medium. The substitution of Kdo₂-myristoyl-lipid IV_A by *P-EtN* and L-Ara4N indicates the normal transport of such pentaacylated LPS. These results support the observed lack of severe growth defects in $\Delta(waaC\ lpxM)$ as compared with $\Delta(waaC\ lpxL)$ derivatives (Table 2).

LPS extracted from $\Delta(waaC\ lpxL\ lpxM)$ confirmed the induction and incorporation of a secondary palmitoleate residue due to LpxP activity, resulting in the presence of both Kdo₂-lipid IV_A and Kdo₂-palmitoleate-lipid IV_A with peaks at 1845.0 and 2081.2 Da, respectively (Figs. 6D and 7D). Furthermore, both of these LPS species were also found to be modified by single *P-EtN* residues with peaks at 1968.0 and 2204.2 Da, respectively (Fig. 7D).

The authenticity of the suppressor-free strain $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ was established by the presence of only the tetraacylated form with Kdo₂-lipid IV_A, corresponding to the main peak at 1845.0 Da, without any hexa- or pentaacylated derivatives. Remarkably, $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ LPS also contains a single (1968.0 Da) as well as two *P-EtN* additions (2091.0 Da) (Fig. 7E). The presence of 2 *P-EtN* residues could be due to stronger induction of the RpoE-regulated *eptB* gene, as shown by our transcriptional induction of the *rpoE* regulon in such mutants (Fig. 10). Because 121 medium contains 2 mM CaCl₂, the addition of *P-EtN* is more likely to occur on the outer Kdo residue, which was not observed in M9 medium, which contains only 0.1 mM CaCl₂. However, a fraction of lipid A $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ might contain two *P-EtN* on lipid A as seen in fragmentation spectra (data not shown). Overall, these analyses confirm the absence of L-Ara4N in the LPS of $\Delta(waaC\ lpxL)$, $\Delta(waaC\ lpxL\ lpxM\ lpxP)$, although *P-EtN*-modified derivatives could be observed (Fig. 7, B, D, and E). Thus, modification with L-Ara4N serves as a more stringent marker than by the *P-EtN* for poor translocation efficiency of tetraacylated lipid A.

The Origin of *P-EtN* and L-Ara4N in $\Delta waaC$ and Its Derivatives—We expected in $\Delta waaC$ lipid A, as observed in the wild-type lipid A, both *P-EtN* and L-Ara4N substitution in 121 medium growth conditions. However, LPS of $\Delta waaC$ contained mostly L-Ara4N substitution on the lipid A (Figs. 7 and 8). The *P-EtN* substitution in $\Delta waaC$, giving rise to peaks at 2360.3 and 2491.4 Da, seemed to arise mostly from the *P-EtN* addition to the Kdo region. We addressed the presence of *P-EtN* either on Kdo or lipid A, and L-Ara4N in *waaC* derivatives. Thus, LPS from strains $\Delta(waaC\ eptA)$, $\Delta(waaC\ eptB)$, and $\Delta(waaC\ basR\ eptB)$ were analyzed. LPS of $\Delta waaC$ and its derivatives revealed a common occurrence of species with a peak at 1928.2 Da, corresponding to L-Ara4N substitution on lipid A_{hexa} (Fig. 8). In the LPS of $\Delta(waaC\ eptB)$ peaks at 1920.2 Da could arise due to *P-EtN* substitution on lipid A, consistent with the presumed function of EptA phosphoethanolamine transferase. This mass peak is missing in the LPS of $\Delta(waaC\ eptA)$,

FIGURE 6. Charge deconvoluted ESI FT-ICR mass spectra in negative ion mode of LPS isolated from *waaC* deletion (A) and its derivatives with deletions in genes encoding late acyltransferases (B–E) grown in permissive conditions at 23 °C in M9 minimal medium. The origin of the lipid A species by the addition of lauroyl, myristoyl, and palmitoleate are drawn schematically. Mass numbers are the monoisotopic masses of major peaks. Minor peaks, mostly corresponding to the substitution by phosphate or sodium adducts, are not labeled.



consistent with lack of EptA phosphoethanolamine transferase in this strain. Hence, these data suggest, depending on the presence of Ca^{2+} in growth medium (34) and genetic composition, *P-EtN* can be present on both lipid A and Kdo. However, *P-EtN* substitution on Kdo in $\Delta waaC$ is favored due to increased σ^E -dependent transcription of the *eptB* gene in $\Delta waaC$. Quite surprising is the presence of a peak at 1928.2 Da in $\Delta(waaC\ eptB\ basR)$ LPS, which is ascribed to the L-Ara4N substitution. These results argue that genes, whose products are involved in L-Ara4N synthesis and transfer, can also be induced independent of *basS/R* regulon in 121 medium.

Analysis of unspecific fragmentation, leading to cleavage of labile lipid A Kdo linkage of these LPS, revealed the peak at 2140.2 Da in $\Delta(waaC\ eptB)$ (Fig. 8). This can arise from the parent molecule with a mass of 2360.3 Da (Kdo_2 -lipid A_{hexa} -*P-EtN*) by loss of 1 Kdo residue. These results support the presence of *P-EtN* on lipid A in $\Delta(waaC\ eptB)$, because phosphoethanolamine transferase EptB, which mediates *P-EtN* addition to the outer Kdo, is absent. This analyses revealed peaks at 2237.3 and 2368.9 Da, corresponding to Kdo_2 -lipid A_{hexa} and Kdo_2 -lipid A_{hexa} -L-Ara4N, respectively, in all 4 samples. Another peak at 2360.3 Da was present in all, except $\Delta(waaC\ basR\ eptB)$. This could arise from substitution with *P-EtN* to Kdo_2 -lipid A_{hexa} either on lipid A or Kdo in $\Delta waaC$. However, in $\Delta(waaC\ eptB)$ this species can only be attributed to the addition of *P-EtN* to lipid A. In contrast, this species in $\Delta(waaC\ eptA)$ represents the presence of *P-EtN* on the Kdo. Thus, depending upon the relative induction and activation of EptB in *waaC* derivatives, *P-EtN* can be found either on Kdo or the lipid A, or both.

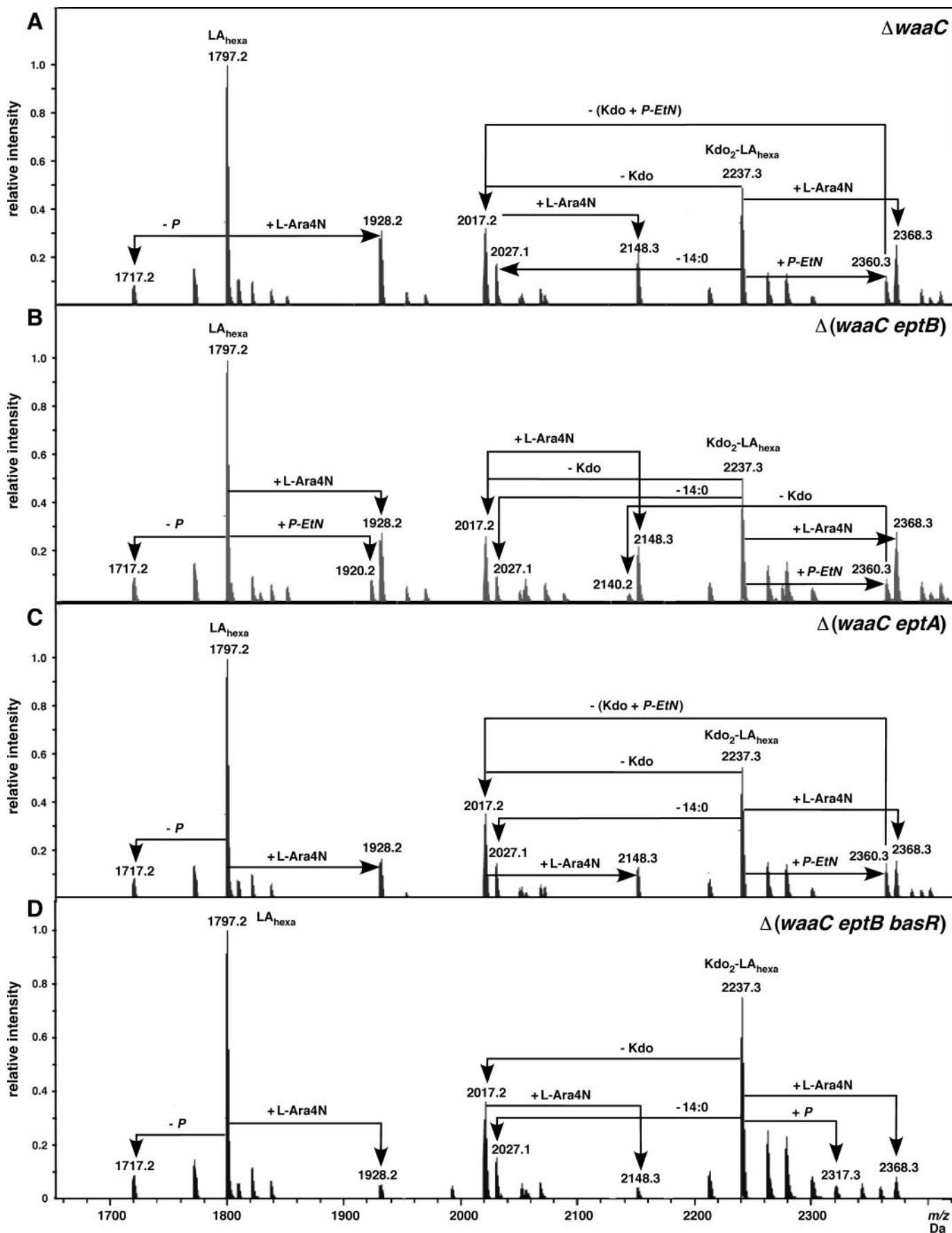
LPS with Tetraacylated Lipid A Leads to Alteration of the Core Glycoform Composition in Modifying Medium—Wild-type *E. coli* K-12 is known to contain multiple glycoforms of the core oligosaccharide (35). One minor form, which contains 3 Kdo residues, has been described that is associated with truncation of the outer core after the Gal residue (35, 36). Here, we found that LPS of the $\Delta(lpxL\ lpxM\ lpxP)$ strain, deleted for all three late acyltransferases, hence synthesizing tetraacylated lipid A, also exhibited changes in the LPS core structure in 121 medium, but not in M9 medium (Fig. 9A). In 121 medium, LPS of $\Delta(lpxL\ lpxM\ lpxP)$ mutants possessed mostly derivatives of the glycoform containing 3 Kdo residues with rhamnose and up to 4 *P-EtN* residues, revealed by peaks at 3556.3, 3679.4, 3802.3, and 3925.4 Da (Fig. 9A). However, as expected from the lack of L-Ara4N in the lipid A part, no glycoform derivatives with substitution by L-Ara4N were found. The 4 *P-EtN* residues could arise from 2 substitutions in lipid A, one on Kdo and one on Hep I. Further additional compositional changes, which are common to 121 medium for the wild-type, were also observed in $\Delta(lpxL\ lpxM\ lpxP)$.

Because *msbA* overexpression suppressed the conditional lethality of $\Delta(lpxL\ lpxM\ lpxP)$, we also examined LPS composition of such derivatives. Under *MsbA* overproducing condi-

tions, lipid A could be substituted by L-Ara4N as is evident from the peak at 2222.0 Da, corresponding to species with the presumed structure of [Kdo_2 -*P-EtN*-lipid IV_A] with 1-residue *P-EtN* and L-Ara4N molecules (Fig. 9B). These results are in accordance with more efficient transport of tetraacylated lipid A under the conditions of *MsbA* overproduction. In the presence of *msbA* overexpression, LPS extracted from the $\Delta(lpxL\ lpxM\ lpxP)$ triple mutant contained glycoforms with 2 as well as 3 Kdo residues. Tetraacylated LPS glycoforms containing 2 Kdo residues corresponded to peaks at 3544.3, 3667.3, and 3790.4 Da, each with 1 *P-EtN* residue. The peak at 3798.3 Da corresponded to the incorporation of L-Ara4N, consistent with the data from the lipid A part. These glycoforms with 2 Kdo residues contained a complete core. However, the derivatives with 3 Kdo had characteristic truncation of the outer core (36). Some new glycoforms were also found with the predicted structure of lipid A_{tetra} - Kdo_2 -Hep₂-Hex₂-P₁ and 1 or 2 *P-EtN* with peaks at 2756.1 and 2879.2 Da, respectively. This seemed to act as precursor for the addition of 1 Hex (3041.2 Da) and 1 *P-EtN* (3164.2 Da) as well as its modification to the glycoform containing 3 Kdo residues and rhamnose. The restoration of synthesis of glycoforms with 2 Kdo and a complete core by *msbA* overexpression in the $\Delta(lpxL\ lpxM\ lpxP)$ mutant confirms the suppression of other growth defects.

Synergistic Induction of the CpxR/A and σ^E Pathways of Extracytoplasmic Signal Transduction in *waaC* and Its Derivatives—The physiological response to the synthesis of Kdo_2 -lipid A_{hexa} , Kdo_2 -lipid A_{penta} , or Kdo_2 -lipid IV_A was determined by following the effects on envelope stress responsive pathways under the control of RpoE and CpxR/A two-component system. The responses of these two principal pathways were assayed by measuring transcriptional activities of *rpoHP3-lacZ* and *cpxP-lacZ* promoter fusions. The *rpoHP3* promoter activity reflects the impact on RpoE activity being solely transcribed by $E\sigma^E$ RNA polymerase (26), although the *cpxP* promoter is under the transcriptional control of the CpxR/A two-component system of signal transduction (28). Significantly, $\Delta waaC$ mutants showed constitutive 3-fold elevated levels of the *rpoHP3* promoter even at the permissive temperature of 30 °C and in growth conditions that do not result in any known stress (M9 medium), indicating constitutive induction of the RpoE pathway (Fig. 10A). However, deletion of neither *lpxL* nor *lpxM* genes alone resulted in any significant increase in the activity of the *rpoHP3* promoter, which is different from an earlier report (37) (Fig. 10A). Only a small increase in *rpoHP3-lacZ* activity (up to 30%) was observed in $\Delta(lpxL\ lpxM)$ or $\Delta(lpxL\ lpxM\ lpxP)$ mutants (Fig. 10A). Thus, underacylation of the lipid A in *E. coli*, synthesizing the complete LPS core, does not induce σ^E -dependent transcriptional response significantly, but LPS core truncation due to *waaC* deletion causes strong σ^E induction.

FIGURE 7. Charge deconvoluted ES FT-ICR mass spectra in negative ion mode of LPS from *waaC* and its derivatives grown under LPS-modifying conditions in 121 medium. A, $\Delta waaC$, proposed structure of Kdo_2 -lipid A with assignment of *P-EtN* and L-Ara4N addition. B, $\Delta(waaC\ lpxL)$, the predicted composition of Kdo_2 -lipid IV_A and Kdo_2 -lipid IV_A with the addition of myristoyl and palmitoleate residues with *P-EtN* additions are shown. C, $\Delta(waaC\ lpxM)$, predicted structure of *P-EtN* and L-Ara4N modified Kdo_2 -myristoyl-lipid IV_A . D, $\Delta(waaC\ lpxL\ lpxM)$ assignments of Kdo_2 -lipid IV_A -*P-EtN* and Kdo_2 -palmitoleate-lipid IV_A with *P-EtN*. E, $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ with two *P-EtN* residues. Note the attachment of *P-EtN* in B–D can also occur on the outer Kdo.



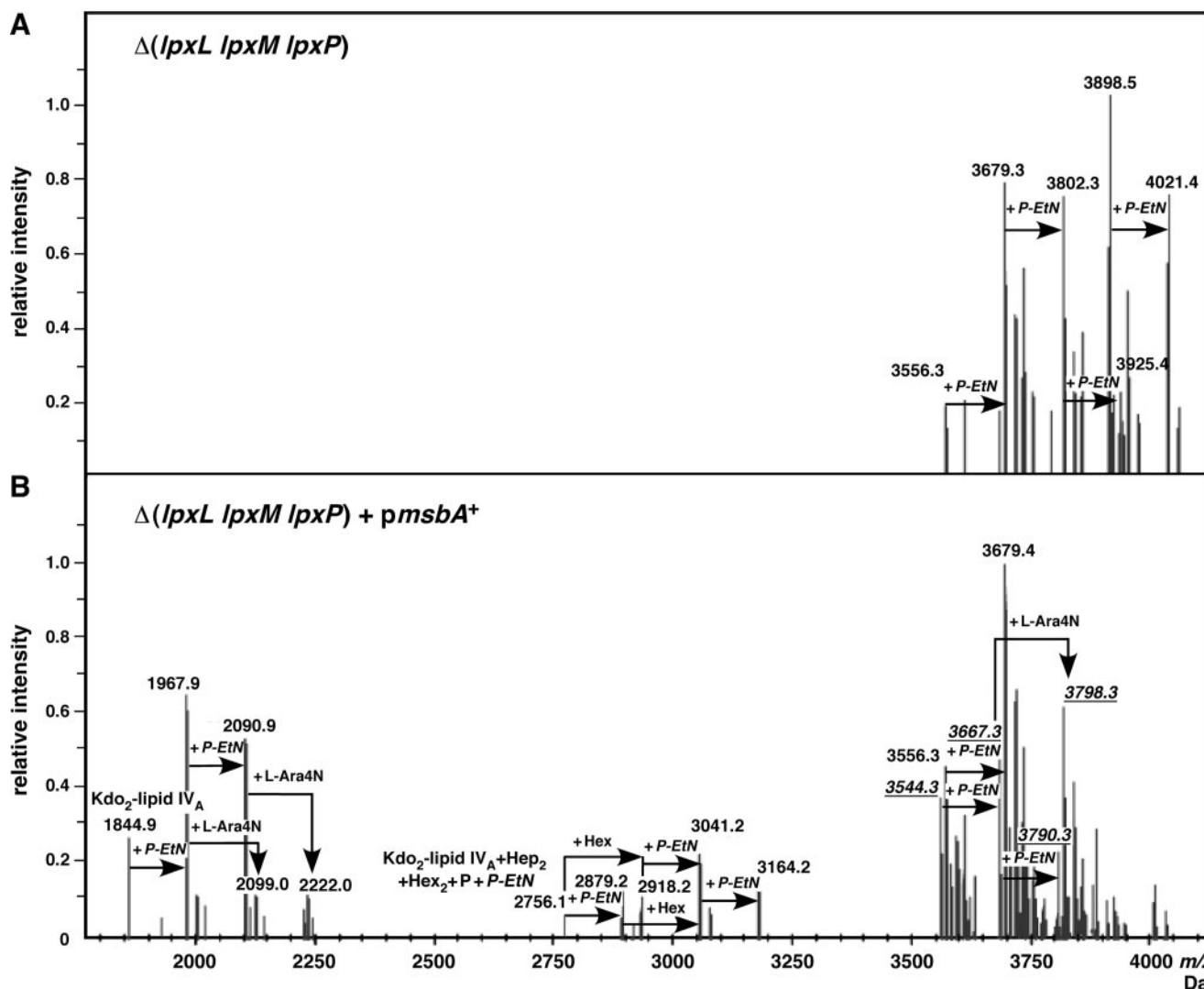


FIGURE 9. **Multicopy suppression leading to addition of L-Ara4N in the LPS of mutant synthesizing tetraacylated lipid A.** Charge deconvoluted ES FT-ICR mass spectra in negative ion mode of native LPS of strain SR7781 $\Delta(lpxL lpxM lpxP)$ (A) and SR7781/*pmsbA*⁺ (B). Cultures were grown in 121 medium at 30 °C and LPS was extracted. *Mass numbers* refer to monoisotopic peaks with predicted composition, correlating with varying numbers of the substitution of *P-EtN* and/or L-Ara4N on the basic structure lipid IV_A-3Kdo-Rha with 3Hep-3Hex are presented in **bold**. The *mass numbers in italics and underlined in panel B* refer to species corresponding to tetraacylated derivatives of glycoform II with 2 Kdo residues (35, 36), but with varying *P-EtN* and/or L-Ara4N substitutions. Both the spectra for panels A and B were recorded under the same conditions. In panel B accumulation of various intermediates with various substitutions following MsbA overexpression is depicted.

However, strains synthesizing tetraacylated lipid IV_A with Re LPS $\Delta(waaC lpxL)$ exhibited a synergistic additive induction of RpoE activity. This was reflected by more than 4-fold elevated transcriptional activity of the *rpoHP3* promoter (Fig. 10A). Curiously, the activity of the *rpoHP3* promoter in $\Delta(waaC lpxM)$, synthesizing Kdo₂-lipid A_{pentar}, remained at the same level as that of the *waaC* mutant alone with a 3-fold increase as compared with the isogenic wild-type or the $\Delta lpxM$ mutant alone. Taken together, these findings explain the severe growth defects and constitutive elevated levels of RpoE activity in strains carrying $\Delta(waaC lpxL)$ deletion combinations synthesizing primarily Kdo₂-lipid IV_A derivatives.

Regarding Cpx two-component system activation, deletion of the *waaC* gene or *lpxL*, or *lpxM*, did not significantly induce *cpxP* promoter activity (Fig. 10B). Only a modest increase of about 30% in the basal level activity of the *cpxP* promoter was observed in $\Delta waaC$. However, a significant 3- to 4-fold induction was observed when either $\Delta lpxL$ or $\Delta lpxM$ were combined with the *waaC* deletion (Fig. 10B). The maximal increase again with *cpxP* promoter activation, like in case with the σ^E -dependent promoter, was observed in $\Delta(waaC lpxL)$ mutational combination. Overall these results revealed a strong induction of the CpxR/A pathway of signal transduction in mutants that synthesize Kdo₂-lipid IV_A or Kdo₂-lipid

FIGURE 8. **Origin of *P-EtN* in *waaC* LPS.** Charge deconvoluted ES FT-ICR mass spectra in negative ion mode of Re LPS derivatives with moderate unspecific fragmentation, showing the complete structure as well as lipid A fragments. Spectra of LPS from isogenic $\Delta waaC$ (A), $\Delta(waaC eptB)$ (B), $\Delta(waaC eptA)$ (C), and $\Delta(waaC basR eptB)$ (D). All cultures were grown at 30 °C in 121 minimal medium. The *mass numbers* given are those of monoisotopic peaks. In all spectra the major peak with a mass of 1797.2 Da corresponds to the hexaacylated 1,4'-bisphosphate lipid A. In panel B the substitution of lipid A with both *P-EtN* and L-Ara4N is indicated. Fragmentation schemes along with the observed masses are indicated. Unlabeled peaks mostly correspond to Na⁺ adducts.

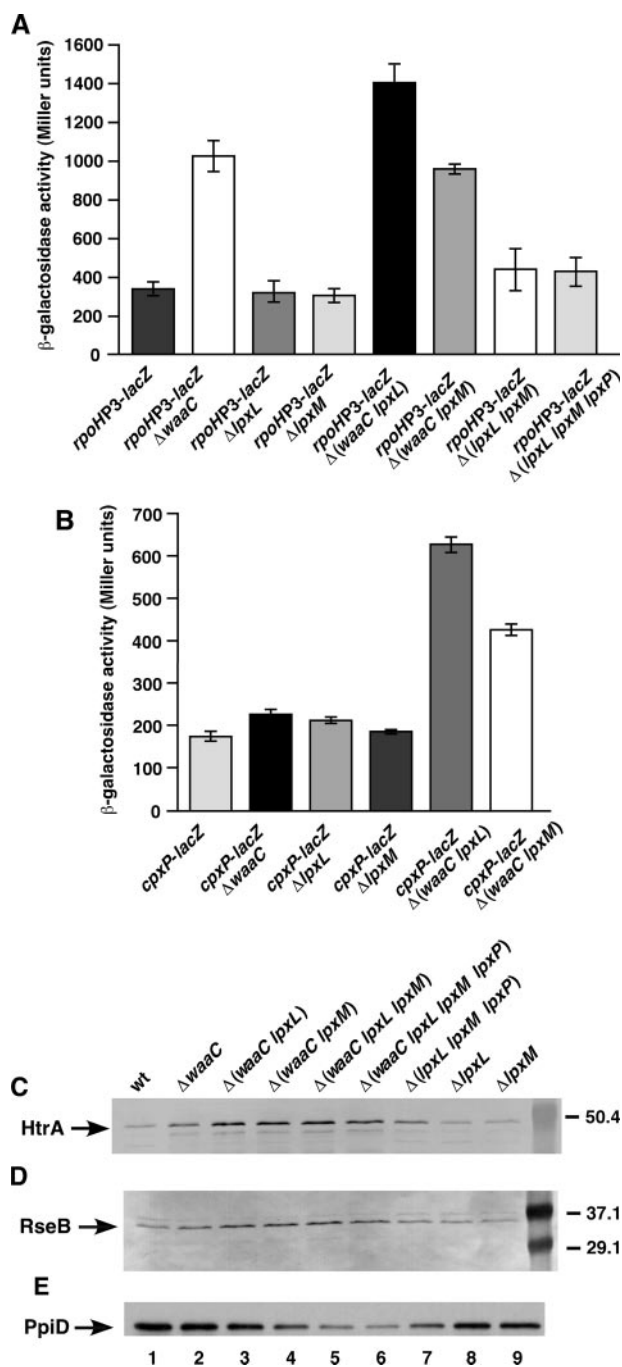


FIGURE 10. Induction of RpoE and CpxR/A pathways. Exponentially grown cultures of wild-type K-12 strain W3110 or its derivatives lacking *waaC*, *lpxL*, and *lpxM* alone and their deletion combinations with *lpxP*, carrying either *rpoHP3-lacZ* (A) or *cpxP-lacZ* fusion (B) on the chromosome were grown in M9 minimal medium under permissive growth conditions. Cultures were diluted to an A_{600} of ~ 0.02 and grown at 30 °C in the same medium. Aliquots of samples were drawn and analyzed for β -galactosidase activity. Error bars represent S.E. of four independent measurements. C–E, cultures of wild-type, Δ *waaC*, and its derivatives were grown at 23 °C in M9 medium to logarithmic phase, harvested by centrifugation, and equivalent amounts of cells were used to prepare whole cell lysates. Proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with antiserum raised against HtrA (C), RseB (D), and PpiD (E). The genotype of each strain used is indicated.

A_{penta} , due to a combination of *waaC* deletion with either *lpxL* or *lpxM* deletions. However, individual lack of either *lpxL* or *lpxM* genes did not significantly induce either Cpx or RpoE pathways.

These results were further corroborated by Western blot analysis of two downstream indicator proteins (HtrA and RseB) of these two pathways of signal transduction. Transcription of the *htrA* gene is induced upon induction of both RpoE pathways as well as the CpxR/A two-component system (26). RseB serves as an indicator for the RpoE pathway alone, because its encoding gene is cotranscribed as the *rpoE-rseArseBrseC* operon, which is positively autoregulated by RpoE (38, 39). Even under permissive growth conditions of 23 °C in M9 medium, Δ (*waaC lpxL*), Δ (*waaC lpxM*), and Δ (*waaC lpxL lpxM lpxP*) showed a significant increase in the levels of HtrA and RseB proteins (Fig. 10, C and D), further strengthening our conclusions based on transcriptional assays with reporter promoter fusions. The higher levels of HtrA, as compared with that of RseB, are consistent with the synergistic control of *htrA* transcription by σ^E and CpxR/A pathways, leading to its additive induction. Increase in RseB only reflects activation of the RpoE operon. It should be noted that levels of RpoE operon are tightly regulated not only positively at the transcriptional level, but also negatively by RseA and RseB (38, 39).

Induction of Signal Transduction in Δ *waaA*—Because the LPS of the Δ *waaA* mutant comprised either mostly free lipid IV_A precursor or lipid IV_A derivatives with late secondary acyl chains incorporated, depending upon the growth medium and temperature, we analyzed its impact on envelope responsive RpoE and Cpx pathways. The Δ *waaA* suppressor-free deletions were constructed in strains containing σ^E - and CpxR/A-regulated promoter fusions. Under the permissive growth conditions, both in M9 and LB medium, at either 21 or 30 °C, pronounced 3–6-fold induction of the σ^E -dependent *rpoHP3* promoter was observed (Fig. 11A). Of significance is the more enhanced magnitude of RpoE induction (6-fold) in Δ *waaA* grown in LB medium under the conditions when mostly lipid IV_A precursor accumulated without any further acylation by late acyltransferases. However, at lower temperature (21 °C), when not only tetra- but also penta- and hexaacylated lipid IV_A derivatives were observed, induction of the σ^E -dependent promoter *rpoHP3* was somewhat dampened, although still more than 3-fold higher than the wild-type. Given the activity of the *rpoHP3* promoter in the Δ (*lpxL lpxM lpxP*) mutant strain was hardly induced by 30–40% (Fig. 10) as compared with a 3–6-fold induction in Δ *waaA*, further reinforcing the requirement of Kdo and glycosylation of lipid A derivatives, which is Kdo-dependent, for proper outer membrane function. This induction of RpoE in Δ *waaA* mutants was further supported by the increased accumulation of products of RpoE regulon members such as HtrA (Fig. 11D).

Despite the increased complexity of lipid A composition in M9 medium-grown *waaA* mutants, as compared with that grown in LB medium at 21 °C due to the incorporation of C16:0 palmitoyl acyl chain, no further increase in σ^E activation was observed (Fig. 11A). Because no such lipid IV_A modification by palmitoylation was observed in LB medium either at 21 or 30 °C (Fig. 5) indicates that σ^E activity is not induced by PagP-dependent lipid A palmitoylation. Thus, in *waaA* mutants RpoE activation results from accumulation of free lipid IV_A derivatives lacking any glycosylation.

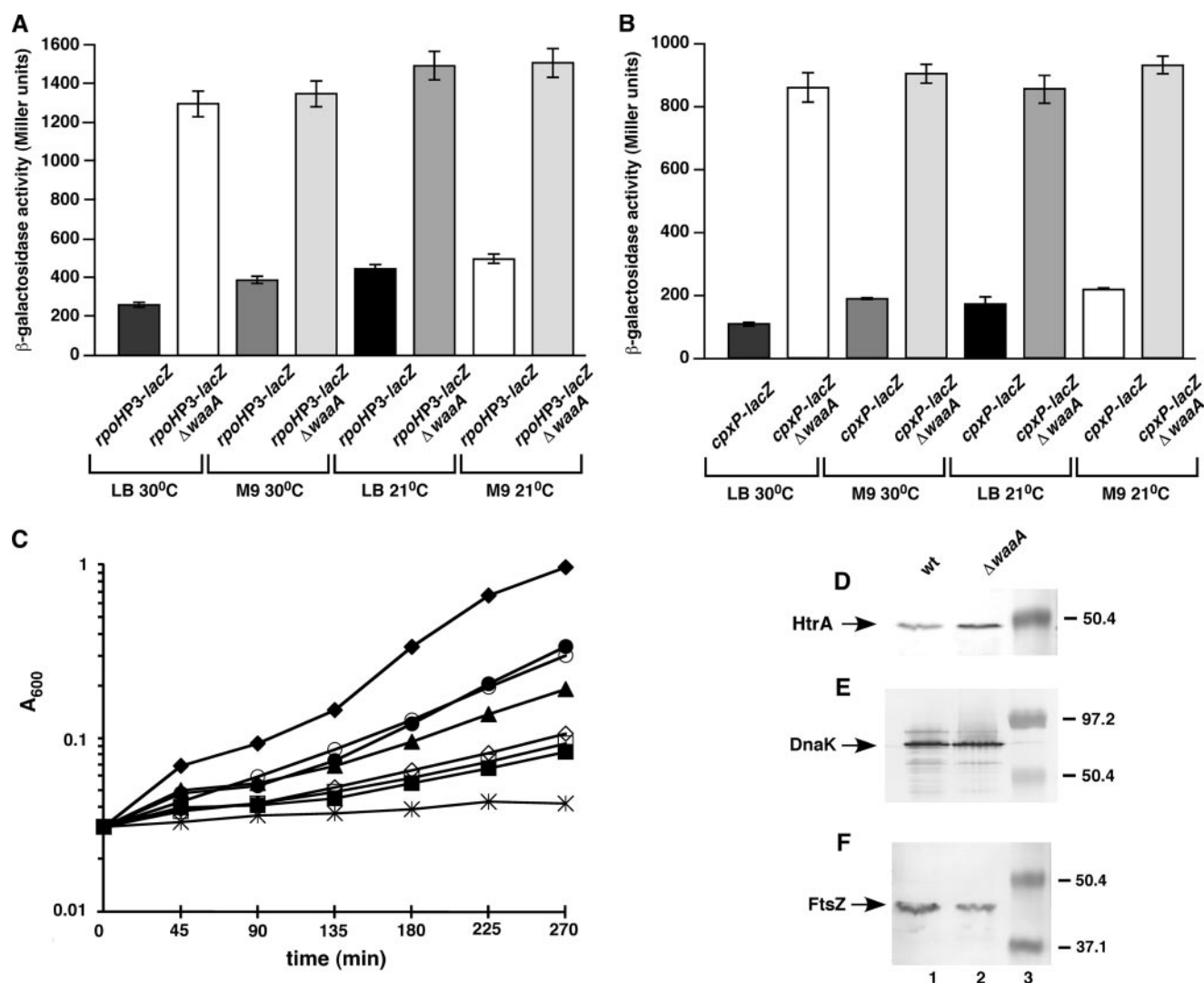


FIGURE 11. Induction of stress response pathways upon deletion of the *waaA* gene. Isogenic bacterial cultures of $\Delta waaA$ and its parental wild-type carrying either single-copy chromosomal *rpoHP3-lacZ* fusion (A) or *cpxP-lacZ* fusion (B) were grown at 21 °C in M9 medium to early log phase. Cultures were washed and diluted to an A_{600} of ~ 0.02 and grown at either 21 or 30 °C in LB and M9 medium and analyzed for β -galactosidase activity as described in the legend to Fig. 10. Average of four independent measurements is shown. Error bars represent S.E. of four independent measurements. C, the same cultures used in A were simultaneously monitored for bacterial growth by measuring optical density at A_{600} . Symbols: ◆, wild-type 30 °C LB; ▲, $\Delta waaA$ 30 °C LB; ●, wild-type 30 °C M9; ■, $\Delta waaA$ 30 °C M9; ○, wild-type 21 °C LB; □, $\Delta waaA$ 21 °C LB; ◇, wt 21 °C M9; ☆, $\Delta waaA$ 21 °C M9 indicate the respective strains and growth conditions. D–F, cultures of wild-type and $\Delta waaA$ were grown at 21 °C in M9 medium to logarithmic phase, harvested by centrifugation, and equivalent amounts of cells were used to prepare whole cell lysates. Proteins were resolved by 12% SDS-PAGE and transferred to nitrocellulose membranes. Blots were probed with antiserum raised against HtrA (D), DnaK (E), and FtsZ (F). The genotype of each strain used is indicated on the top of panel D. The position of molecular weight standards from pre-stained molecular markers (Bio-Rad) is indicated in D–F (lane 3).

The response of CpxR/A-dependent *cpxP* promoter was even more pronounced in $\Delta waaA$ mutants. The *cpxP* promoter was activated in LB medium, ~ 8 -fold at 30 °C and around 5-fold at 21 °C. In M9 medium $\Delta waaA$ also exhibited ~ 4.5 –5-fold induction of the *cpxP* promoter at either 21 or 30 °C (Fig. 11B). This massive induction of the *cpxP* promoter in $\Delta waaA$ mutants under permissive growth conditions is unprecedented in *E. coli* upon a single gene deletion. As a control, the amounts of the RpoH-regulated heat shock protein DnaK were not significantly altered (Fig. 11E), indicating that the cytoplasmic misfolding responsive RpoH pathway is not significantly affected in $\Delta waaA$. Overall these results show that $\Delta waaA$ mutation, resulting in the synthesis of free lipid IV_A precursor and its derivatives without any glycosylation, alters the primarily envelope responsive stress pathways and not the cytoplasmic

protein misfolding pathway represented here by the lack of induction of the RpoH-dependent DnaK heat shock protein.

Requirement of SurA for the Viability of *waaC* Mutants—Deep-rough mutants are known to have impaired OM, including reduced amounts of OMP content (11, 13). To identify the limiting factors that confer this phenotype, we constructed a series of mutational combinations with null mutations in genes whose products are involved in maintaining the correct OMP content. These include mutations in the genes encoding periplasmic folding factors with a presumed role in OMP maturation (*skp*, *surA*, *ppiD*, and *fkpA*), one of the key components of the OMP biogenesis complex (*yfgL*), periplasmic protease (*htrA*), and OMP synthesis regulators like *hfq* and small non-coding RNA gene *rybB*. Among these, the combination Δ (*surA waaC*) turned out to be a uniquely lethal combination both at

Tetraacylated LPS of *E. coli* K-12

30 and 37 °C in medium supporting slow (minimal medium) or rapid growth (LB medium). A double null chromosomal $\Delta(\textit{surA waaC})$ mutant could be constructed only if the *surA* gene was present in *trans* on a plasmid. To authenticate these results, two different null alleles of *waaC* and *surA* each were tested in reciprocal transductions (Table 3). These results provide an explanation for our previous isolation of the *surA* gene as a multicopy suppressor in restoring the proper amounts of OMP in a deep-rough *rfaD* mutant (13). In contrast, a *surA* null mutation could be transduced at a normal frequency in $\Delta(\textit{lpxL lpxM lpxP})$ (Table 3), arguing a more critical requirement of SurA in *waaC* mutants rather than in strains synthesizing tetraacylated LPS. Interestingly, $\Delta(\textit{htrA waaC})$ and $\Delta(\textit{yfgL waaC})$ could be constructed at both temperatures, although the colony size at 37 °C was significantly reduced. Deletion of *yfgL* results in compromised OMP, but does not seem to be as crucial as the SurA requirement under the conditions that effect LPS biogenesis. These results indicate a primary role for SurA in maturation of OMP and being a limiting factor in deep-rough $\Delta\textit{waaC}$.

Because the transcription of *surA*, *htrA*, and *skp* genes, encoding various envelope-folding factors, is positively regulated by σ^E and/or CpxR/A systems, the amounts of their products also increased in $\Delta\textit{waaC}$ and its combinations with $\Delta\textit{lpxL}$ (Fig. 10). To gain further insights in limiting factors, we looked at the amounts of other periplasmic folding factors that might become limiting in $\Delta\textit{waaC}$ and its derivatives. Western blot analyses of total cell extracts from cultures grown under permissive conditions was performed and probed for the amounts of various proteins. This analyses revealed that PpiD levels were reduced in deletion derivatives of the *waaC* gene, particularly in $\Delta(\textit{waaC lpxL lpxM})$ and $\Delta(\textit{waaC lpxL lpxM lpxP})$ (Fig. 10E, lanes 5 and 6). As controls $\Delta\textit{lpxL}$ or $\Delta\textit{lpxM}$ mutants exhibited PpiD levels comparable with the wild-type (Fig. 10E). These results are in agreement with *in vitro* binding of PpiD with peptide libraries derived from OMP.⁵ This reduction in PpiD amounts can partly explain the reduction in OMP amounts in Re mutants coupled with synthetic lethality of *waaC surA*. PpiD has also been recently shown to bind early nascent secretory precursors as they emerge from the Sec translocon (40).

DISCUSSION

Up to now, suppressor-free *E. coli* strains that have minimal LPS structure are either mutants that synthesize Re LPS, *i.e.* Kdo₂ hexaacylated lipid A, or strains with tetraacylated lipid A having a complete LPS core. In this work, we constructed several Re mutants ($\Delta\textit{waaC}$ derivatives) that lack one or more of the late acyltransferases under slow-growth conditions (minimal medium at 23 °C) to avoid accumulation of extragenic suppressors. This led to the construction of viable suppressor-free $\Delta(\textit{waaC lpxL lpxM lpxP})$, which synthesize only Kdo₂-lipid IV_A LPS. To further identify the minimal LPS structure required for viability, we constructed strains deleted for the *waaA* gene, which encodes Kdo transferase, under the same slow-growth conditions. Such suppressor-free $\Delta\textit{waaA}$ deletion strains were viable up to 30 °C and did not require under such growth con-

ditions additional copies of the lipid A transporter MsbA and were shown to have no suppressor mutations. The structure of LPS of such strains was confirmed by mass spectrometric analyses and shown to lack Kdo and at 30 °C primarily synthesized lipid IV_A. Thus, acyl-oxyacyl residues within the lipid A, Kdo attachment to lipid A, as well as further core sugar substituents that are on Kdo, are not absolutely essential for growth under slow-growth conditions of minimal medium at low temperatures. This would be up to now the minimal LPS structure that does not require either extra copies of the *msbA* gene or any suppressors for sustaining *E. coli* viability. However, $\Delta(\textit{waaC lpxL lpxM lpxP})$ and $\Delta\textit{waaA}$ exhibited a very narrow growth range, with defects in cell division. Such suppressor-free strains could only be constructed in slow-growth conditions of minimal medium at low temperatures. $\Delta(\textit{waaC lpxL lpxM lpxP})$, even at the low temperature of 23 °C, did not grow at wild-type rates unless extra copies of the *msbA* gene were provided.

$\Delta\textit{waaA}$ mutants were predicted to synthesize only the free lipid IV_A precursor species, without the incorporation of late secondary acyl chains. Indeed at 30 °C, $\Delta\textit{waaA}$ accumulated only lipid IV_A precursors without any noticeable amounts of late secondary acyl chains. However, mass spectrometric analyses of LPS from $\Delta\textit{waaA}$ grown at 21 °C revealed incorporation of σ^E -dependent cold shock-inducible (12 °C), acyl carrier protein-dependent palmitoleate (C16:1) in the tetraacylated lipid IV_A precursor. This modified lipid IV_A derivative with palmitoleate served *in vivo* as a precursor to generate hexaacylated species with lipid IV_A + palmitoleate and myristate derivatives. Presence and incorporation of the unsaturated C16:1 palmitoleate acyl chain indicates that Kdo attachment to lipid IV_A may not be absolutely required for its incorporation. However, such a Kdo-independent incorporation of the C16:1 acyl chain seems to be restricted to conditions that lead to its substantial induction, due to transcriptional activation of σ^E regulation including the *lpxP* gene in $\Delta\textit{waaA}$ and LpxP activity at temperatures below 30 °C. These results support the concept of unsaturated fatty acid incorporation in homeoviscous adaptation (41). Because such a pentaacylated derivative of free lipid IV_A precursor with palmitoleate in place of laurate also seemed to be a *in vivo* substrate for addition of myristate, argues that this late acylation step also does not require the absolute presence of Kdo at low temperatures. Furthermore, mass peaks corresponding to laurate C12:0 acyl chain incorporation were also observed in the free lipid IV_A precursor at 21 °C, particularly in slow-growth conditions of minimal medium, but not at 30 °C. These results indicate that under slow-growth conditions at temperatures around 21 °C, late acylation can occur without attachment of Kdo to the lipid IV_A precursor. Because we observed the lipid IV_A precursor to be modified by *P-EtN* when LPS-modifying growth conditions of 121 medium were used, implies that lipid IV_A can be translocated at basal levels of MsbA under slow-growth conditions to support OM biogenesis without accumulation of any suppressors. Thus, our suppressor-free *waaA* mutants should be ideal for further detailed studies on the *in vivo* and *in vitro* lipid A trafficking and kinetics of late acyltransferase-dependent incorporation of secondary acyl chains.

⁵ M. Liebscher, C. Schiene-Fischer, G. Fischer, and S. Raina, unpublished results.

Growth of suppressor-free $\Delta waaA$ in minimal medium at low temperature also caused additional lipid IV_A modification. This is reflected by acylation with the secondary palmitate chain, which occurs at position 2 on the proximal glucosamine. This lipid A modification occurs in the outer membrane by the PagP enzyme, which uses glycerophospholipids as its acyl donor (42). Because the PagP enzyme in *E. coli* is usually latent and is mostly activated upon breach of the OM permeability barrier indicates the increased presence of phospholipid patches in the OM of $\Delta waaA$ when grown at low temperatures in minimal medium. However, $\Delta waaA$ revealed palmitoyl modification only in minimal medium at low temperatures, but not in LB medium either at 21 or 30 °C indicating influence of growth conditions.

LPS of $\Delta(waaC lpxM)$ was found to be substituted by both *P-EtN* and L-Ara4N in 121 medium. However, under the same conditions, LPS of $\Delta(waaC lpxL)$, $\Delta(waaC lpxL lpxM)$, $\Delta(waaC lpxL lpxM lpxP)$, $\Delta(lpxL lpxM lpxP)$, and free lipid IV_A derivatives from $\Delta waaA$ did not show L-Ara4N addition, although *P-EtN* substitution occurred. This lack of L-Ara4N substitution in tetraacylated lipid A or lipid IV_A precursors reflects the main growth defect due to their presumed retarded translocation to the outer membrane, because L-Ara4N addition occurs only in the periplasm, supports our results, and is consistent with the requirement of MsbA for translocation. These results provide evidence that our Kdo₂-lipid IV_A and *waaA* mutants do not contain any extragenic suppressors, which allowed rapid transport of the lipid A. However, L-Ara4N could be incorporated in the tetraacylated lipid A of $\Delta(lpxL lpxM lpxP)$ mutants when extra copies of the *msbA* gene were provided in *trans*. Taken together our results suggest that the addition of L-Ara4N define the more stringent requirement for rapid translocation of lipid IV_A to the periplasm than *P-EtN*, because both modifications are presumed to occur in the periplasm.

Consistent with a requirement for lipid IV_A translocation to be necessary for viability of *E. coli*, we showed that near wild-type growth could be restored when *msbA* is overexpressed in $\Delta(lpxL lpxM lpxP)$ mutants. Furthermore, even the growth of $\Delta(waaC lpxL lpxM lpxP)$ mutants could be partially restored in fast-growing conditions, when expression of the *msbA* gene was enhanced from an exogenous-controlled promoter. Interestingly, we could isolate plasmid-born suppressor mutations in the *msbA* gene, which showed a better suppression of $\Delta(waaC lpxL lpxM lpxP)$ mutants. Three such alterations, Asp-498 to Val-498, Ala-510 to Pro-510, and Leu-279 to Val-279 changes were characterized. Among these, Val-498 mutation in a single chromosomal copy was found to effectively restore growth of $\Delta(waaC lpxL lpxM lpxP)$ mutants even at 37 °C in both minimal and rich medium. The Asp-498 residue is part of the linker region to Walker box II and is highly conserved in a majority of MsbA homologues. The other two residues are also relatively well conserved. Leu-279 is located in EL loop 3, which connects TM5 and TM6 and is in close vicinity of residue Ala-270. Mutation of residue Ala-270 to Thr-270 in MsbA has been shown to cause a block in the lipid A transport (43). Ala-510 is located within the Walker box II (44). Further *in vitro* work will be required to understand their exact mechanism of suppression and properties of such MsbA variants.

Analyses of LPS of $\Delta waaC$, $\Delta(waaC eptB)$, $\Delta(waaC eptA)$, and $\Delta(waaC basR eptB)$ mutants revealed that *P-EtN* substituents can occur both on lipid A or Kdo in $\Delta waaC$ from the structural point of view. However, $\Delta waaC$ have elevated RpoE activity, hence the synthesis of EptB is favored, thus explaining the preferred Kdo-*P-EtN* in $\Delta waaC$. Curiously, lipid A of $\Delta(waaC basR eptB)$ mutants revealed the presence of L-Ara4N from LPS extracted from bacteria grown in 121 medium. Thus, in *E. coli* L-Ara4N modification of lipid A can occur in part without induction of the BasS/R two-component system and may involve an additional mechanism of regulation.

Interestingly, $\Delta waaA$ mutants synthesizing free lipid IV_A precursor and its unglycosylated derivatives and $\Delta waaC$ derivatives with Kdo₂-lipid IV_A or Kdo₂-lipid A_{penta} LPS, exhibited a dramatic basal level induction of envelope responsive signal transduction pathways, even under permissive growth conditions. The most striking was the induction of the CpxR/A two-component system, because its activation had not been linked to either truncations in LPS or underacylation of LPS. Absence of either LpxL or LpxM, or WaaC alone did not induce significantly the CpxR/A two-component system. However, either combination of $\Delta(waaC lpxL)$ or $\Delta(waaC lpxM)$ caused strong induction of this pathway of signal transduction. Among these, the most significant induction was observed in $\Delta(waaC lpxL)$, consistent with their more severe growth defects. Furthermore, $\Delta waaC$ mutant alone also exhibited constitutively elevated levels of the RpoE pathway. However, a further additive effect in $\Delta(waaC lpxL)$ combinations, but not in $\Delta(waaC lpxM)$, was observed. This induction mirrors the growth defects of these corresponding mutants, known ability of pentaacylated lipid A translocation, and underlies the severe envelope defects in Kdo₂-lipid IV_A mutants. Consistent with the principal mechanism of OMP sensing by RpoE (27) and lack of any known major OMP content/folding defects, individual *lpxL* or *lpxM* mutants did not show elevated RpoE activity. However, strains lacking all the late acyltransferases, $\Delta(lpxL lpxM lpxP)$ mutants, revealed a mild 30 to 40% increase in RpoE activity, however, to a much lower extent than the *waaC* mutant, which have known major defects in OMP maturation.

$\Delta waaA$ mutants grown at 30 °C in LB medium exhibited up to 8-fold strong induction of both RpoE and CpxR/A pathways, conditions under which only free lipid IV_A precursor without any penta- and hexaacylated derivatives accumulated. Although at 21 °C, overall RpoE- and CpxR/A-regulated promoter activities increased, but the fold-induction was reduced from 8- to 5-fold and 6- to 3-fold for *cpxP* and *rpoHP3* promoters, respectively, in comparison to their activity at 30 °C. This suppression can be due to incorporation of palmitoleate secondary acyl chain and further acylation by myristate, generating penta- and hexaacylated lipid IV_A derivatives, which could confer some adaptive advantage. Still the 3- to 5-fold increase in these stress regulons, even at 21 °C growth conditions, is significantly more than that observed in $\Delta(lpxL lpxM lpxP)$ under similar growth conditions, indicating that accumulation of free lipid A derivatives and lack of glycosylation induces this hyperinduction of envelope stress response. Furthermore, conditions leading to PagP-dependent palmitoyl modification of lipid IV_A derivatives did not induce σ^E activity more than that observed, when no such lipid A modifi-

cation was observed in $\Delta waaA$. Overall, these results demonstrate that both RpoE and Cpx signal transduction pathways are highly induced upon accumulation of free lipid IV_A precursors lacking any glycosylation due to *waaA* deletion.

An additional aspect of signal transduction induction observed in this study revealed that $\Delta(lpxL\ lpxM\ lpxP)$, synthesizing tetraacylated lipid A but with the intact *waaC* gene, exclusively synthesized the glycoform with 3 Kdo with a characteristic truncation in the outer core (35, 36). However, synthesis and accumulation of this glycoform was observed only when $\Delta(lpxL\ lpxM\ lpxP)$ were grown in 121 medium, but not in the phosphate-rich M9 medium. This indicates that modifications of tetraacylated lipid A also influence core structure alterations that promote incorporation of a third Kdo and biosynthesis. Furthermore, overexpression of MsbA was found to restore the accumulation of the usual glycoform with 2 Kdo residues as well as incorporation of L-Ara4N.

We also investigated the limiting factors in *waaC* mutants and potential reasons for defects in OMP maturation in deep-rough mutants. Construction of a series of *waaC* null combinations with null mutations in known periplasmic folding factors or those that regulate them, revealed that the *waaC surA* null combination is synthetically lethal, whereas $\Delta(waaC\ htrA)$, $\Delta(waaC\ skp)$, $\Delta(waaC\ ppiD)$, and $\Delta(waaC\ yfgL)$ were viable. These results support our earlier isolation of the *surA* gene as a multicopy suppressor of the *rfaD* mutant, which like *waaC* mutants synthesize Re LPS (13). Thus, one of the factors that is limiting and causing OMP defects could be due to the requirement of higher amounts of SurA, presumably to accelerate folding of porin monomers. Another reason for *waaC surA* synthetic lethality could result from constitutively alleviated levels of RpoE and the consequent increase of HtrA amounts in *waaC* mutants. Increased HtrA protein levels could cause higher turnover of intermediates of OMP folding, causing a decrease in overall amounts of OMP. Our results also suggest that known decreased OMP amounts could partly be due to a decrease in the levels of PpiD, a periplasmic OMP folding factor. Taken together, these results suggest both, SurA, PpiD for accelerating OMP folding/maturation, and MsbA for LPS export, are limiting in bacteria that synthesize Re LPS with tetraacylated lipid A. Such mutants should allow further insight into the mechanisms and factors involved in lipid A export and OMP maturation. *waaA* and *waaC* mutants could be further exploited to search for additional components that are required for OMP maturation. $\Delta waaA$ and $\Delta(waaC\ lpxL\ lpxM\ lpxP)$ mutants should also be of potential use in identifying novel antibiotics. Given our results, Kdo-independent late acylation of the lipid IV_A precursor in $\Delta waaA$ mutants will be useful for further investigation of *in vivo* and *in vitro* studies to examine specificity of these acyltransferases. It will be of interest to obtain extragenic suppressors that restore wild-type growth and in converse to identify lethal combinations with other mutation(s) in $\Delta waaA$ background.

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