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Molecular Basis of Lipopolysaccharide Heterogeneity in *Escherichia coli*

ENVELOPE STRESS-RESPONSIVE REGULATORS CONTROL THE INCORPORATION OF GLYCOFORMS WITH A THIRD 3-DEOXY-α-D-MANNO-OCT-2-ULOSONIC ACID AND RHAMNOSE[S]

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Background: LPS is essential for viability, although it is highly heterogeneous.
Results: Synthesis of different glycoforms is regulated by differential expression of WaaZ (KdoIII transferase) and WaaR (glycosyltransferase). RpoE-transcribed *rybB* sRNA represses WaaR synthesis, and ppGpp is required for KdoIII incorporation in RpoE-inducing conditions.
Conclusion: RpoE induction causes truncation of outer core and rhamnose addition to KdoIII.
Significance: LPS alterations are crucial for outer membrane function.

Mass spectrometric analyses of lipopolysaccharide (LPS) from isogenic *Escherichia coli* strains with nonpolar mutations in the *waa* locus or overexpression of their cognate genes revealed that *waaZ* and *waaS* are the structural genes required for the incorporation of the third 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo) linked to Kdo disaccharide and rhamnose, respectively. The incorporation of rhamnose requires prior sequential incorporation of the Kdo trisaccharide. The minimal *in vivo* lipid A-anchored core structure $Kdo_2Hep_2Hex_2P_1$ in the LPS from Δ*waaO* (lacking α-1,3-glucosyltransferase) could incorporate Kdo_3Rha, without the overexpression of the *waaZ* and *waaS* genes. Examination of LPS heterogeneity revealed overlapping control by RpoE σ factor, two-component systems (BasS/R and PhoB/R), and ppGpp. Deletion of RpoE-specific anti-σ factor *rseA* led to near-exclusive incorporation of glycoforms with the third Kdo linked to Kdo disaccharide. This was accompanied by concomitant incorporation of rhamnose, linked to either the terminal third Kdo or to the second Kdo, depending upon the presence or absence of phosphoethanolamine on the second Kdo with truncation of the outer core. This truncation in Δ*rseA* was ascribed to decreased levels of WaaR glycosyltransferase, which was restored to wild-type levels, including overall LPS composition, upon the introduction of *rybB* sRNA deletion. Thus, Δ*waaR* contained LPS primarily with Kdo_3 without any requirement for lipid A modifications. Accumulation of a glycoform with Kdo_3 and 4-amino-4-deoxy-L-arabinose in lipid A in Δ*rseA* required ppGpp, being abolished in a Δ(ppGpp^0 *rseA*). Furthermore, Δ(*waaZ lpxLMP*) synthesizing tetraacylated lipid A exhibited synthetic lethality at 21–23°C pointing to the significance of the incorporation of the third Kdo.

Lipopolysaccharides (LPS) are the major amphiphilic constituents of the outer leaflet of the outer membrane (OM)[2] of Gram-negative bacteria, including *Escherichia coli*. Although LPS are highly heterogeneous in composition, they share a common architecture composed of a membrane-anchored phosphorylated and acylated β(1→6)-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. The core oligosaccharide can be further divided into the inner and outer core. The lipid A part and the inner core are generally conserved in structure but often have nonstoichiometric substitutions. The 3-deoxy-α-D-*manno*-oct-2-ulosonic acid (Kdo)- lipid A portion of LPS defines the minimal structure required to support growth of *E. coli* up to 42 °C and is the most conserved part in the LPS of Gram-negative bacteria (1–3). After the synthesis of lipid IV_A precursor, the transfer of Kdo to it is a critical and essential step in the LPS biosynthesis. This Kdo incorporation ensures the incorporation of secondary laurate and myristate chains, resulting in the synthesis of hexaacylated lipid A and further extension by sequential addition of heptoses and hexoses (1–3). Thus, the gene encoding Kdo transferase can be deleted only under slow growth conditions of minimal medium at/or below 21 °C without requirement of any suppressor mutations (4). Under such slow growth conditions at low temperatures, such mutants exhibit a limited incorporation of laurate and myristate chains

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[2] The abbreviations used are: OM, outer membrane; Kdo, 3-deoxy-α-D-*manno*-oct-2-ulosonic acid; OMP, outer membrane proteins; P-EtN, phosphoethanolamine, L-Ara4N, 4-amino-4-deoxy-L-arabinose; ESI FT, electrospray ionization Fourier transform-ion cyclotron; ppGpp, guanosine 3′,5′-bispyrophosphate.

(4). Thus, overexpression of either the lauroyl- or the myristosyltransferase in a *waaA* deletion could restore growth at 30 or 37 °C but not above (5).

The biosynthesis of *E. coli* K-12 LPS is relatively well studied, and the main genetic and structural determinants are known (1, 2). However, the overall composition of LPS is quite heterogeneous due to several nonstoichiometric substitutions (Fig. 1). Among the nonstoichiometric substitutions commonly observed in lipid A part are the addition of phosphoethanolamine (P-EtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) (3). Such substitutions are not needed for growth under laboratory conditions but can have adaptive advantage in specific niches, because they confer resistance to cationic peptides like polymyxin B (3). The inner core structure can also be substituted nonstoichiometrically by residues, including phosphate, rhamnose (Rha), P-EtN, and additional Kdo (3). Two independent studies addressed structural aspects of substitution of additional Kdo linked to Kdo disaccharide (6, 7). In one study, overexpression of the *E. coli* K-12 *waaZ* gene in an *E. coli* isolate with R1 core, which lacks the *waaZ* gene, was found to lead to the synthesis of increased amounts of Kdo(2→4)Kdo(2→4)Kdo trisaccharide in the inner core, which was accompanied by a truncation of the outer core (7). However, given the heterogeneity and overall complex composition of *E. coli* K-12 LPS, no obvious differences could be observed between a *waaZ* null as compared with the wild type, and only upon overexpression could differences be observed. In another study, using LPS from an *E. coli* K-12 strain, four different LPS glycoforms were purified, and one of the minor forms was found to contain an Kdo(2→4)[α-L-Rha(1→5)]Kdo(2→4)Kdo-branched tetrasaccharide connected to lipid A, and it is designated as glycoform IV (6). This glycoform was found to have a truncation of the outer core, in which the terminal disaccharide L-α-D-Hep(1→6)α-D-Glc was missing. Both of these studies suggest that incorporation of additional (2→4)α-Kdo on α-Kdo(2→4)α-Kdo disaccharide causes truncation of the outer core, but its molecular basis remained unknown. Furthermore, the structural gene required for the addition of Rha to Kdo remained to be identified, and the functional and regulatory mechanisms that contribute to the presence of several glycoforms remained unaddressed.

We earlier showed that LPS of strains with tetraacylated lipid A due to lack of LpxL, LpxM, and LpxP late acyltransferases exhibited increased accumulation of glycoform IV with predicted presence of Rha, and P-EtN on the second Kdo in phosphate-limiting growth conditions (4). It is now established that the main function of the RpoE regulon is to ensure a correct assembly of outer membrane proteins (OMP) as well as to regulate functions involved in LPS translocation to the OM (8, 9). Furthermore, RpoE not only responds to OMP misfolding, but also to the synthesis of defective LPS composed of either Kdo_2-lipid IV_A or of lipid IV_A derivatives devoid of any glycosylation (4). However, whether LPS heterogeneity, including synthesis of LPS glycoform IV, is also regulated by this stress-responsive signal transduction is not known, because a Δ(*lpxL lpxM lpxP*) strain also exhibits mild RpoE induction (4). Furthermore, it is not clear to what extent two-component systems other than known BasS/R-mediated lipid A modifications contribute to LPS heterogeneity. The importance of LPS core composition is further manifested in the discovery showing that out of the 51 genes, which are essential for bacterial growth at critical high temperature, products of 8 such genes are involved in LPS core assembly (10). Thus, in this study, we first identified structural genes that are required for the synthesis of glycoform with the third Kdo and Rha and their functional significance, using a panel of isogenic mutants with nonpolar mutation in various genes encoded in the *waa* locus. Next, we addressed the role of various regulators involved in sensing outer membrane alterations. This included analyses of LPS from strains with mutations in genes whose products are involved in lipid A modifications and the main regulatory control elements of the extracytoplasmic stress pathway under the control of RpoE/RseA signal transduction. Furthermore, we addressed if any molecular switches control the relative abundance of different glycoforms and their impact on the OMP profile.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media—Bacterial strains and plasmids used in this study are described in Table 1. M9 phosphate-rich medium contained 42 mM Na_2HPO_4, 24 mM KH_2PO_4, 9 mM NaCl, 19 mM NH_4Cl_2, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.01 mM $FeCl_3$, 0.2% glucose, 0.05% casamino acids, and 0.5 μg/ml thiamine. Phosphate-limiting 121 medium (11) was composed of 126 mM Tris-HCl, 18 mM Tris-base, 1 mM $MgCl_2 \cdot 6H_2O$, 2.5 mM Na_2SO_4, 80 mM NaCl, 20 mM KCl, 20 mM NH_4Cl_2, 2 mM $CaCl_2$, 0.02 mM $FeCl_3$, 0.02 mM $ZnCl_2$, 0.2% glucose, 0.1% casamino acids, and 0.25 μg/ml thiamine. When necessary, media were supplemented with ampicillin (100 μg ml^{-1}), tetracycline (10 μg ml^{-1}), kanamycin (50 μg ml^{-1}), spectinomycin (50 μg ml^{-1}), or chloramphenicol (20 μg ml^{-1}).

Generation of Null Mutations and Construction of Their Combinations—Nonpolar antibiotic-free deletion mutations of various genes were generated using the λ Red recombinase/FLP-mediated recombination system (12). 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 (12), and recombined on the chromosome of BW25113 containing the λ Red recombinase-encoding plasmid pKD46. Gene replacements and their exact chromosomal locations were verified by PCR and further transduced in W3110. All the deletions were verified to be nonpolar. Construction of deletion derivatives of the *rseA*, *rseB*, *rpoE*, *rybB*, *basS*, *eptB*, and *eptA* genes and strains lacking all the late acyltransferases in W3110 were described previously (4). To construct Δ(*lpxL lpxM lpxP waaZ*), Δ*waaZ* deletion was introduced into Δ(*lpxL lpxM lpxP*) strain on M9 medium at 21 or 30 °C in the presence or absence of the *lpxL*-bearing plasmid. Because a null mutation in the *waaU* gene has not been described in *E. coli* K-12, a nonpolar deletion derivative was constructed using the λ Red recombinase/FLP-mediated recombination system in BW25113 and transduced in W3110 at 30 and 37 °C, both on LB and M9 minimal medium. To construct *relA spoT* derivatives, relevant mutations were transduced into W3110 and into isogenic Δ*rseA* on LA-rich medium. The presence of the suppres-

TABLE 1
Bacterial strains and plasmids used in this study

Strains/plasmids	Relevant characteristic	Ref. or source
Strains		
W3110	λ^-, *IN*(*rrnD-rrnE*)*1*, *rph-1*	*E. coli* Genetic Stock Center, Yale
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	12
BL21(DE3)	F$^-$ *ompT hsdSB*(*rB*$^-$ *mB*$^-$) *gal dcm* (DE3)	Invitrogen
GK1111	W3110 Δ*lac*	4
SR8129	GK1111 ϕ (*rpoH*P3-*lacZ*)	4
GK1162	W3110 *eptB*<>*aph*	4
GK1181	W3110 *eptB*<>*frt*	This study
GK1395	W3110 *eptA*<>*aph*	4
GK1806	W3110 *eptA*<>*frt*	This study
GK1400	W3110 *basR*<>*aph*	4
SR8044	W3110 *basR*<> *frt*	This study
SR9008	GK1111 ϕ (*waaZ-lacZ*)	This study
GK1913	W3110 *waaZ*<>*aph*	This study
GK3037	W3110 *waaZ*<> *frt*	This study
SR8236	W3110 *waaZ*<>*cat*	This study
GK2031	GK1913 (p*waaZ*$^+$ cm^R)	This study
GK2004	W3110 *waaS*<>*aph*	This study
SR7936	W3110 *waaO*<>*aph*	This study
GK2201	SR7936 (p*waaZ*$^+$ cm^R)	This study
SR9037	W3110 *waaB waaO*<>*frt*	This study
SR9043	SR9037 (p*waaZ*$^+$ cm^R)	This study
GK1917	W3110 *waaB*<>*aph*	This study
SR6584	W3110 *waaR*<>*aph*	This study
KK371	Δ*relA* Δ*spoT*	40
SR7890	KK371 *rseA*<>*aph*	This study
SR8289	W3110 *rseA*<>*aph*	This study
SR8286	W3110 *rseB*<>*aph*	This study
SR8686	W3110 *ydcQ*<>*aph rpoE*<>*cat*	This study
GK2576	W3110 *mgr*<>*ada*	This study
SR9071	W3110 *waaU*<>*aph*	This study
SR9176	W3110 *waaR*::3×FLAG<>*aph*	This study
SR9184	W3110 *waaR*::3×FLAG<>*aph rseA*::Tn*10* tet	This study
GK2388	W3110 *waaR*::3×FLAG<>*frt rybB*<>*aph*	This study
GK2390	W3110 *waaR*::3×FLAG<>*frt rseA*::Tn*10* tet *rybB*<>*aph*	This study
SR9060	W3110 *waaZ*::3×FLAG<>*aph*	This study
GK2295	W3110 *waaZ*::3×FLAG<>*aph rseA*::Tn*10* tet	This study
GK2037	W3110 *phoB*<>*aph*	This study
GK2282	W3110 *basR*<>*frt phoB*<>*aph*	This study
SR9188	GK2282 *rseA*::Tn*10* tet	This study
SR6582	W3110 *waaL*<>*aph*	This study
SR8522	W3110 *micA*<>*cat*	This study
SR8265	W3110 *rybB*<>*aph*	This study
GK2626	BW25113 *lpxM*::3×FLAG<>*aph*	This study
GK2660	BW25113 *lpxM*::3×FLAG<>*frt waaO*<>*aph*	This study
GK2664	BW25113 *lpxM*::3×FLAG<>*frt waaB waaO*<>*aph*	This study
GK2551	W3110 *hfq*<>*ada*	This study
GK2268	W3110 *rybB*<>*aph rseA*::Tn*10* tet	This study
SR8739	W3110 *rseA*::Tn*10* tet *micA*<>*cat*	This study
SR8965	W3110 *rseA*::Tn*10* tet *waaZ*<>*cat*	This study
SR8505	W3110 *eptA*<>*frt rseA*<>*aph*	This study
GK2468	BL21(DE3) (p*waaZ*$^+$)	This study
GK2472	BL21(DE3) (p*waaS*$^+$)	This study
GK2476	BL21(DE3) (p*waaZ*$^+$ *waaS*$^+$)	This study
SR8129	GK1111 ϕ *rpoH*P3-*lacZ*	(4)
GK2590	SR8129 *waaY*<>*aph*	This study
GK2567	SR8129 *waaR*<>*aph*	This study
SR7781	W3110 *lpxM*<>*frt lpxP*<>*frt lpxL*<>*aph*	4
SR9020	SR7781 *waaZ*<>*cat*	This study
SR7465	W3110 *lpxP*<>*frt lpxM*<>*frt*	4
SR8985	SR7465 *waaZ*<>*aph*	This study
Plasmids		
pCP20	Contains temperature-sensitive replicon and Thermally inducible FLP recombinase	*E. coli* Genetic Stock Center, Yale
pKD3	*oriR6K*$_\gamma$, *bla*(Amp^R), *kan*, *rgnB*(Ter), *cat*	*E. coli* Genetic Stock Center, Yale (12)
pKD13	*oriR6K*$_\gamma$, *bla*(Amp^R), *kan*, *rgnB*(Ter)	*E. coli* Genetic Stock Center, Yale (12)
pKD46	*araBp-gam-bet-exo*, *bla*(Amp^R), *repA101*(ts) *oriR101*	*E. coli* Genetic Stock Center, Yale (12)
pRS551	*lacZYA* transcriptional fusion vector Kan^R	
pSR8969	pRS551 (p*waaZ-lacZ)*	This study
pSR8974	pRS551 (p*waaY-lacZ*)	This study
pSUB11	3×FLAG vector	14
pSR7960	*waaZ* amp^R in pET16b	This study
pGK2055	*waaS* in pET24b	This study
pSR9298	*waaZ waaS* kan^R in pET24b	This study
JW3599	*waaZ* cm^R	13

sor-free *relA spoT* combination was verified by their inability to grow on amino acid-free M9 medium.

For protein induction, the minimal coding sequence of *waaZ* and *waaS* genes was cloned in pET16b and pET24b expression vectors. To co-express *waaZ* and *waaS*, the *waaZ* gene under T7 transcription control was subcloned from pET16b (pSR7960) into pGK2055 (pET24b *waaS*) in the BglII site, resulting in plasmid pSR9298, wherein both the genes are expressed from individual T7 promoter (Table 1). For controlled complementation and mild induction (0.15 mM isopro-

pyl 1-thio-β-D-galactopyranoside), the *waaZ* gene was expressed in pCA24N. In this vector, the minimal coding sequence is under the tight p*tac* promoter (13).

Protein Purification—Expression of hexa-His-tagged WaaZ variants was induced in *E. coli* BL21 strain by the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside at an absorbance of 0.1 at 600 nm in a 1-liter culture. After an induction for 4 h at 37 °C, cells were harvested by centrifugation at 7,000 rpm for 20 min. The pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole (buffer A)) and supplemented with lysozyme to a final concentration of 200 μg ml^{-1}. The mixture was incubated on ice for 20 min, sonicated, and centrifuged at 45,000 × *g* for 30 min at 4 °C. Soluble proteins (15 ml) were applied over nickel-nitrilotriacetic acid beads (Qiagen), washed, and eluted with buffer A containing 100 mM imidazole.

Construction of Chromosomal C-terminal FLAG Derivatives—Using pSUB11 as template (14) and oligonucleotides listed in supplemental Table S1, C-terminal 3×FLAG-tagged *waaZ*, *waaR,* and *lpxM* PCR products with *aph* marker were generated. PCR products were electroporated to generate chromosomal recombinants of BW25113 containing the λ Red recombinase-encoding plasmid pKD46. Correct chromosomal exchanges were verified by PCR and sequencing of PCR products from chromosomal FLAG-tagged derivatives. Whether the expression of the concerned gene was not altered was verified by the presence of the wild-type phenotype. The 3×FLAG derivatives were transduced into W3110, followed by pCP20-mediated excision of *aph* cassette. Whenever required, the appropriate deletion mutations were further introduced into these FLAG epitope derivatives, and expression was revealed by Western blotting using FLAG-specific M2 monoclonal antibodies from Sigma (F3165).

LPS Extraction and Growth Analysis—Cultures of isogenic bacteria were grown in a rotary shaker at 190 rpm in phosphate-rich M9 medium or in phosphate-limiting medium until an absorbance of 0.8–1.0 at 600 nm with appropriate antibiotic at a permissive temperature depending upon the mutation(s). Four hundred-ml cultures were harvested by centrifugation at 7,000 rpm for 30 min and dried. LPS was extracted by the phenol/chloroform/petroleum ether procedure (15) and lyophilized. For the LPS analysis, lyophilized material was dispersed in water by sonication and resuspended at a concentration of 2 mg ml^{-1}.

Mass Spectrometry—Electrospray ionization Fourier transform ion cyclotron (ESI FT-ICR) mass spectrometry was performed in negative ion mode using an APEX QE (Bruker Daltonics) equipped with a 7-tesla actively shielded magnet and dual ESI-Maldi. LPS samples were dissolved at a concentration of ~10 ng μl^{-1} and analyzed as described previously (4, 16). Mass spectra were charge deconvoluted, and the mass numbers given refer to the monoisotopic peaks. Mass calibration was done externally using well characterized similar compounds of known structure (16).

Western Blot Analysis—To detect and estimate changes in the levels of WaaZ, WaaR, and LpxM, 25-ml cultures of C-terminally 3×FLAG-tagged derivatives were grown with shaking at 37 °C in LB or 121 medium. Absorbances at 595 nm were measured at different intervals, and aliquots were drawn at different stages of bacterial growth as indicated. Samples were harvested by centrifugation at 3,000 × *g* for 10 min. Protein amounts were measured by BCA kit, and 20 μg of each sample was used. Samples were resuspended in SDS lysis buffer and were applied to 12% SDS-PAGE. After the electrophoresis, proteins in the gel were blotted to PVDF membrane. To determine any differences in OMP composition because of the switch from glycoform I to glycoform V LPS, whole cell lysates from cultures of strains with remarkable differences in the accumulation of either of the two glycoform were prepared. Cultures were grown up to identical absorbances, harvested by centrifugation as described above, and lysed in SDS lysis buffer. Equivalent amount of total protein (5 μg) was subjected to 12% SDS-PAGE. The relative amounts of OmpA, OmpC, and OmpF were revealed by Western blotting, using corresponding antibodies as described previously (17–19).

β-Galactosidase Assays—To measure the activity of the *waaZ* promoter, single copy chromosomal promoter fusions to the *lacZ* were constructed. The induction of the RpoE pathway was monitored in strains carrying the *rpoH*P3 promoter, whose construction has been previously described (20). Putative promoter region of the *waaZ* gene was amplified by PCR, using specific oligonucleotides (supplemental Table S1). After PCR amplification, gel-purified DNA was digested with EcoRI and BamHI, cloned in pRS551 vector, and transferred to chromosome in single copy by recombination with λRS45, selecting for Kan-resistant lysogens as described previously for other promoter fusions (8, 20, 21). To measure β-galactosidase activity, isogenic bacterial strains carrying promoter fusions were grown with appropriate antibiotics at 37 °C. Cultures were harvested by centrifugation and diluted to an A_{595} of 0.02. Cultures were allowed to grow for another 90 min at 37 °C, and β-galactosidase activity was measured at different growth intervals. At least four independent cultures were assayed for each mutant and isogenic parent.

RESULTS

Growth of E. coli in Phosphate-limiting Medium Induces Synthesis of Glycoform IV—We previously reported that growth of *E. coli* K-12 in phosphate-limiting 121 medium induces lipid A modifications because of the induction of BasS/R two-component system leading to the substitution of P-EtN and L-Ara4N (4). Growth in this medium also favors addition of P-EtN to the second Kdo (4). In this study, we analyzed in depth the molecular and structural basis of the incorporation of the third Kdo and any other alteration in the LPS inner and outer core. Comparison of LPS of the *E. coli* K-12 wild-type strain W3110 obtained from phosphate-rich (M9) *versus* phosphate-limiting (121) medium revealed several differences. They are manifested by the presence of mass peaks predicted to contain either the usual glycoform I or the relatively rare glycoform IV and non-stoichiometric substitutions by P-EtN and L-Ara4N (Fig. 1). Mass peaks predicted to be derivatives of glycoform IV were preponderant species in LPS samples obtained from growth in phosphate-limiting medium. They are represented by mass peaks at 3948.7, 4079.8, 4202.8, and 4298.9 Da (supplemental Fig. S1). These mass peaks can be explained by the presence of

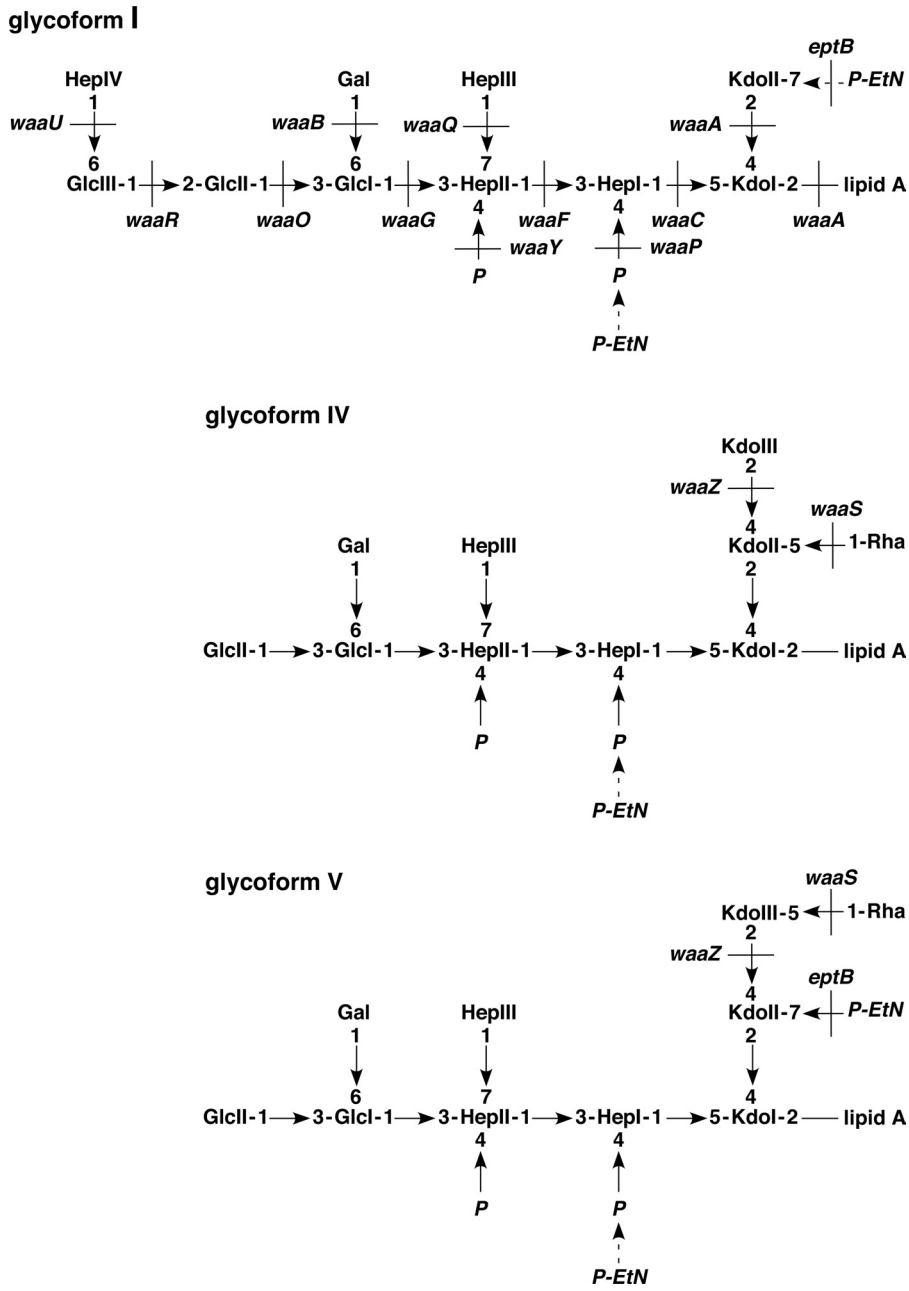


FIGURE 1. **Proposed LPS structures from *E. coli* K-12 in phosphate-limiting growth conditions of 121 minimal medium.** Schematic drawing of LPS glycoform I, IV, and V compositions with various nonstoichiometric substitutions in the LPS core region is presented. Glycoform IV has Rha addition on the second Kdo. Glycoform V differs because Rha is added on the terminal third Kdo with P-EtN on the second Kdo. The cognate genes, whose products are involved at different steps, are indicated.

a third Kdo and Rha linked to the Kdo disaccharide with a concomitant truncation of the outer core with a predicted composition $LA_{hexa}(Kdo_3RhaHep_3Hex_3P_2)$ (supplemental Fig. S1, *A* and *B*) (6, 7). The mass differences among the above described mass peaks can be explained by additional substitution with P-EtN and L-Ara4N. Additional mass peaks at 3936.7, 4059.8, 4190.8, and 4286.9 Da are predicted to be derivatives of glycoform I. These mass peaks can be explained as $LA_{hexa}(Kdo_2Hep_4Hex_4P_2)$ accompanied by additional substitutions with P-EtN and L-Ara4N as indicated. As expected, we also observed a mass peak of 4489.9 Da, which can be explained by the addition of GlcNAc to glycoform I (supplemental Fig. S1*B*). This addition of GlcNAc is derived from the O-antigen biosynthetic pathway (1). In contrast, LPS obtained from M9 medium revealed mass peaks at 3915.7 Da and several of its derivatives, predicted to correspond to typical glycoform I, with additional sodium and phosphate adducts (supplemental Fig. S1*A*). Further mass peak at 3927.7 Da can be explained to be a derivative, corresponding to glycoform IV, with additional Na and P adducts (supplemental Fig. S1*A*). No mass peaks with predicted substitution of P-EtN and L-Ara4N were detected in the LPS from M9 growth conditions.

The predicted modification of the inner core with the presence of the third Kdo and Rha, corresponding to glycoform IV

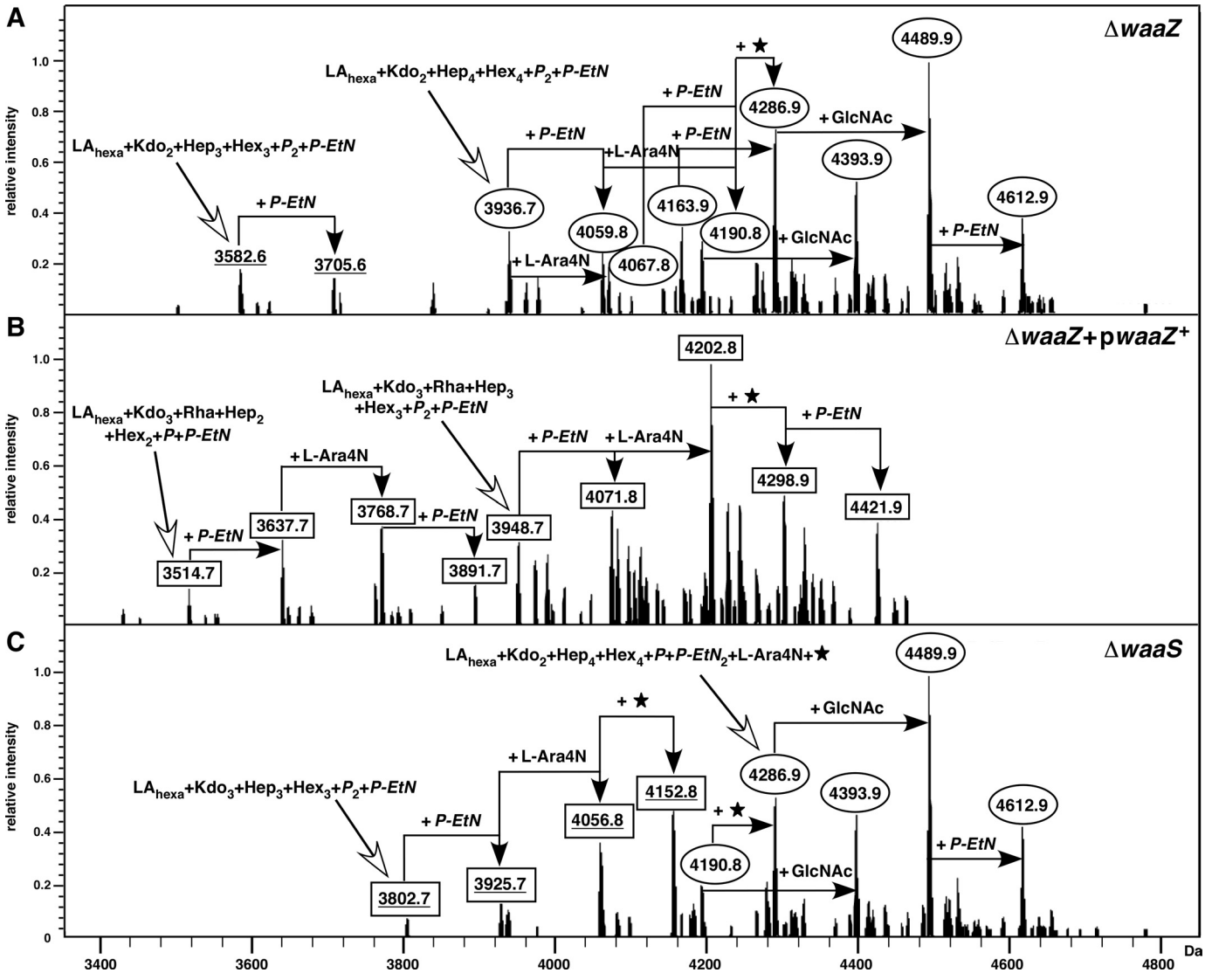


FIGURE 2. **WaaZ and WaaS are required for the incorporation of the third Kdo and Rha, respectively.** Charge deconvoluted ESI FT-MS spectrum in negative ion mode of LPS from isogenic Δ*waaZ* (*A*), Δ*waaZ* + p*waaZ*$^+$ (*B*), and Δ*waaS* (*C*) strains. LPS was extracted from cultures grown at 37 °C in 121 medium growth conditions. The *mass numbers* refer to monoisotopic peaks. The predicted composition with varying number of substitutions of P-EtN and with L-Ara4N substitution is indicated. Mass peaks corresponding to glycoforms IV and V, containing the third Kdo, are shown as *rectangular boxes* and glycoform I with complete core derivatives as *circles*. All mass peaks marked with a *black star* differ by 96 Da. The details of the composition of major mass peaks are described in supplemental Table S2.

derivatives, was further supported by MS/MS analyses of isolated mass peaks corresponding to mass peaks at 3948.7, 4071.8, and 4202.8 Da (see below). Because substitution of P-EtN and L-Ara4N is indicative of correct translocation of LPS and contributes toward structural heterogeneity (3), we analyzed LPS of several isogenic nonpolar mutant derivatives to address the molecular basis of the third Kdo incorporation, using phosphate-limiting growth conditions.

waaZ and waaS Are the Structural Genes for Incorporation of the Third Kdo and Rha, Respectively—Previous studies with overexpression of *E. coli* K-12 *waaZ* gene in *E. coli* strain with R1 LPS core suggested that the *waaZ* gene could be the structural gene encoding KdoIII transferase (7). Given the high complexity of LPS in standard LB medium, the structural details of LPS from a strain with chromosomal deletion of the *waaZ* gene and its individual contribution could not be analyzed in *E. coli* K-12 derivatives (7). However, mass spectrometric analyses of LPS extracted from the wild-type strain grown in 121 medium revealed clear distinction between glycoform I and glycoform IV (supplemental Fig. S1). Thus, LPS from Δ*waaZ* and individual nonpolar deletion derivatives of all the genes in *waa* locus were analyzed. Significantly, LPS obtained from a Δ*waaZ* derivative lacked typical mass peaks corresponding to complete glycoform IV with ions at 3948.7 Da. Such mass peaks at 3948.7 Da representing glycoform IV are present in the LPS obtained under the same growth conditions from the isogenic wild type. However, mass peaks at 3582.6 Da and its derivatives corresponding to LPS with a truncation of the outer core causing loss of terminal heptose and hexose are present. Such mass peaks are explained as LA$_{hexa}$(Kdo$_2$Hep$_3$Hex$_3$$P_2$) derivatives (Fig. 2*A*). These mass peaks are characteristic of glycoform IV, but without the third Kdo and Rha. The presence of mass peaks at 3582.6 Da with the same truncation of the outer core as observed in the wild type with glycoform IV LPS and its deriv-

atives substituted with P-EtN and L-Ara4N suggests that the incorporation of the third Kdo may not be the reason for the truncation under these defined growth conditions. All such mass peaks also lacked Rha, as revealed by the loss of 146.1 Da, indicating that the incorporation of Rha required a prior addition of the third Kdo. The other mass peaks at 3936.7 Da and its derivatives represent typical ions corresponding to glycoform I with complete core and varying numbers of substitutions by P-EtN and L-Ara4N (Fig. 2*A*).

To confirm that the loss of the incorporation of the third Kdo was due to lack of WaaZ function, LPS from Δ*waaZ* transformed with *waaZ* complementing plasmid was analyzed. In this plasmid the *waaZ* gene is expressed under the tight control of p*tac* promoter (13). As shown in Fig. 2*B*, typical mass peaks at 3948.7, 4071.8, and 4202.8 Da correspond to the predicted presence of glycoform IV derivatives with the third Kdo, as seen previously, in the LPS from plasmid-free wild type grown in 121 medium. Interestingly, mild induction of the *waaZ* gene product also revealed mass peak at 3514.7 Da and its derivatives. Such mass peaks correspond to predicted core containing $LA_{hexa}(Kdo_3RhaHep_2Hex_2)$, indicating that *in vivo* even these smaller structures can support the incorporation of the third Kdo. This was further supported by results from Δ*waaO* mutants.

Analyses of LPS from Δ*waaS* mutant revealed the presence of mass peaks at 3802.7 Da and its derivatives with a predicted incorporation of the third Kdo but lacking Rha (Fig. 2*C*). Furthermore, LPS of Δ*waaS* also contained typical mass peaks corresponding to glycoform I derivatives with complete core ($Hep_4Hex_4P_2$) with or without additional predicted GlcNAc. Such mass peaks at 4286.9, 4489.9, and 4612.9 Da corresponding to glycoform I were also present in the isogenic wild type. The loss of Rha in the LPS of Δ*waaS*, as compared with its presence, was also verified by GC/MS analyses (data not shown). Taken together, these results suggest that the incorporation of the third Kdo does not require prior incorporation of Rha, whereas Rha addition does require the addition of the third Kdo to the Kdo disaccharide. Furthermore, because the LPS extracted from Δ*waaS* mutant contained otherwise normal glycoform I, the changes in composition are only specific to the loss of Rha incorporation in the inner core.

Addition of Rha in Inner Core Requires Prior Incorporation of the Third Kdo—Earlier work has shown that the LPS of *E. coli* B strains lack galactose and its core contains fewer heptoses and hexoses than other core types (22). However, the genetic basis of these LPS changes is not known. Furthermore, the confirmed presence of either Rha or the third Kdo is not clear in *E. coli* B. We first sequenced the *waa* region of *E. coli* B derivative BL21. DNA sequence analyses revealed that it has an IS element in the *waaT* gene with an overall chromosomal organization resembling the *E. coli* strains with R1 core. The *waaT* gene in the R1 core region encodes α-1,2-galactosyltransferase adding hexose at the same place where *E. coli* K-12 WaaR acts (23). Furthermore, both *waaZ* and *waaS* genes were found to be absent. Thus, this served as a good tool to further substantiate *in vivo waaZ*- and *waaS*-dependent modification of the inner core. Examination of LPS of three different *E. coli* B derivatives (BL21) revealed similar mass peaks, and the data for one representative are presented (Fig. 3*A*). The mass peak at 3420.6 Da can be explained as $LA_{hexa}(Kdo_2Hep_3Hex_2P_2P\text{-EtN})$. Additional mass peaks at 3516.6, 3551.6, and 3647.7 Da, and their derivatives correspond to further modification by P-EtN, L-Ara4N, and also a derivative with additional 96 Da (Fig. 3*A*).

The lack of the third Kdo and Rha in BL21 LPS, even under growth conditions that favor their incorporation, is consistent with the absence of *waaZ* and *waaS* genes in its genome. Next, a plasmid containing either *E. coli* K-12 *waaZ* alone (pSR7960) or *waaS* (pGK2055) or *waaZwaaS* (pSR9298) with T7 polymerase-based expression system vectors was introduced in the same parental BL21 strain. The overexpression of the *waaS* gene alone revealed mass peaks like that of the parental BL21 strain without any new mass peaks (Fig. 3*B*). However, the induction of the *waaZ* gene expression resulted in new mass peaks, as represented by ions at 3859.7, 3982.7, and 4113.8 Da, which are predicted to arise from the addition of the third Kdo. This is in contrast to the presence of only Kdo_2 LPS forms in the parental strain or in *waaS*-overexpressing strains (Fig. 3*C*). However, co-overexpression of *waaZ* and *waaS* genes from the plasmid containing both genes behind individual T7 promoter revealed mass peaks with predicted incorporation of the third Kdo and also those with further addition of Rha. Thus, the addition of the third Kdo can explain mass peaks at 3859.7 and 3990.8 Da as indicated (Fig. 3*C*). The predicted further addition of Rha can explain the mass peak at 4136.8 Da. However, no mass peaks with the predicted addition of Rha without prior addition of KdoIII could be detected by mass spectrometric analysis. Thus, the incorporation of Kdo_3 + Rha in *E. coli* B upon co-overexpression of both *waaS* and *waaZ* genes complements our results from Δ*waaS* or Δ*waaZ E. coli* K-12 derivatives. Thus, we conclude that *waaS* and *waaZ* genes are the structural genes required for the incorporation of Rha and the third Kdo linked to the Kdo disaccharide. Furthermore, these results also show that the incorporation of Rha requires prior and sequential addition of the third Kdo.

Structural Requirements for Incorporation of the Third Kdo in Vivo—We showed earlier the preference of glycoform IV LPS in strains synthesizing tetraacylated lipid A, however, with intact genes for LPS core biosynthesis (4). Here, we systematically analyzed isogenic in-frame nonpolar deletions in structural genes encoding different glycosyltransferases with respect to the ability to incorporate the third Kdo and Rha to define the minimal core structure required for the addition of the third Kdo. LPS extracted from Δ*waaC*, Δ*waaF*, Δ*waaG*, and Δ*waaP* mutants were found to lack the third Kdo and Rha. Even overexpression of the *waaZ* gene product from a plasmid could not reveal incorporation of the additional third Kdo using immunostaining with Kdo-specific antibodies and chemical and mass spectrometric analyses (data not shown). However, deletion derivatives of *waaL* and *waaU* genes, encoding O-antigen ligase and putative heptosyltransferase IV, respectively, were found to have both glycoform I and IV derivatives, indicating that their products do not effect overall the incorporation of the third Kdo. Concerning the minimal *in vivo* LPS structure that can allow the addition of the third Kdo and Rha, LPS of Δ*waaO* mutant, even without any additional *waaZ* plasmid-borne overexpression, was found to incorporate the third Kdo and

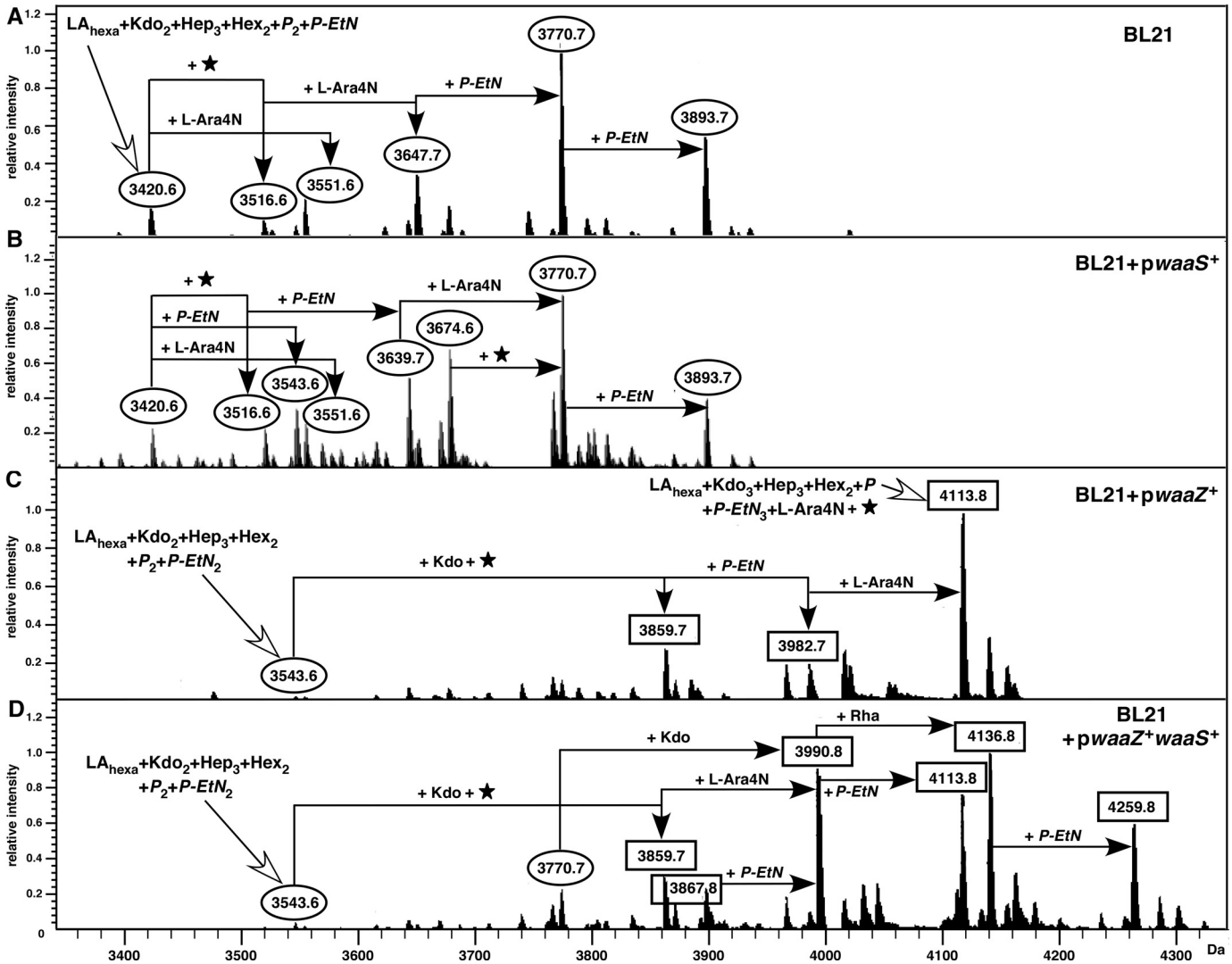


FIGURE 3. **Incorporation of the third Kdo and Rha in *E. coli* BL21 strain upon co-overexpression of *waaZ* and *waaS*.** Mass spectra of LPS isolated from cultures of *E. coli* B derivative BL21 grown in 121 medium (*A*) or BL21 with induction of *E. coli* K-12 *waaS* gene alone (*B*), with induction of *E. coli* K-12 *waaZ* gene alone (*C*), or BL21 with co-overexpression of *waaZ* and *waaS* genes (*D*). Charge deconvoluted ESI FT-MS spectrum in negative ion mode is presented, and mass peaks refer to monoisotopic peaks. All mass peaks marked in *rectangular boxes* are explained to arise by the addition of the third Kdo with or without Rha. The *black star* indicates differences of 96 mass units.

Rha as revealed by the presence of mass peaks at 3768.7 Da. This mass peak can be explained as hexaacylated LPS with $Kdo_3RhaHep_2Hex_2P$ with L-Ara4N and 2P-EtN residues (supplemental Fig. S2*A*). Furthermore, LPS from strain overexpressing the *waaZ* gene in Δ*waaO* background revealed the presence of several mass peaks with the predicted incorporation of the third Kdo and Rha in both pentaacylated and hexaacylated derivatives (supplemental Fig. S2*B*). This is exemplified by the presence of mass peaks with ions at 3304.5, 3514.7, and 3637.7 Da. Such accumulation of pentaacylated mass peaks with the third Kdo and Rha in Δ*waaO* derivative suggests that LPS forms lacking myristoyl secondary chain can serve as substrate for the incorporation of the third Kdo. This was further supported by the examination of LPS by mass spectroscopy of Δ*lpxM* mutant with the intact core biosynthetic pathway. Such a mutant with only pentaacyl lipid A contains LPS derivatives of both glycoform I and IV (data not shown). Examination of LPS of either Δ*waaP* or Δ*waaG* strains with or without the *waaZ* gene overexpression did not reveal any mass peaks corresponding to the substitution with the third Kdo and Rha. These results suggest that both phosphorylation of HepI and a minimal outer core structure with two Hep and two Hex residues is required for the synthesis of derivatives with the third Kdo and Rha *in vivo* consistent with results of Δ*waaO* mutants.

Galactose Addition Is Required for Incorporation of the Third Kdo in the Absence of WaaO—Because the LPS of *E. coli* K-12 contains Gal substitution on GlcI, we analyzed LPS of Δ*waaB* mutants. The *waaB* gene in *Salmonella enterica* serovar Typhimurium has been shown to encode the glucosyl LPS 1,6-galactosyltransferase (24). Examination of LPS from Δ*waaB* revealed mass peaks corresponding to two derivatives as follows: one with a predicted addition of WaaO-dependent glucose and another without such glucose incorporation, causing a truncation. The truncated derivatives correspond to Hep_2Hex in the core region and lacked at the same time the third Kdo and Rha. However, mass peaks with a predicted core composition of

Hep_3Hex_2 were found to be predominantly substituted by the third Kdo and Rha (data not shown). Such derivatives can be explained to arise from the incorporation of the WaaO-dependent GlcII and the WaaQ-dependent side chain heptose in *waaB* mutants.

Because LPS of Δ*waaB* exhibited considerable complexity because of variable amounts of Hep and Hex residues, a double nonpolar chromosomal Δ(*waaB-waaO*) mutant was constructed to further investigate the minimal structure *in vivo*. This was necessary to answer the question whether prior incorporation of galactose and/or the second glucose is required for the incorporation of the third Kdo. Examination of its LPS revealed mass peaks predicted to correspond to LPS with two Kdo, two or three Hep, and a single Hex (supplemental Fig. S2*D*). The mass peak at 3258.5 Da is predicted to contain the side chain heptose HepIII and the phosphorylated HepII (supplemental Table S2). Additional derivatives with mass peaks at 3389.6 and 3512.6 Da can be explained with additional L-Ara4N and further P-EtN substitutions. Most significantly, LPS of Δ(*waaB-waaO*) did not reveal any mass peaks containing the third Kdo and Rha. Such a mutant did not confer the ability to incorporate the third Kdo even upon overexpression of the *waaZ* gene. These results thus suggest that the simultaneous lack of Gal and the GlcII does not allow the third Kdo incorporation. This could be due to structural constraints leading to reduced affinity of available minimal acceptor for WaaZ. Thus, the minimal *in vivo* structure able to accept the third Kdo is the form with two Hep residues in the inner core and two Hex residues in the outer core as shown with Δ*waaO* mutant. Furthermore, the synthesis of glycoform IV and V does not require the presence of Gal attached to GlcI, when *waaO* is functional. These conclusions are supported by the results obtained from *E. coli* B derivatives, which lack Gal but can incorporate a third Kdo when the *waaZ* gene from *E. coli* K-12 was overexpressed (Fig. 3*C*).

As shown above, LPS of Δ*waaO*, Δ*waaB*, and Δ(*waaB-waaO*) mutant strains contained several mass peaks with pentaacylated lipid A (supplemental Fig. S2). This could be either due to reduced amounts/activity of LpxM or due to structural preferences. Thus, we examined levels of LpxM-FLAG in *waaO* and *waaB* mutants as compared with the wild type. No significant differences were observed (data not shown), and this regulation (defect) needs further study.

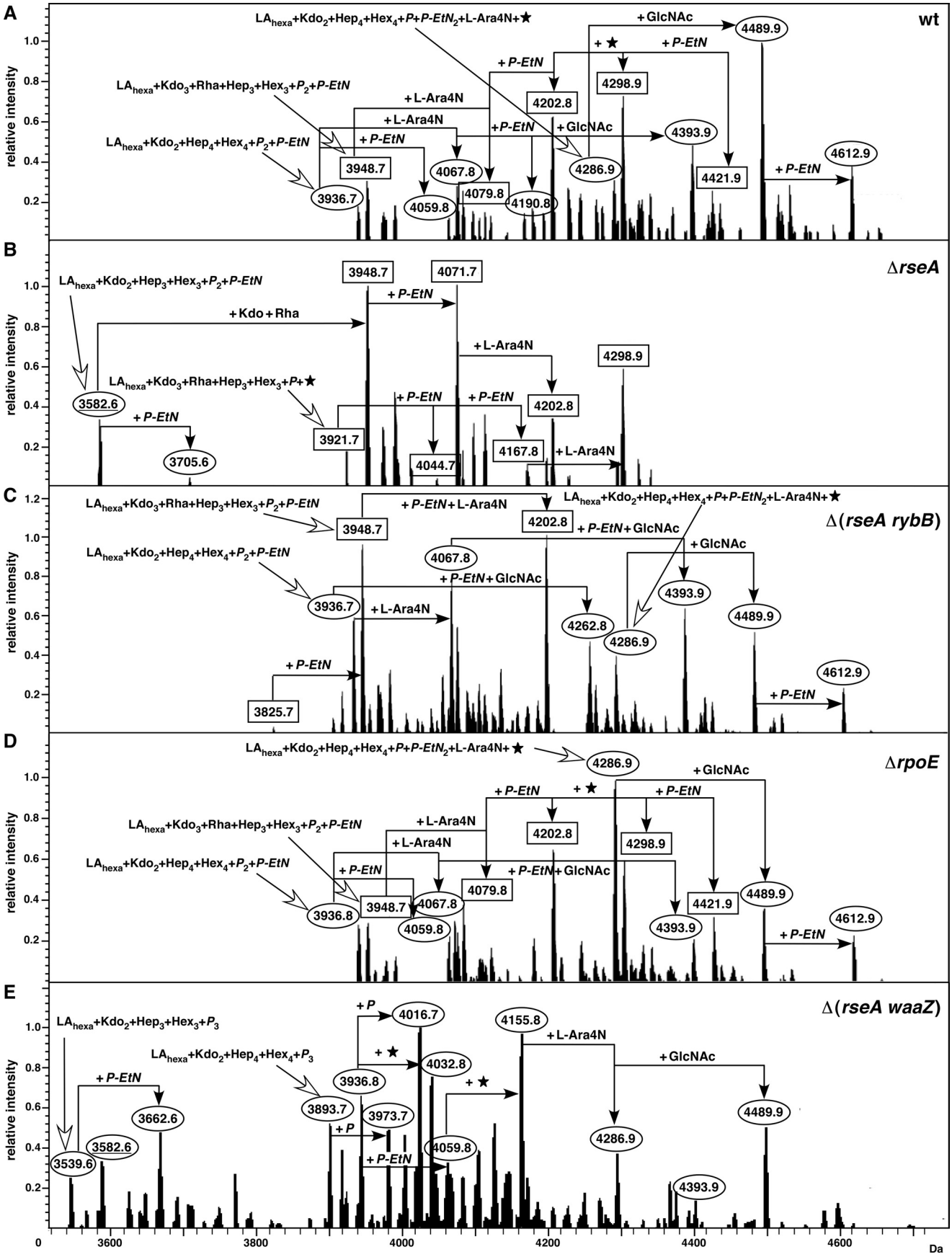
Side Chain Heptose Addition Does Not Require WaaO and WaaB Function—Our data also revealed that neither the Gal addition (WaaB-dependent) nor the GlcII addition (WaaO-dependent) are prerequisite for the HepIII incorporation. This is evident from the presence of a mass peak at 3674.6 Da in the LPS from Δ*waaO* mutant (supplemental Fig. S2*A*). Similarly, the mass peak at 3258.5 Da can be explained to include the phosphorylated HepII and additional substitutions with or without additional P-EtN and L-Ara4N as indicated (supplemental Fig. S2*D*).

Control of waaZ Expression—The molecular basis of the incorporation of the third Kdo was addressed by measuring *waaZ-lacZ* promoter activity and accumulation of chromosomal FLAG-tagged WaaZ by Western blot analyses. Cloning of regions upstream of the *waaZ* coding sequence in single copy promoter probe vectors identified an additional promoter located between the *waaY* and *waaZ* ORFs. The shift of the culture conditions from phosphate-rich M9 medium to phosphate-limiting medium revealed an increase by about 2-fold of the *waaZ-lacZ* promoter activity (Fig. 10*A*). These results were further supported by a nearly similar observed increase in the accumulation of WaaZ-FLAG upon Western blot analysis (Fig. 8*C*).

Induction of RpoE Leads to Preferential Accumulation of LPS Glycoform IV and V with the Third Kdo—The main function of the RpoE σ factor is to control OMP biogenesis and some of the steps of LPS translocation (8). RpoE in turn responds to outer membrane perturbations. This includes signals like imbalance in OMP composition and defects in the early steps of LPS biosynthesis (4, 8, 17, 25). Thus, we analyzed LPS from mutants with either constitutively induced RpoE or strains with mutations in genes predicted to regulate LPS biosynthesis/modifications. Several isogenic nonpolar deletion derivatives were constructed, including strains deleted for the main negative regulators of RpoE like *rseA* and *rseB* (26, 27). Deletion derivatives of *hfq*, *mgrR*, *rpoS,* and *yrbC/D* genes were also constructed and their LPS analyzed. Among these, *hfq* mutants show up-regulated RpoE activity, besides alterations in several functions controlled by noncoding RNAs and RpoS σ factor. Products of *yrb*/*mla* locus have been implicated in phospholipid migration to OM (28). *mgrR* encoding noncoding RNA has been implicated in the translational repression of the *eptB* gene product (phosphoethanolamine transferase for P-EtN addition to the second Kdo) (29). Among all of these, the most dramatic alteration in the LPS composition was that obtained from *rseA* mutants (Fig. 4). Δ*rseA* mutants elicit constitutive induction of the *rpoE* regulon, given the known function of RseA acting as an anti-σ factor for RpoE. In contrast, LPS of other mutants, including *rseB*, *mgrR*, *rpoS* and *hfq*, depicted mass peaks quite like that obtained from the wild type and were found to contain both glycoform I and IV derivatives (Fig. 5).

Mass spectrometric analyses of LPS from the Δ*rseA* mutant revealed nearly the exclusive presence of mass peaks corresponding to the predicted incorporation of the third Kdo and Rha and a characteristic truncation with a loss of terminal Hep-Glc disaccharide. The expected glycoform IV with the third Kdo and Rha derivatives can be assigned to mass peaks at 3921.7, 3948.7, 4071.7, 4167.8, 4202.8, and 4298.9 Da (Fig. 4*B*). For example, mass peak with ion at 3948.7 Da can be assigned to LPS containing $LA_{hexa}(Kdo_3RhaHep_3Hex_3P_2$P-EtN). The other mass peaks can be attributed to arise due to additional substitutions with one or two additional P-EtN and L-Ara4N residue(s). Incorporation of P-EtN and L-Ara4N was also observed from predicted and observed lipid A mass spectrometric analyses (data not shown). The mass peaks at 3921.7, 4167.8, and 4298.8 Da can be explained as new derivatives with an addition of 96 mass units. The origin of this modification cannot be explained on the basis of known *E. coli* LPS structures. This new modification seems to arise because of HexA substitution on HepIII accompanied by the loss of phosphate on HepII[3] (see under "Discussion").

[3] G. Klein, B. Lindner, H. Brade, and S. Raina, unpublished results.



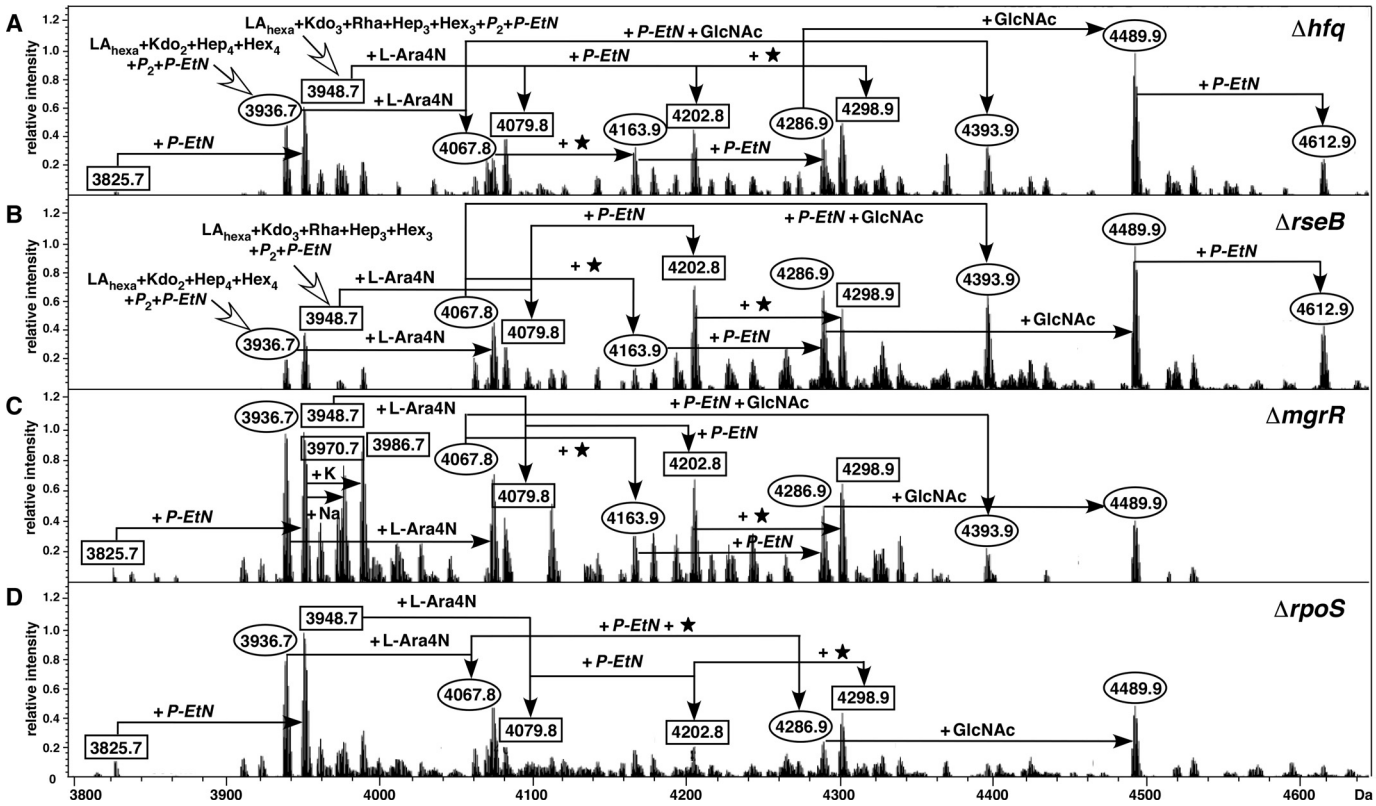


FIGURE 5. **Mass spectrometric analyses of LPS in various stress regulatory mutants.** Charge deconvoluted ESI FT-MS spectrum in negative ion of LPS obtained from 121 medium and from strains lacking either Hfq RNA-binding protein regulating various sRNAs (*A*), negative regulator RseB (*B*), noncoding sSRNA *mgrR* (*C*), and stationary phase σ factor RpoS (*D*) is shown. The mass peaks corresponding to glycoform I, but with varying P-EtN and/or L-Ara4N substitutions, are marked with *circles*. Mass peaks corresponding to predicted glycoforms IV and V with varying substitutions of P-EtN and/or L-Ara4N are *boxed*. Mass peaks with a *black star* arise because of addition of 96 mass units.

Interestingly, only mass peaks with predicted hexaacylated LPS derivatives containing two Kdo residues in Δ*rseA* background are represented by mass peaks at 3582.7 and 3705.6 Da. These can be explained as precursors of glycoform IV with characteristic absence of the terminal Hep-Hex disaccharide and the lack of the third Kdo and Rha with an overall composition of $LA_{hexa}(Kdo_2Hep_3Hex_3P_2$P-EtN) (Fig. 4*B*). The lack of any major structural differences in Δ*rseB* mutants as compared with dramatic changes in Δ*rseA* has important implications. RseB is a minor negative regulator as compared with major negative control by RseA of RpoE σ factor (26, 27). Thus the maximal induction of the RpoE regulon is required for near exclusive synthesis of glycoforms IV and V as observed in *rseA* mutants.

To ascertain that the observed synthesis of primarily glycoform IV was WaaZ-dependent, a double mutant Δ(*waaZ rseA*) was constructed, and its LPS was analyzed. Data presented in Fig. 4*E* revealed mainly mass peaks corresponding to predicted glycoform I derivatives, lacking the third Kdo; these are represented by ion peaks from 3893.7 to 4489.9 Da (Fig. 4*E*). However, analyses of LPS of Δ(*waaZ rseA*) also revealed the presence of mass peaks predicted to be precursors of glycoform IV without the third Kdo and Rha, with the truncation of the terminal Hep-Glc disaccharide. Such mass peaks resemble LPS derivatives of a Δ*waaR* mutant, represented by the mass peak at 3582.6 Da and its derivatives. Furthermore, LPS of Δ(*waaZ rseA*) unexpectedly was found to contain mass peaks, which correspond to the predicted addition of phosphate residues as revealed by peaks at 3893.7, 3973.7, and 4016.7 Da. The accumulation of such mass peaks of glycoform I derivatives in Δ(*waaZ rseA*) LPS might be a compensatory mechanism for the loss of the third Kdo in such a background. Thus, the overall induction of RpoE because of Δ*rseA* mutation seemed to result in reduced amounts or activity of WaaR, resulting in the accumulation of LPS with truncation after HexIII and activation of *waaZ*-dependent pathway for glycoform IV and V biosynthesis.

Position of Rha Dictated by the Presence of P-EtN on the Second Kdo—As shown above, the isolation of LPS from phosphate-limiting growth conditions led to a significant shift in the accumulation of glycoform with Kdo_3Rha incorporated. This shift to Kdo_3Rha is however most dramatic and pronounced in RpoE-inducing conditions as shown in the case of *rseA* mutants. We previously showed that 121 medium containing 2 mM Ca^{2+} is sufficient to allow a nonstoichiometric substitution

FIGURE 4. **Incorporation of the third Kdo upon constitutive induction of RpoE σ factor in Δ*rseA* mutants is regulated by noncoding *rybB* sRNA.** Charge deconvoluted ESI FT-MS spectrum in negative ion mode of LPS obtained from *E. coli* strain W3110 phosphate-limiting growth conditions (*A*) and its isogenic derivatives carrying *rseA* mutation (*B*), Δ(*rseA rybB*) combination (*C*), Δ*rpoE* null strain (*D*), or Δ(*rseA waaZ*) mutations (*E*) are shown. The *mass numbers* refer to monoisotopic peaks with predicted composition. Mass peaks representing glycoforms IV and V are *boxed* with *rectangles* and those corresponding to glycoform I in *circles*. Mass peaks marked with a *black star* differ by 96 mass units.

of P-EtN on the second Kdo (4). EptB is known to require Ca^{2+} for its activity (30). Furthermore, its relative incorporation depends on the induction of the *eptB* gene encoding phosphoethanolamine transferase specific to the second Kdo. The transcription of the *eptB* gene is induced upon RpoE up-regulation. Until now, the structure assignment of glycoform IV is based on the analysis of an oligosaccharide, which lacked P-EtN on the second Kdo (6). Thus, we analyzed in detail the KdoRha linkage under the conditions of the presence or the absence of P-EtN on the second Kdo. It needs to be emphasized that the second Kdo is also a site for the nonstoichiometric substitution by P-EtN. To address it, we analyzed composition of several isolated mass peaks predicted to contain KdoRha from LPS obtained from strains either lacking the *eptB* gene or when its expression is favored like in *rseA* mutants.

To demonstrate this switch, triply charged molecular ions from LPS obtained from either the wild-type ($eptB^+$) or *rseA* mutants were isolated and fragmented by the collision-induced dissociation. Main fragment ions result from the cleavage of the labile LA-Kdo linkage, comprising single charged LA [LA-H^+]$^{1-}$ species and doubly charged core oligosaccharide fragment ions [Core-H_2O-$2H^+$]$^{2-}$ and a group of triply charged fragments, which provide information on the Kdo substitution. MS/MS spectra with parental ion at 4071.7 and 4202.8 Da revealed mass peaks at 3705.6 and 3836.7 Da, which can be explained by simultaneous loss of 366.3 Da. They are explained to arise by Kdo + Rha cleavage from the branched Kdo_3-Rha tetrasaccharide (Fig. 6, *B* and *C*). This indicates that Rha is linked to the terminal KdoIII. Additional mass peaks from MS/MS spectra of parental ions at 4071.7 and 4202.8 Da revealed ion peaks at 3362.6 and 3493.6 Da, respectively. These are explained to arise from further simultaneous loss of (KdoP-EtN) residues from ions at 3705.6 and 3836.7 Da, respectively. These new LPS forms are hence designated as glycoform V (Fig. 1 and supplemental Fig. S1). Data in Fig. 6, *inset*, are charge deconvoluted mass spectra showing the most relevant features of fragmentation. These results and examination of several other peaks, for example ion peaks at 4079.7 and 4298.8 corresponding to glycoform V derivatives with P-EtN on the second Kdo, always were found to have KdoIIIRha as shown here for parental ions at 4071.7 and 4202.8 Da. This interesting change caused by the substitution point of P-EtN on KdoII determining the presence of Rha on KdoIII was not observed when the ion at 3948.7 Da was subjected to MS/MS analysis. This parental ion was not found to have P-EtN on the second Kdo and can explain ions arising at 3728.7 Da by the loss of the third Kdo and further loss of Rha leading to the mass peak at 3582.6 Da (Fig. 6*A*). Thus, the presence of P-EtN on KdoII leads to the addition of Rha to the third Kdo. Because incorporation of Rha was found to require prior addition of the third Kdo, the most favored position for Rha is the third Kdo under the conditions of EptB-dependent P-EtN addition on KdoII. Induction of RpoE, as is the case with Δ*rseA* mutants, is known to exhibit constitutive transcriptional induction of the *eptB* gene (4, 31).

To further substantiate the above results, the MS/MS analysis of isolated mass peaks at 3948.7, 4071.7, 4079.7, and 4202.8 Da were carried out from LPS obtained from a Δ*eptB* strain. Fragmentation spectra of such ion peaks revealed that in all such cases the terminal third Kdo was cleaved without Rha, which can explain mass peaks at *m/z* 1241.9, 1286.2, and 1326.9 interpreted as $(M - H)^{3+}$ ions (Fig. 7). This is in contrast to fragmentation spectra of isolated ion peaks of 4071.7 and 4202.8 Da from $eptB^+$ strains, which revealed simultaneous loss of Kdo + Rha from the branched Kdo_3Rha tetrasaccharide (Fig. 6, *B* and *C*). Fragmentation spectra of all four parental ion peaks from Δ*eptB* showed that predicted Rha cleavage occurs only after the loss of the terminal third Kdo. Furthermore, no ion peaks corresponding to fragmentation of KdoP-EtN was observed in the fragmentation spectra of ion peaks from *eptB* mutants. Because EptB is known to function as P-EtN transferase specific to the second Kdo, we can conclude that the incorporation of P-EtN is required to synthesize glycoform V with a switch of Rha addition to the terminal third Kdo.

RpoE-dependent Enhanced Glycoform IV and V Accumulation—One possible explanation for the nearly exclusive presence of glycoform IV and V derivatives in Δ*rseA* could be that $E\sigma^E$ RNA polymerase initiates the *waaZ* transcription. In such a scenario, Δ*rpoE* mutants are expected not to contain glycoform IV and V derivatives and have LPS resembling that of Δ*waaZ* mutants. However, analyses of LPS from a Δ*rpoE* strain revealed the presence of characteristic mass peaks at 3948.7, 4079.8, and 4202.8 Da with predicted incorporation of the third Kdo and Rha (glycoform IV and V) and not like that of LPS from Δ*waaZ* mutants (Fig. 4*D*). As can be seen from the mass spectrometric analysis of LPS obtained from Δ*rpoE*, several mass peaks with predicted glycoform I with or without the addition of HexNAc and the nonstoichiometric addition of P-EtN and L-Ara4N are also present. Thus, the overall distribution of mass peaks in Δ*rpoE* LPS resembled more or less that of the isogenic wild type. Mapping of the transcriptional initiation site further supported these results. In such an analysis, no obvious RpoE-regulated promoter upstream of the *waaZ* gene region was detected (data not shown). Thus, the overall LPS composition with preferential synthesis of either glycoform I or IV derivatives is not due to direct RpoE-dependent transcriptional initiation of the *waaZ* gene.

RpoE-regulated Noncoding Small RNA rybB-mediated Regulation of Glycoform IV and V Accumulation—To further understand the molecular basis of LPS changes upon the RpoE induction, we analyzed LPS of several mutants with either individual deletions in RpoE regulon members or their deletion combinations with Δ*rseA*. The RpoE σ factor is known to be induced upon imbalance or misfolding of porins (17, 25). We studied the potential control by *micA* and *rybB* noncoding sRNAs. These sRNAs are known to control translation of major porins, and synthesis of these sRNAs is regulated by RpoE (32). A deletion of individual *micA* or *rybB* genes did not cause any major alterations in the composition of LPS (data not shown). However, LPS from the Δ(*rseA rybB*) derivative prominently resulted in restoration of the glycoform I presence, resulting in LPS composition quite like the wild type (Fig. 4*C*). LPS of Δ(*rseA micA*) was also found to contain some glycoform I derivatives but overall only a partial suppression of *rseA* phenotype in terms of preponderance of glycoform IV and V content (data not shown). The restoration of glycoform I accumulation as seen by characteristic mass peaks appearance and suppression of glyco-

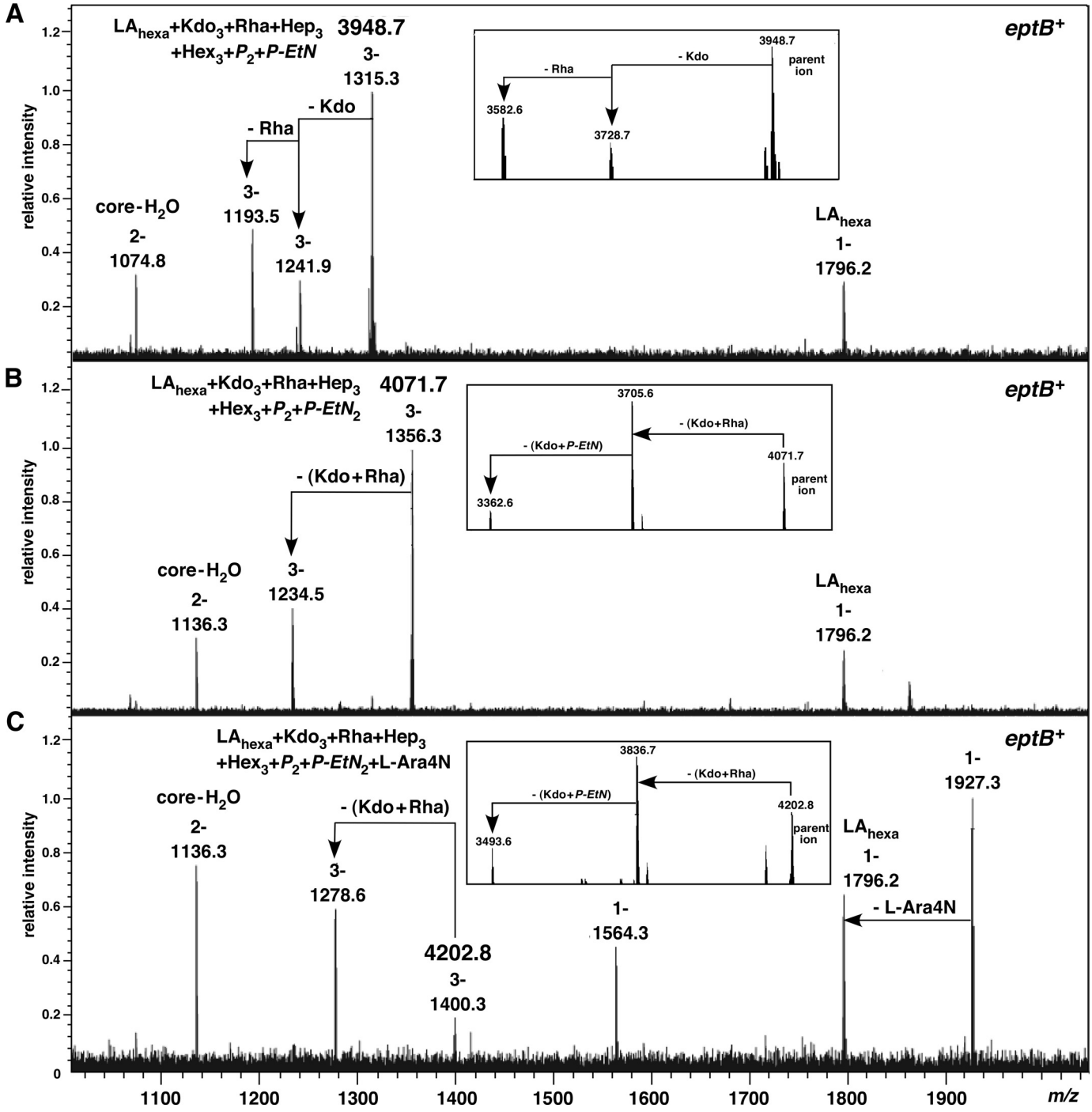


FIGURE 6. **Incorporation of P-EtN on the second Kdo results in switch of Rha addition to terminal third Kdo, leading to synthesis of glycoform V.** *A* corresponds to fragmentation spectra of parent ion at 3948.7 Da representing glycoform IV. In accordance to its published fragmentation behavior (6), a sequential loss of the first Kdo, followed by the cleavage of a Rha, confirms the substitution of Rha at the middle Kdo. *B* and *C* depict fragmentation spectra of parent ions at 4071.7 and at 4202.8 Da, respectively, both representing glycoforms V. Here, instead of the cleavage of one Kdo alone only the cleavage of (Kdo + Rha) is observed. Furthermore, the cleavage of labile linked L-Ara4N and P-EtN can be observed either from the complete molecular ions as from the singly charged LA fragment ion.

form IV and V accumulation with the third Kdo and Rha in Δ(*rseA rybB*) was further addressed by examining levels of WaaZ and other effector proteins.

Glycoform IV and V Accumulation Is Due to rybB-dependent Translational Repression of WaaR in Δ*rseA*—Because the introduction of Δ*rybB* in Δ*rseA* caused suppression of glycoform IV and V accumulation, we examined the levels of WaaZ and WaaR from their chromosomal copy using C-terminal FLAG epitope derivatives. The rationale for this is the commonly observed truncation of the terminal Hep-Glc disaccharide in the outer core upon the incorporation of the third Kdo associated with the presence of glycoform IV (4, 6, 7). First, in-frame chromosomal *waaR*-FLAG and *waaZ*-FLAG were constructed in the wild-type strain and then such FLAG-

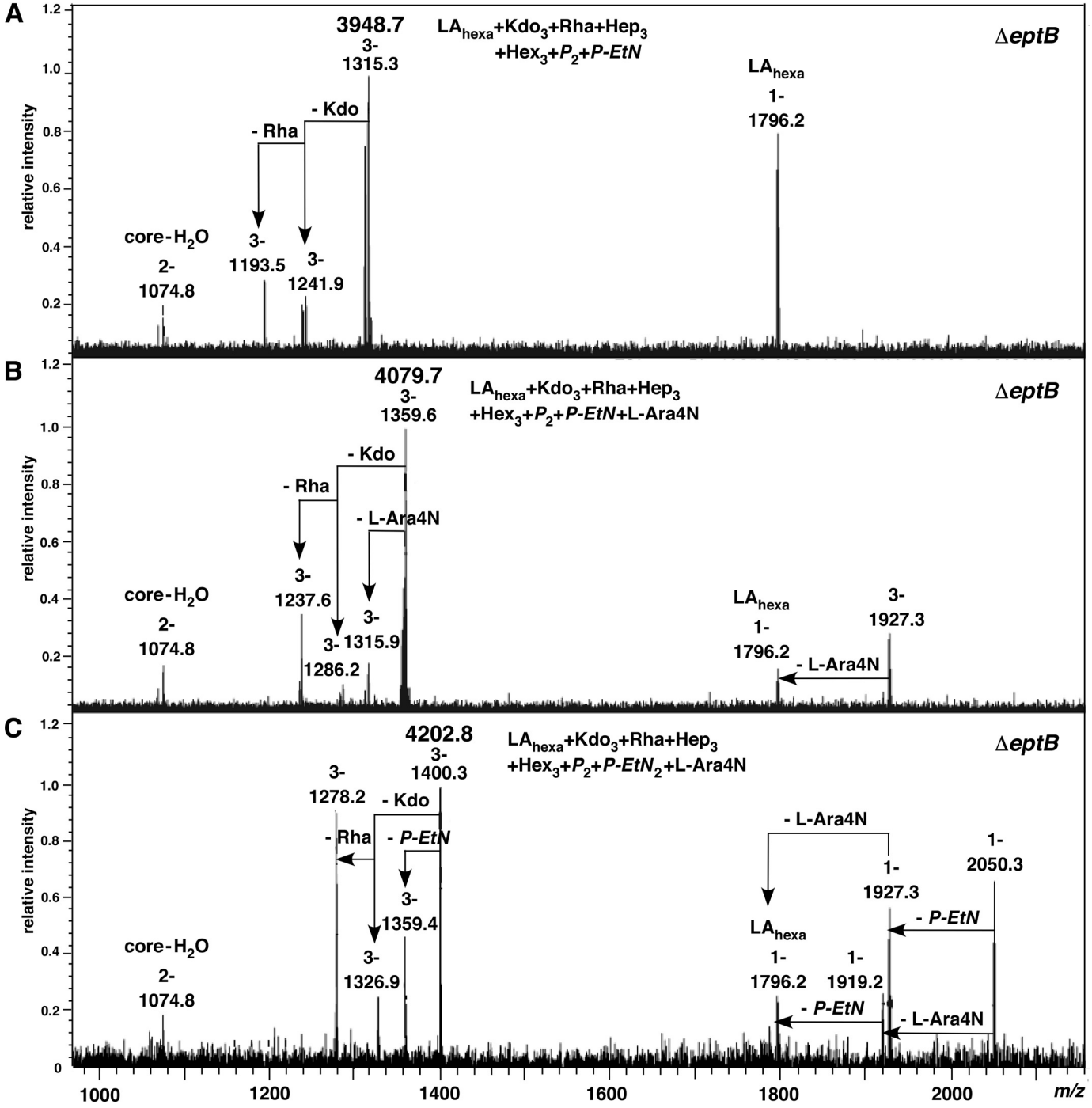


FIGURE 7. ***eptB* mutants lack glycoform V.** ESI FT-ICR mass spectra are shown of the negative ion mode after isolation of parental ions of mass peaks obtained from LPS of *eptB* mutants, which lack P-EtN on Kdo_2. Isolated ions peaks with the third Kdo and Rha were subjected to fragmentation analysis, and mass numbers correspond to $(M - H^+)^-$ ions for lipid A and $(M - H)^{+3}$ for derivatives from cleavage in the Kdo region. *A–C* corresponds to MS/MS spectra from isolated molecular species at 3948.7, 4079.8, and 4202.8 Da, respectively. As depicted in the mass spectra, all selected species include first the cleavage of a Kdo then followed by the cleavage of Rha, indicating glycoform IV.

marked WaaZ and WaaR alleles were transduced in Δ*rseA* and Δ(*rseA rybB*) backgrounds. Whole cell extracts from such isogenic bacteria were prepared and analyzed. Consistent with the requirement of WaaZ for the incorporation of the third Kdo and the nearly exclusive glycoform IV and V presence in Δ*rseA*, the level of WaaZ was higher in the Δ*rseA*. This increase in WaaZ levels was particularly more prominent in the stationary phase, as compared with the WaaZ levels in the isogenic wild type (Fig. 8*C, lanes 3 versus 4*). Thus, in a Δ*rseA* background the incorporation of the third Kdo is at least in part due to WaaZ accumulation in stationary phase, but it is not sufficient to explain LPS composed of mainly glycoform IV and V derivatives.

As shown above, both Δ*rseA* and its isogenic Δ(*rseA waaZ*) mutant derivatives caused accumulation of LPS precursor with truncation of terminal Hex-Hep disaccharide. These results

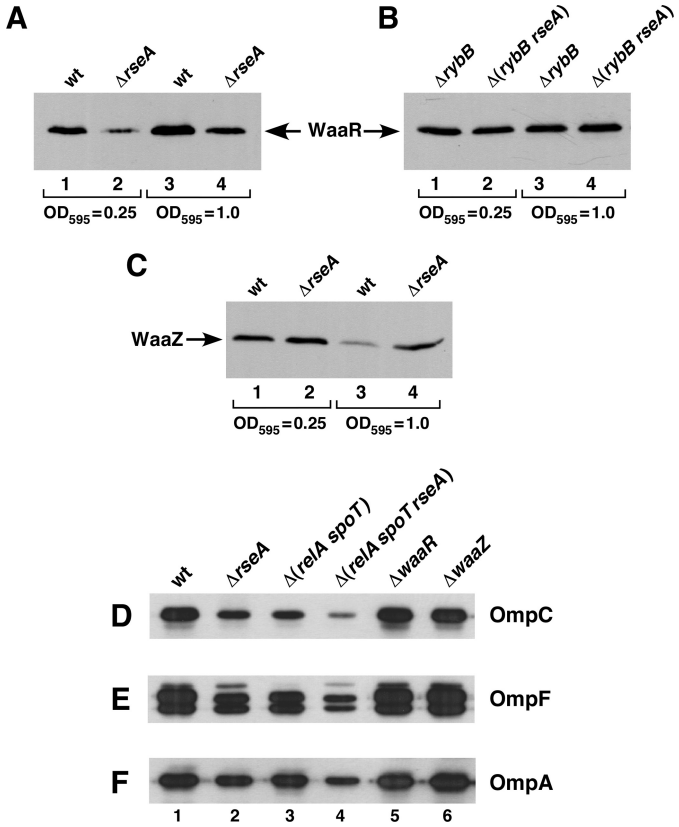
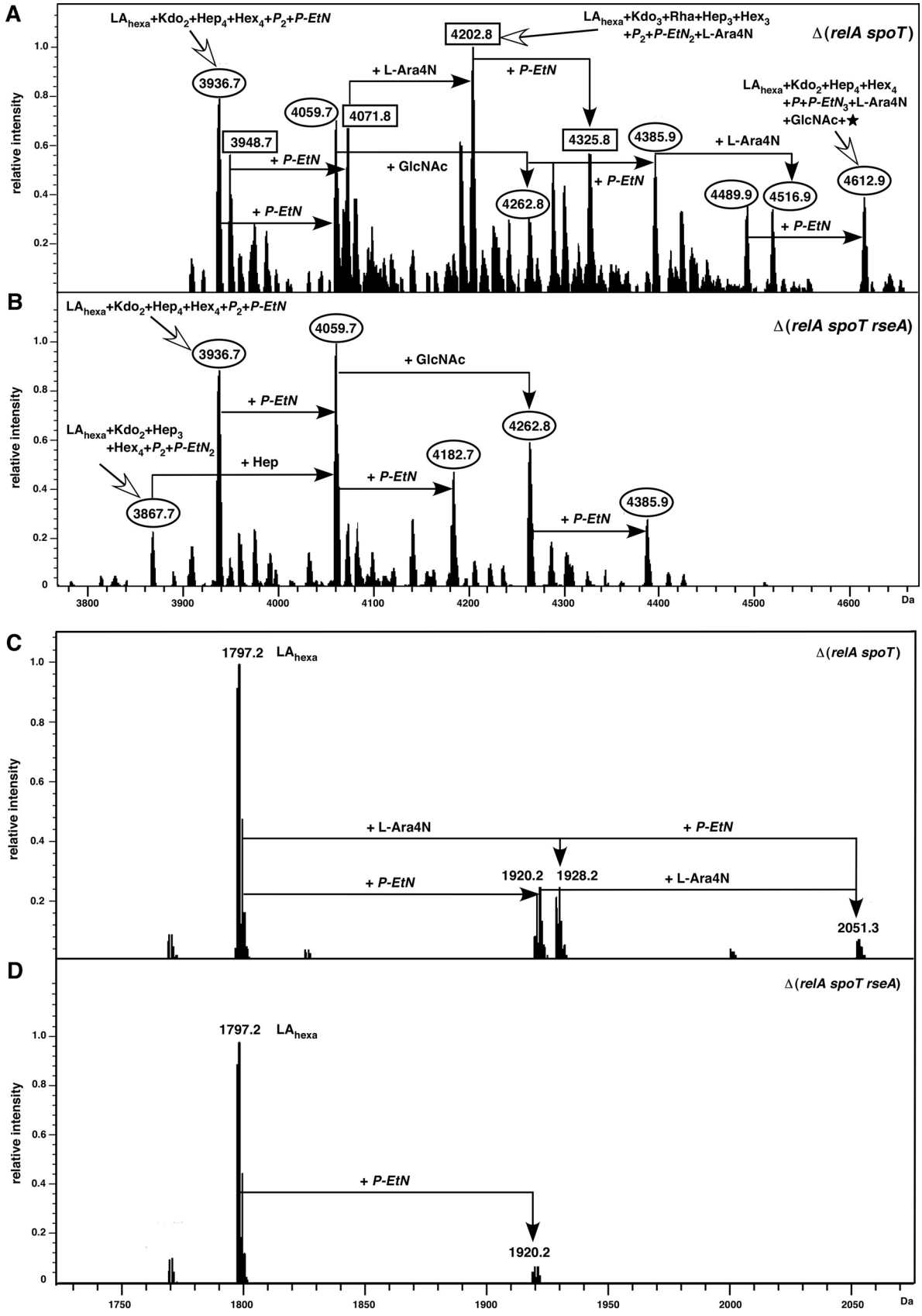


FIGURE 8. **Repression of WaaR and induction of WaaZ induce the third Kdo incorporation but not that of OMP.** Culture of the wild type (*wt*) and its derivatives with or without the C-terminal 3×FLAG tag fusion were grown to early log phase in LB medium at 37 °C, washed, and adjusted to A_{595} of 0.02 in 121 medium growth conditions. Aliquots of samples were drawn at different intervals and harvested by centrifugation. Equivalent amounts of proteins (20 μg) were lysed in SDS sample buffer and resolved on 12% SDS-PAGE. Proteins were transferred to PVDF membrane and probed with specific antibodies. Samples from the wild type and its derivative with in-frame *rseA* deletion, both carrying chromosomal *waaR* replaced by *waaR*-FLAG tag, were probed with monoclonal M2 antibody against FLAG tag (*A*). Samples prepared from WaaR-FLAG-tagged derivatives, carrying mutations in Δ*rybB* and Δ(*rybB rseA*), were prepared and resolved on SDS-PAGE under identical conditions and levels of WaaR-FLAG were determined as described above (*B*). Western blots of samples prepared from the wild-type and isogenic Δ*rseA* strains carrying *waaZ*-FLAG replacement of the chromosomal *waaZ* gene were probed with FLAG-specific antibody (*C*). *D–F* correspond to Western blot analysis to reveal the relative amounts of OmpC, OmpF, and OmpA, respectively, from isogenic strains grown in 121 medium. The relevant genotype of each strain, from which samples were prepared, is indicated on the top of *D*.

argue that in Δ*rseA* mutant accumulation of glycoform IV and V could be due to WaaR defect, which can explain the resulting truncation. Thus, WaaR-FLAG levels were examined by Western blot analyses. As shown in Fig. 8, *A* and *B*, levels of WaaR are reduced in a Δ*rseA* and restored back to near wild-type levels in Δ(*rseA rybB*) derivative. Taken together, these results demonstrate that WaaR synthesis is subjected to translation repression upon RpoE activation via *rybB* sRNA resulting in the incorporation of the third Kdo. Thus, glycoform IV and V accumulation is primarily due to reduction in WaaR amounts at translational level.

*Δ*waaR *Mutants Exhibit Enhanced Incorporation of the Third Kdo Leading to Preferential Accumulation of Glycoform IV and V*—Based on the results showing that reduction in WaaR levels in *rseA* mutants or overall growth conditions in 121 medium is responsible for inducing the synthesis of glycoform IV and V with truncation of the outer core, we investigated LPS of *waaR* mutants. Consistent with the above results, mutant strains lacking glycosyltransferase due to nonpolar *waaR* deletion were found to primarily contain LPS with glycoform IV and V derivatives (supplemental Fig. S2*C*). All the main mass peaks at 3948.7, 4079.8, 4202.8, and 4325.8 Da correspond to characteristic glycoform IV and V derivatives with additional substitutions by P-EtN and L-Ara4N as indicated (supplemental Fig. S2*C*). Other mass peaks with lower molecular masses can be explained as a premature termination of LPS synthesis or as precursors. This is evident from their predicted composition as indicated (supplemental Fig. S2*C*). Results from Δ*waaR* suggest that the lack of the *waaR* gene itself or the presence of presumably preferred precursor with Hep_3Hex_3 composition (mass peaks at 3582.6 Da) highly favors the incorporation of the third Kdo, leading to the synthesis of glycoform IV and V. Analysis of LPS of a Δ*waaR* mutant grown in phosphate-rich growth conditions also revealed several mass peaks with predicted glycoform IV incorporation (supplemental Fig. S2*E*). Thus, *in vivo* LPS from strain Δ*waaR* is the preferred substrate for the incorporation of the third Kdo. Hence, we can conclude that levels of WaaR seem to determine the switch between glycoform I and IV. Also induction of lipid A modifications (BasS/R- and PhoB/R-dependent) is not required in Δ*waaR* for the incorporation of the third Kdo.

Presence of Glycoform IV and V with Three Kdo in Δ*rseA Is Abolished in* Δ*(relA spoT rseA)*—The alarmone ppGpp is a general signal of starvation stress. ppGpp regulates the activation of alternative σ factors upon entry into stationary phase and also competition among various σ factors (33). ppGpp is also known to be involved in regulation of the PhoB/R two-component system and RpoE (33, 34). Because in this study we show that induction of RpoE and PhoB/R controls switches in LPS composition, we analyzed LPS from isogenic $ppGpp^0$ (*relA spoT*) and Δ(*relA spoT rseA*) strains. Examination of the mass spectra of LPS from Δ(*relA spoT*) and the isogenic wild type revealed similar mass peak composition regarding the presence of glycoform I, IV, and V (Fig. 9*A*). However, introduction of Δ*rseA* mutation in Δ(*relA spoT*) ($ppGpp^0$ background) abolished the accumulation of mass peaks corresponding to glycoform IV and V derivatives. This resulted in the lack of detectable amounts of LPS with the third Kdo and Rha, which are predominant in Δ*rseA* parental strain (Fig. 9*B*). Thus, no mass peaks at 3948.7 Da and its derivatives were detected in Δ(*relA spoT rseA*). On the contrary, only mass peaks at 3936.7 Da and its derivatives corresponding to glycoform I are present. These mass peaks are represented by observed ions at 4262.8 and 4385.9 Da. They are predicted to arise upon further substitutions by P-EtN and GlcNAc. Interestingly, not only accumulation of glycoform IV and V was suppressed but also no mass peaks with predicted L-Ara4N incorporation were observed, which are present in both Δ*rseA* as well as Δ(*relA spoT*) (Fig. 9, *C* and *D*). Analyses of mass peaks corresponding to the lipid A part revealed only a mass peak at 1920.2 Da (LA_{hexa}P-EtN) but not that corresponding to L-Ara4N addition. Thus, ppGpp seems to control both LPS core composition and lipid A modification in RpoE-inducing conditions. These data also show that in *E. coli* L-Ara4N incorporation not only requires BasS/R



induction but also seems to be further fine-tuned by ppGpp. Interestingly, until now ppGpp has not been implicated in the control of lipid A or LPS core structural alterations in *E. coli* or in other organisms.

*PhoB/R and BasS/R Induction Is Required for Glycoform IV and V Presence in Δ*rseA *but Not in Wild Type*—We previously showed that phosphate limitation and components like Zn^{2+} and Fe^{3+} contribute to BasS/R-dependent lipid A modifications in 121 growth medium. Because growth of *E. coli* in this medium also caused a pronounced shift to the presence of glycoform IV and V, we analyzed the requirement of either BasS/R and/or PhoB/R two-component systems for this phenotype. Analyses of LPS extracted from isogenic Δ*basR* and Δ*phoB* bacteria grown in 121 medium revealed the presence of glycoform I with complete core and glycoform IV derivatives (supplemental Fig. S3*A*). However, LPS of Δ*basR* mutant bacteria revealed much less heterogeneity as compared with either LPS from the wild-type or Δ*phoB* mutant. This can be explained by the obvious lack of nonstoichiometric addition of P-EtN and L-Ara4N to lipid A (supplemental Fig. S3*A*). The mass peak at 4071.8 Da in Δ*basR* can be assigned to glycoform IV and V with additional two P-EtN residues and that at 4167.8 Da with an additional substitution of 96 mass units. These results show that unlike P-EtN addition on lipid A, which is BasS/R-dependent, nonstoichiometric P-EtN additions on either the second Kdo or HepI are BasS/R-independent. This explains the presence of the mass peak at 4071.7 Da. Examination of LPS obtained from Δ(*basR phoB*) derivative also revealed the presence of mass peaks corresponding to glycoforms I, IV, and V (supplemental Fig. S3*B*). However, significantly LPS of a strain Δ(*basR phoB rseA*) contained mass peaks corresponding to glycoform I, IV, and V LPS derivatives in contrast to presence of mainly glycoform IV and V in Δ*rseA* (supplemental Fig. S3*C*). These results argue that induction of both BasS/R and PhoB/R two-component systems contributes to synthesis/incorporation of glycoform IV and V in the Δ*rseA* derivative, although individually both PhoB/R and BasS/R are dispensable in this switch of glycoforms. In support of these observations, LPS obtained from Δ(*eptA rseA*) and Δ(*eptA arnT rseA*) also revealed restoration of glycoform I synthesis in Δ*rseA* as revealed by the presence of corresponding mass peaks (data not shown). However, this restoration of glycoform I in Δ(*eptA rseA*) and Δ(*eptA arnT rseA*) combinations is not to the wild-type extent as seen in Δ(*rseA rybB*) mutants, arguing overlapping control by lipid A modification system as well as the translational control of WaaR by *rybB* noncoding RNA for the switch to glycoform IV and V.

Preferential Glycoform I or IV and Impact on OMP Levels—It is established that a Δ*rseA* mutant exhibits constitutively elevated RpoE activity leading to reduced amounts of OMPs. This is ascribed to induction of RpoE-regulated noncoding RNAs, which down-regulate porin synthesis (32). The results presented in this work further revealed that such Δ*rseA* bacteria primarily synthesize LPS composed of mainly glycoform IV and V derivatives. Thus, we asked whether the overall switch of glycoform I to glycoform IV and V is also reflected in any *in vivo* alterations of OMP levels. It is compelling because Δ(*rseA rybB*) combination leads to restoration of OMP composition resulting in suppression of several defects of Δ*rseA* (35). We also showed that Δ(*rseA rybB*) exhibit a switch back to glycoform I synthesis. With the construction of panels of several single or various mutational combinations synthesizing either glycoform I or glycoform IV and V, we determined the relevance of the incorporation of the third Kdo (glycoform IV and V) for OMP composition. Whole cell lysates were prepared from the wild type, Δ*rseA*, Δ(*relA spoT*), Δ(*relA spoT rseA*), Δ*waaR,* and Δ*waaZ* mutants. Levels of OmpF, OmpC, and OmpA were compared under identical growth conditions after Western blotting and were revealed using specific antibodies against each protein. Among the strains used, Δ*waaZ* and Δ(*relA spoT rseA*) were shown to have LPS composed mainly of glycoform I derivatives (Figs. 2*A* and 9*B*) and in contrast Δ*rseA* and Δ*waaR* possessed primarily LPS of glycoform IV and V in 121 growth conditions (Fig. 4*B* and supplemental Fig. S2*C*). As expected, *rseA* mutants accumulated reduced levels of major OMPs, but Δ*waaR* mutants with nearly similar LPS composition revealed OMP content (levels) quite like the wild type (Fig. 8, *D–F*). These results argue that the induction or preferential presence of glycoform IV and V does not contribute to OMP reduction. Examination of OMP levels of Δ*waaZ* and Δ(*relA spoT rseA*), however, showed highly reduced levels of OMPs in Δ(*relA spoT rseA*), although Δ*waaZ* possessed OMP levels nearly similar to the wild type, despite the two mutants representing LPS with only glycoform I derivatives. Interestingly, Δ(*relA spoT*) mutants also contained reduced amounts of OMPs, which were not suppressed in Δ(*relA spoT rseA*), although the LPS composition with respect to glycoform I *versus* glycoform IV and V was dramatically different. Taken together, these results suggest that *in vivo* preferential synthesis of either glycoform I or glycoform IV and V does not interfere in the OMP biogenesis significantly. These results are consistent with lack of any RpoE induction in Δ*waaR* mutants (see below).

RpoE Signal Transduction in waaR Mutants, Consequences of Truncation of Outer Core—Because Δ*rseA* and Δ*waaR* revealed nearly similar LPS mass peaks corresponding primarily to glycoform IV and V derivatives with truncation of the outer core, we examined whether Δ*waaR* also exhibits constitutive hyperinduction of the RpoE regulon, which responds to OM dysfunction as seen in Δ*rseA* (26, 35). A defined Δ*rseA* mutant is known to exhibit a 6–10-fold basal level increased transcriptional activity from RpoE-regulated promoters such as *rpoH*P3, *rpoE*P2, and *htrA* (26, 27). Thus, a Δ*waaR* mutation was introduced in the wild-type strain carrying single copy $E\sigma^E$-transcribed *lacZ* promoter fusions. Data consistently revealed that no increase in the activity of a representative RpoE-regulated

FIGURE 9. **Requirement of ppGpp alarmone for glycoform IV and V synthesis and L-Ara4N incorporation in Δ*rseA* derivatives.** Charge deconvoluted ESI FT-ICR mass spectra in negative ion mode depicting spectra mass peaks corresponding to intact LPS obtained from sp. *relA spoT* $ppGpp^0$ mutants (*A*) and its derivative Δ(*relA spoT rseA*) (*B*) are shown. Cultures were grown in lipid A modifying growth conditions at 37 °C as described earlier. Δ(*relA spoT*) (*C*) and Δ(*relA spoT rseA*) (*D*) show a part of negative ion mass spectra of the native LPS after unspecific fragmentation leading to cleavage of the labile lipid A-Kdo linkage. Mass peaks corresponding to hexaacylated lipid A part and its derivatives with substitutions with P-EtN and/or L-Ara4N are marked with *filled arrow*.

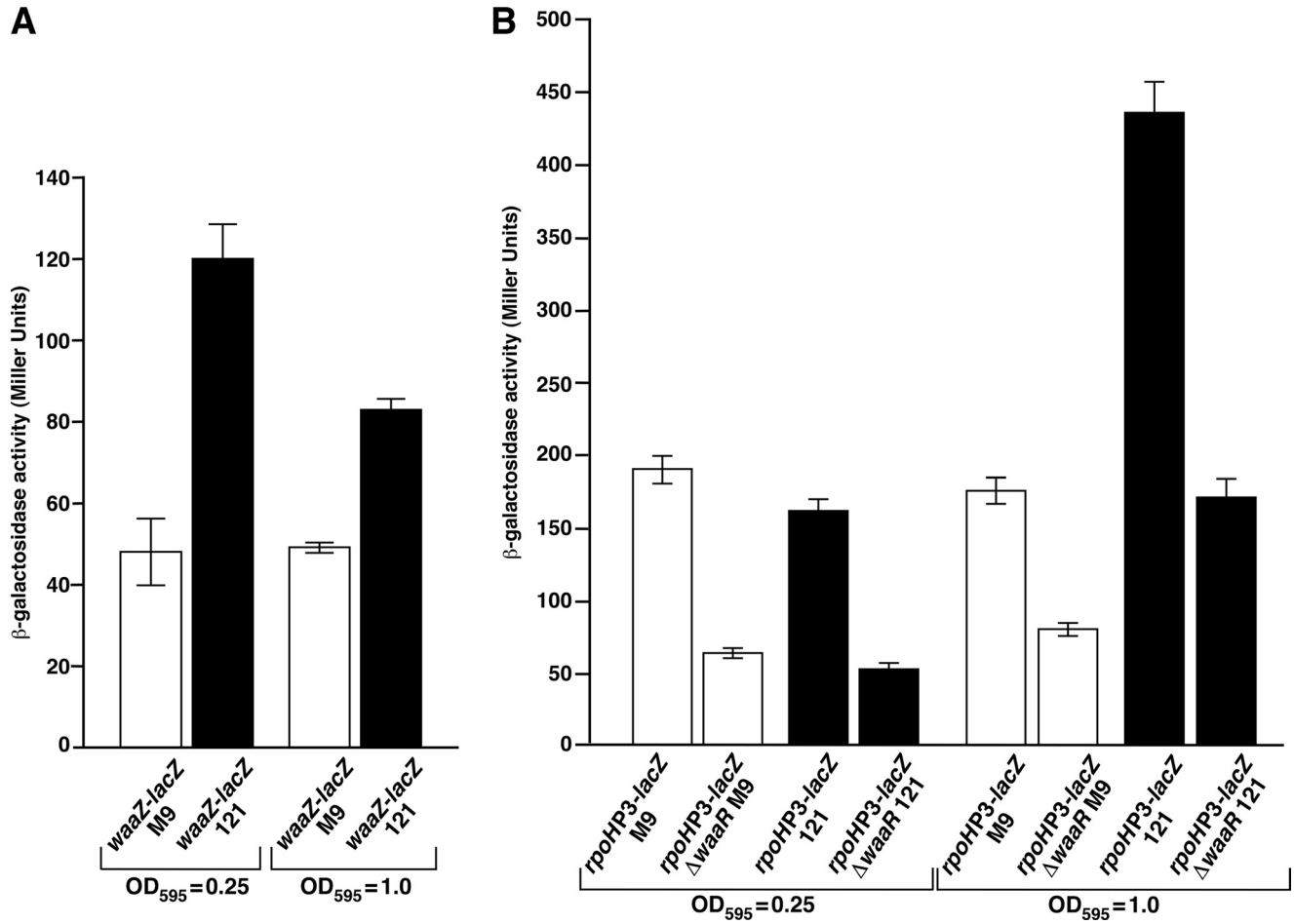


FIGURE 10. **Induction of transcription of the *waaZ* promoter in 121 medium (*A*) and activity of RpoE-regulated signal transduction pathway in Δ*waaR* strains (*B*).** Cultures of *E. coli* strain GK1111 carrying single copy chromosomal *waaZ-lacZ* promoter fusions were grown to early log phase in LB medium at 37 °C, washed, and adjusted to A_{595} of 0.02 in M9 or 121 medium. Aliquots of samples were drawn at different intervals and analyzed for β-galactosidase activity. Data from one representative set of early log phase growth conditions (A_{595} of 0.25) and late log phase (A_{595} of 1.0) are presented (*A*). *B,* cultures of *E. coli* wild-type strain GK1111 carrying single copy chromosomal *rpoH*P3-*lacZ* promoter fusion or its isogenic derivative with Δ*waaR* mutation were grown to early log phase in LB medium at 37 °C. Cultures were washed and adjusted to A_{595} of 0.02 in M9 or 121 medium. Aliquots of samples were drawn at different intervals. The A_{595} was measured and analyzed for β-galactosidase activity as indicated above. *Error bars* represent S.E. of four independent measurements.

promoter fusion (*rpoH*P3-*lacZ*) occurred. Rather, in all tested growth conditions, the activity of *rpoH*P3-*lacZ* was lower in Δ*waaR* mutant than in the isogenic parent (Fig. 10*B*). Thus, despite similar LPS composition of Δ*waaR* and Δ*rseA,* the observed increased presence of glycoform IV and V with the third Kdo does not result in RpoE activation. Thus, the preferential presence of glycoform IV and V can occur independent of RpoE activation as in Δ*waaR,* and this LPS modification (increased accumulation/addition of the third Kdo) is not directly responsible for the RpoE induction.

Requirement of WaaZ in Strains Synthesizing Tetraacylated Lipid A with Intact waaC Gene—In this work, we showed that the constitutive induction of RpoE in Δ*rseA* mutants induces the shift to preferential WaaZ-dependent glycoform IV and V incorporation. Earlier, we showed that strains lacking late acyltransferases, but with intact *waaC*, also showed preponderance of these glycoforms with the third Kdo. Thus, we addressed if this WaaZ-dependent incorporation of a glycoform with the third Kdo has a physiological function. Introduction of Δ*waaZ* in Δ*rseA* background did not confer any noticeable growth defects or suppress dramatic phenotypes like cell lysis or overall RpoE activity. However, a requirement for WaaZ was observed in Δ(*lpxL lpxM lpxP*) mutants. Δ(*lpxL lpxM lpxP*) mutants do not grow at 30 °C on rich medium but can grow on minimal medium (4, 36). In this study, we further show that suppressor-free Δ(*lpxL lpxM lpxP*) mutants can be constructed on rich medium at 21 or 23 °C but not at 30 °C or above (Table 2). Next, to test the requirement of WaaZ defined nonpolar Δ*waaZ* mutation was introduced in a Δ(*lpxL lpxM lpxP*) strain. Thus, Δ(*lpxL lpxM lpxP waaZ*) and Δ(*lpxM lpxP waaZ*) mutants could be constructed at 21 or 23 °C in the presence or absence

TABLE 2

Colony forming ability of Δ(*lpxL lpxM lpxP*) derivatives in transductional combinations with Δ*waaZ*

	M9		LA		
	21 °C	30 °C	21 °C	30 °C	37 °C
Δ(*lpxM lpxP*)	++[a]	++	++	++	++
Δ(*lpxM lpxP*) + Δ*waaZ*	++	++	++	++	+[b]
Δ(*lpxM lpxP*) + Δ*lpxL*	++	++	++	−[c]	
Δ(*lpxL lpxM lpxP*) + Δ*waaZ*	++	+[b]	−	−	
Δ(*lpxL lpxM lpxP*) + Δ*waaZ* + p*lpxL*$^+$	++	++	++	++	

[a] ++ indicates ≥500 colonies.
[b] + indicates 100–500 colonies but is small in size.
[c] − indicates inability to support colony forming capacity.

of plasmid covering one of these genes by T4-mediated transductions on minimal M9 medium. However, viable Δ(*lpxL lpxM lpxP waaZ*) were not obtained without complementing plasmid on rich medium at either 21 or 23 °C (Table 2). Taken together, these results showed that WaaZ indeed has a physiological role in *E. coli* K-12 strains with tetraacylated lipid A with intact LPS core region.

DISCUSSION

In this study, using phosphate-limiting growth conditions, we found a pronounced shift to the presence of LPS derivatives with the third Kdo and Rha linked to lipid A-anchored Kdo disaccharide with a characteristic truncation of the outer core designated as glycoform IV. This glycoform is usually a minor component of LPS, whereas the major glycoform with a complete core is designated as glycoform I ($LA_{hexa}Kdo_2Hep_4Hex_4P_2$) (6). We demonstrated that the *waaZ* gene is the structural gene required for addition of the third Kdo to the Kdo disaccharide linked to lipid A. Accordingly, LPS of a Δ*waaZ* strain contained only typical glycoform I and no glycoform IV derivatives with the third Kdo. However, mass peaks predicted to correspond to precursor(s) of glycoform IV with truncation of the terminal Hep-Hex without the third Kdo were found. These results suggested that the truncation of the outer core is not directly due to incorporation of the third Kdo. Interestingly, no mass peaks predicted to contain Rha were found in Δ*waaZ.* Furthermore, we show that the addition of Rha requires WaaS function. Δ*waaS* strain could add the third Kdo to Kdo disaccharide without the addition of Rha. These results argue that addition of Rha occurs only after the third Kdo is incorporated. Consistent with these findings, we showed that *E. coli* B strains, naturally lacking *waaZ* and *waaS* genes, can incorporate Rha in the LPS inner core only after WaaZ-dependent addition of the third Kdo.

We also showed that the presence of P-EtN on the second Kdo (penultimate Kdo) in branched Kdo_3Rha tetrasaccharide leads to a switch of Rha addition to the third Kdo, hence defining glycoform V. The change of the position of Rha to the third Kdo was more pronounced under the conditions of RpoE induction, such as in a Δ*rseA* mutant. MS/MS analysis of several isolated mass peaks corresponding to glycoform V with P-EtN on the second Kdo revealed Rha only on the third Kdo. Kdo_2P-EtN-dependent switch of Rha substitution was confirmed by MS/MS analysis of isolated mass peaks of LPS from a Δ*eptB* mutant. EptB is known to be required for the transfer of P-EtN to the second Kdo (30). Indeed all the mass peaks with the third Kdo in Δ*eptB* were found to contain Rha substitution on the second Kdo, thus corresponding to only glycoform IV. In conclusion, P-EtN on the second Kdo dictates Rha addition to the third Kdo.

Structural and compositional analyses of LPS obtained from various mutants allowed us to demonstrate that *in vivo* the minimal LPS structure that can support the incorporation of the third Kdo to generate Kdo_3Rha is $Kdo_2Hep_2Hex_2$ with requirement for phosphorylation of HepI. The second Hex residue can be either Gal or Glc. These results were authenticated by the analysis of LPS from Δ(*waaB-waaO*), which could not support the incorporation of the third Kdo even upon overexpression of the *waaZ* gene product. This is in contrast to *waaO* mutants with intact *waaB*, containing LPS with a Kdo_3Rha branched tetrasaccharide and hence the core with $Hep_2GlcGal$. Because Δ*waaB,* as well as *E. coli* B which naturally lack Gal, can incorporate LPS substituted with Kdo_3Rha, showed that Gal addition in LPS is not a prerequisite for this modification.

We addressed the question whether any molecular switch regulates preferential synthesis of glycoform I or glycoform IV and V. This switch in phosphate-limiting growth conditions was in part ascribed to increased transcription of the *waaZ* gene. Because growth in this medium induces both PhoB/R and BasS/R two-component systems, LPS from individual Δ*phoB*, Δ*basR* and double Δ(*basR phoB*) was analyzed. Individual Δ*phoB* or Δ*basR* mutants were found to contain both glycoform I and glycoform IV and V. LPS composition was enriched in glycoform IV in a Δ*waaR* mutant even in growth conditions that are repressing for PhoB/R and BasS/R. This is an argument that the induction of PhoB/R and BasS/R systems is not absolutely essential for the synthesis of glycoform IV. Thus, these results suggest that additional control mechanisms for the incorporation of the third Kdo exist, particularly at the control of WaaZ *versus* WaaR levels.

Because several cell envelope functions including the amounts of OMPs are regulated by RpoE σ factor (8) and LPS is the major component of OM, we analyzed LPS from strains, which exhibit constitutive induction of RpoE. Interestingly, Δ*rseA* mutants contained primarily LPS with glycoform V derivatives. RseA acts as RpoE-specific anti-σ factor. Thus, Δ*rseA*, genes which are transcribed by $E\sigma^E$ RNA polymerase, are constitutively up-regulated, although the synthesis of several OMP is repressed (8, 26, 37). However, Δ*rpoE* was found to contain glycoforms I, IV, and V derivatives quite like the wild-type strain. This led us to investigate whether any RpoE-induced gene product might be responsible for the preponderance of glycoform IV and V in Δ*rseA*. Because RpoE does not positively regulate *waaZ* transcription, it argued for potential regulation via a negative control process under the control of RpoE. As RpoE-inducible noncoding RNAs of *rybB* and *micA* are known to repress the synthesis of OMPs, LPS of Δ(*rseA rybB*) and Δ(*rseA micA*) strains was analyzed. Interestingly, introduction of Δ*rybB* mutation in Δ*rseA* suppressed the Δ*rseA*-dependent glycoform IV and V preponderance. Thus, LPS of Δ(*rseA rybB*) strain contained both glycoform I and glycoform IV and V derivatives quite like the wild type. Thus, *rybB* seems to down-regulate a step that promotes WaaZ synthesis and hence the incorporation of the third Kdo. This was shown to occur at repression of WaaR synthesis in Δ*rseA* mutants and its restoration to wild-type levels in Δ(*rseA rybB*). Consistent with the induction of glycoform IV and V synthesis in Δ*rseA*, related to reduction in WaaR amounts, a defined *waaR* mutant synthesized LPS composed of mainly glycoform IV derivatives. Thus, the increase of WaaZ synthesis and reduction in WaaR levels are the main factors that cause a switch to the synthesis of glycoform IV as seen in Δ*rseA* mutants.

Another mechanism of regulation of RpoE-dependent increased synthesis of glycoform IV and V derivatives with the third Kdo was discovered. This was found to occur via the alarmone ppGpp. ppGpp is known to alter the specificity of several

alternative σ factors. Further ppGpp and phosphate starvations have many overlapping functions (37). Analyses of LPS of ppGpp^0 mutants revealed no significant differences as compared with the wild type. However, introduction of Δ*rseA* in ppGpp^0 mutants abolished accumulation of glycoform IV and V presence and virtually resulted in LPS composed of only glycoform I. Such a switch to exclusive synthesis of glycoform I was observed only in Δ*waaZ*. Furthermore, Δ(*relA spoT rseA*) mutants did not incorporate L-Ara4N on lipid A, although P-EtN could be found. These results for the first time implicate previously unknown overlap of control of RpoE/ppGpp with lipid A modification system under phosphate starvation conditions as well as inner core alterations specific to the incorporation of the third Kdo.

Although lipid A modifications by P-EtN and L-Ara4N are known to confer resistance to cationic peptides, the significance of the switch from glycoform I to glycoform IV and V is not known other than synthesis of LPS lacking the O-polysaccharide (smooth to rough variation). Because we constructed several derivatives, which synthesize either only glycoform I (Δ*waaZ*, Δ(*rseA relA spoT*)) or primarily glycoform IV and V (Δ*rseA*, Δ*waaR*), we examined total OMP content specifically of OmpA, OmpF, and OmpC. Consistent with the OMP defect of *rseA* mutants, they exhibited reduced levels of all such OMP. However, *waaR* mutants, despite their similar LPS composition compared with the *rseA* mutants, showed no defect in OMP content. These results were further supported by the lack of any induction of the RpoE pathway in *waaR* mutants. We also show that Δ(*relA spoT*) mutants have reduced OMP content despite normal LPS, but they exhibit a drastic reduction in OMP content and a total dramatic switch to glycoform I in Δ(*relA spoT rseA*) derivative. However, it cannot be due to this LPS switch, because Δ*waaZ* have the wild-type level of OMP despite the synthesis of only glycoform I. Thus, no direct relationship is obvious between switches in glycoforms and OMP content, although both are controlled via RpoE.

Finally, we found *waaZ* is required for growth of Δ(*lpxL lpxM lpxP*) mutants synthesizing tetraacylated lipid A on rich medium at 21–23 °C. A deletion derivative lacking all late acyltransferases Δ(*lpxL lpxM lpxP*) cannot grow on rich medium at 30 °C (4, 36). We show that such a derivative can grow at lower temperatures but requires *waaZ* for growth in rich medium at 21–23 °C. However, Δ(*lpxL lpxM lpxP waaZ*) can be constructed at such lower temperatures but only on minimal medium. These results assume significance, because Δ(*lpxL lpxM lpxP*) mutants synthesize predominantly glycoform V in lipid A modification conditions. Thus, *waaZ* induction confers a subtle growth advantage to Δ(*lpxL lpxM lpx*P) derivatives and may explain its conservation in *E. coli* K-12.

In this work, we also found that LPS of *E. coli* K-12 and *E. coli* B contains a new modification resulting in an addition of 96 mass units in the core region. Our initial results show that this modification arises because of the incorporation of a HexA residue (176.01 Da) on HepIII with an accompanying loss of phosphate residues on HepII. This explains the observed additional 96 mass units presence. HepII is known to require WaaY for phosphorylation, and WaaQ is thought to be a HepIII transferase (38, 39). The rationale for HexA addition on HepIII was supported by the lack of this novel modification in Δ*waaQ* mutants and its enhanced presence in Δ*waaY* derivatives.[3] We have identified a new gene responsible for this modification and is currently under further intensive investigation.

In conclusion, we show induction of PhoB/R, BasS/R, RpoE, and ppGpp jointly control structural alterations of both lipid A and core oligosaccharide composition. Out of these roles of RpoE induction, requirement of ppGpp and modulation of WaaR levels by *rybB* noncoding RNA in controlling LPS composition are addressed and described for the first time. Because Δ*waaR* mutants do not exhibit elevated RpoE induction, have normal OMP content, and yet can have LPS with three Kdo residues (glycoform IV) even without lipid A modifications argue for major control in glycoform IV synthesis at WaaR levels. We also showed that the synthesis of glycoform V results from P-EtN presence on the second Kdo, which leads to a switch of Rha addition to the third Kdo.

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