

Release of the Lipopolysaccharide Deacylase PagL from Latency Compensates for a Lack of Lipopolysaccharide Aminoarabinose Modification-Dependent Resistance to the Antimicrobial Peptide Polymyxin B in *Salmonella enterica*[∇]

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Salmonella enterica modifies its lipopolysaccharide (LPS), including the lipid A portion, to adapt to its environments. The lipid A 3-O-deacylase PagL exhibits latency; deacylation of lipid A is not usually observed in vivo despite the expression of PagL, which is under the control of a two-component regulatory system, PhoP-PhoQ. In contrast, PagL is released from latency in *pmrA* and *pmrE* mutants, both of which are deficient in aminoarabinose-modified lipid A, although the biological significance of this is not clear. The attachment of aminoarabinose to lipid A decreases the net anionic charge at the membrane's surface and reduces electrostatic repulsion between neighboring LPS molecules, leading to increases in bacterial resistance to cationic antimicrobial peptides, including polymyxin B. Here we examined the effects of the release of PagL from latency on resistance to polymyxin B. The *pmrA pagL* and *pmrE pagL* double mutants were more susceptible to polymyxin B than were the parental *pmrA* and *pmrE* mutants, respectively. Furthermore, introduction of the PagL expression plasmid into the *pmrA pagL* double mutant increased the resistance to polymyxin B. In addition, PagL-dependent deacylation of lipid A was observed in a mutant in which lipid A could not be modified with phosphoethanolamine, which partly contributes to the PmrA-dependent resistance to polymyxin B. These results, taken together, suggest that the release of PagL from latency compensates for the loss of resistance to polymyxin B that is due to a lack of other modifications to LPS.

The outer membrane of pathogenic gram-negative bacteria, including *Salmonella enterica*, functions as a barrier to harmful host-derived compounds, such as antimicrobial peptides, gastric secretions, bile, and reactive oxygen. The protection is a consequence of strong lateral interaction between lipopolysaccharide (LPS) molecules, which occupy exclusive areas of the outer leaflet of the outer membrane (reviewed in reference 30). LPS consists of a hydrophobic membrane anchor portion known as lipid A and a nonrepeating core oligosaccharide coupled to a distal polysaccharide (O antigen) that extends from the bacterial surface (reviewed in reference 33). The prototype lipid A structure synthesized by different enteric bacteria, such as *S. enterica* serovar Typhimurium and *Escherichia coli*, is relatively conserved (Fig. 1A). In response to environmental conditions, *S. enterica* covalently modifies its lipid A through palmitoylation, deacylation, the formation of a 2-hydroxymyristate group (hydroxylation), and the addition of 4-amino-4-deoxy-L-arabinose (aminoarabinose) or phosphoethanolamine (7, 33) (Fig. 1B). Similar modifications occur in other gram-negative bacteria, including *E. coli* (44), *Yersinia pestis* (22), and *Pseudomonas aeruginosa* (8), and the modifications are thought to be conserved among gram-negative bacteria for adaptation to their environments.

In *S. enterica* serovar Typhimurium, the two-component regulatory system PhoP-PhoQ induces the expression of genes involved in modifying lipid A (18). PhoQ is a sensor histidine kinase that responds to environmental conditions, including those within mammalian tissues, which are mimicked by magnesium-limited growth medium (9, 10, 14, 28). In response to specific environmental signals, PhoQ phosphorylates PhoP, leading to the activation of *pagL* and *pagP* encoding lipid A 3-O-deacylase and lipid A palmitoyltransferase, respectively (2, 3, 19, 39). Both PagL and PagP are outer membrane enzymes (3, 39), indicating that these enzymes modify mature LPS molecules that are transported to the outer membrane. Lipid A 3-O-deacylation and palmitoylation reduce the ability of lipid A to activate the host Toll-like receptor 4, suggesting that these modifications help pathogens to avoid innate immune recognition (23).

Activation of PhoP-PhoQ leads to the activation of a second two-component regulatory system, PmrA-PmrB (16, 21), which promotes the attachment of aminoarabinose and phosphoethanolamine to phosphate groups on lipid A (Fig. 1). The modification with aminoarabinose is essential for resistance to cationic antimicrobial peptides including polymyxin B (15, 16, 27), which is a cyclic antimicrobial lipopeptide produced by the soil bacterium *Paenibacillus polymyxa* (32). Cationic polymyxin B binds initially to the anionic surface of gram-negative bacteria, in particular to LPS (40), and then invades hydrophobic membranes. The modification of lipid A with aminoarabinose reduces the net anionic charge at this position and the electrostatic repulsion between neighboring LPS molecules (30), and

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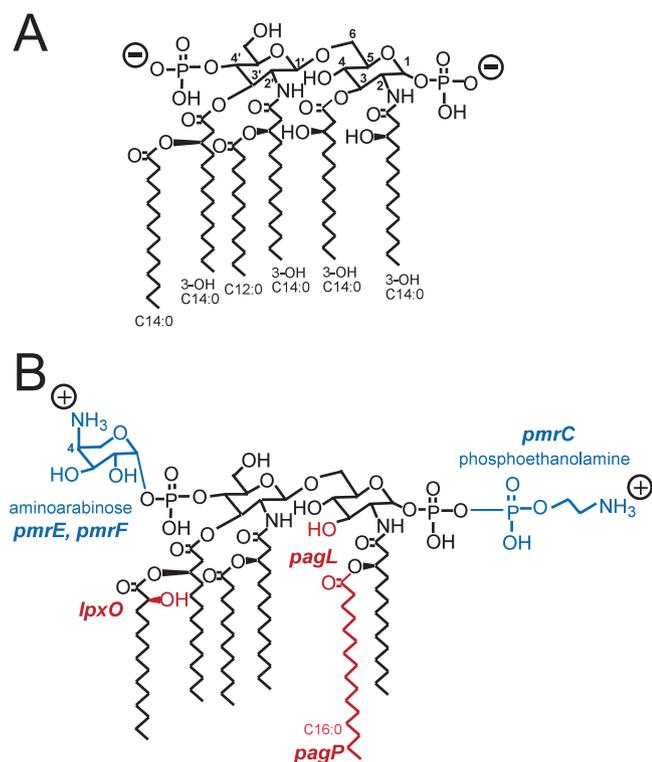


FIG. 1. PhoP-PhoQ- and PmrA-PmrB-regulated lipid A modifications in *S. enterica* serovar Typhimurium. (A) Prototype lipid A of *S. enterica* serovar Typhimurium. (B) Modified lipid A of *S. enterica* serovar Typhimurium. The phosphate residues and acyl chains of lipid A of *S. enterica* can be derivatized in a PhoP-PhoQ- or PmrA-PmrB-regulated manner (reviewed in reference 7). Aminoarabinose and/or phosphoethanolamine groups (shown in blue) can be attached to phosphate residues, under the control of PmrA-PmrB (18, 45). Minor species were present in which the locations of the aminoarabinose and phosphoethanolamine groups were reversed or in which both phosphates were modified with the same substituent (45). Both the *pmrF* operon and *pmrE* are necessary for the PmrA-PmrB-regulated attachment of aminoarabinose to lipid A (15, 45). *pmrC* mediates the PmrA-PmrB-regulated attachment of phosphoethanolamine to lipid A (27). The addition of the palmitate chain is catalyzed by PagP (3, 19), the formation of the 2-hydroxymyristate group requires LpxO (13), and the deacylation at position 3 of lipid A is catalyzed by PagL (39) (shown in red). The *pagL* and *pagP* genes are regulated by PhoP-PhoQ (2), and the *lpxO* gene is partly regulated by PhoP-PhoQ (12, 13). PhoP-PhoQ also activates PmrA-PmrB; therefore, the aminoarabinose and phosphoethanolamine modifications occur under PhoP-PhoQ-activating conditions (18, 44).

these changes are essential for resistance to polymyxin B (15, 27, 31). In a previous study, PmrA-PmrB-regulated lipid A modifications were required for maximal virulence in BALB/c mice infected with *S. enterica* via the oral route (17), suggesting that the changes to the membrane, which were represented by resistance to polymyxin B, were involved in the virulence of salmonellae.

Previous studies demonstrated that deacylated lipid A species were not detected despite the presence of the lipid A deacylase PagL in *S. enterica* (24, 39); therefore, PagL is thought to be latent in the outer membrane under these conditions (24). In contrast, PagL-dependent deacylation of lipid A was detected in the *pmrA*, *pmrE*, and *pmrF* mutants, which are deficient in aminoarabinose-modified lipid A (Fig. 1B)

(24). These results suggest that the modification with aminoarabinose is involved in the latency of PagL, although its biological significance is not clear (24).

In this study, we examined the effects of PagL-dependent deacylation of lipid A using *pmrA pagL* and *pmrE pagL* double mutants. The PagL-null strains were more susceptible to polymyxin B than were the parental strains. These results indicate that the release of PagL from latency compensates for the loss of inducible polymyxin B resistance that is dependent on the modification of lipid A with aminoarabinose.

MATERIALS AND METHODS

Materials. All chemicals were of reagent grade or better. Restriction endonucleases and DNA-modifying enzymes were from New England Biolabs (Beverly, MA) and TAKARA BIO (Ohtsu, Japan). Polymyxin B was from Sigma-Aldrich (St. Louis, MO). Oligonucleotides were prepared commercially by Invitrogen Japan (Tokyo, Japan) and Texas Genomics Japan (Tokyo, Japan). Prestained molecular weight standards were from Apro Science (Naruto, Japan).

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *S. enterica* serovar Typhimurium strain 14028s (American Type Culture Collection, Manassas, VA) was used as the wild-type strain in this study.

Bacteria were grown at 37°C with aeration in N-minimal medium [5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 0.28% (vol/vol) glycerol, 0.1% (wt/vol) Casamino Acids, 2 mg of thiamine/liter, and 0.1 M Tris/HCl (pH 7.4 or 5.8)] supplemented with 10 μM MgCl₂ (10, 24, 27, 29). Ampicillin (100 μg/ml) was used for the cultivation of strains transformed with the plasmid pBAD24 (20) and its derivatives, and ampicillin (10 μg/ml) was used for the cultivation of strains transformed with the low-copy-number vector pWKS30 (41) and its derivatives. Bacterial colonies were picked out and grown overnight at 37°C in 1 to 10 ml of growth medium. The overnight cultures were diluted 1:10 with fresh growth medium and then grown at 37°C for 24 h. After the cultivation, stationary-phase cells were used for further analysis.

Bacterial genetic and molecular biology techniques. Phage P22-mediated transduction was performed as described previously (6). Plasmid DNA was introduced into bacterial strains by electroporation using *E. coli* Pulser (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Recombinant DNA techniques were performed according to standard protocols (37).

Construction of nonpolar *pagL* or *pmrC* deletion mutants. Nonpolar deletion mutant strains were generated according to the method established by Datsenko

TABLE 1. *S. enterica* serovar Typhimurium strains and plasmids

Strain or plasmid	Description	Reference or source
<i>S. enterica</i> serovar Typhimurium		
ATCC 14028s	Wild type	ATCC
JSG421	<i>pmrA</i> ::Tn10d	16
KCS041	<i>pmrE1</i> ::Tn10d	24
KCS178	Δ <i>pmrC</i> ::Kan	This work
KCS180	Δ <i>pmrC</i>	This work
KCS202	Δ <i>pagL</i> ::Kan	This work
KCS203	<i>pmrA</i> ::Tn10d Δ <i>pagL</i> ::Kan	This work
KCS204	<i>pmrE1</i> ::Tn10d Δ <i>pagL</i> ::Kan	This work
KCS205	Δ <i>pmrC</i> Δ <i>pagL</i> ::Kan	This work
KCS208	<i>pmrA</i> ::Tn10d Δ <i>pagL</i>	This work
KCS209	<i>pmrE1</i> ::Tn10d Δ <i>pagL</i>	This work
KCS210	Δ <i>pmrC</i> Δ <i>pagL</i>	This work
KCS216	Δ <i>pagL</i>	This work
Plasmid		
pWKS30	Low-copy-number cloning vector	41
pWLP23	pWKS30 containing <i>pagL</i> including 79-bp upstream region	39
pBAD24	Inducible expression plasmid	20
pKK28	pBAD24 containing <i>pmrC</i> -coding region	This work
pWLP21	pBluescript KS II+ containing <i>pagL</i>	39
pKK7	pEt15b containing <i>pagL</i> , which bears His ₆ epitope instead of 1 to 18 amino acid residues at N terminus	This work

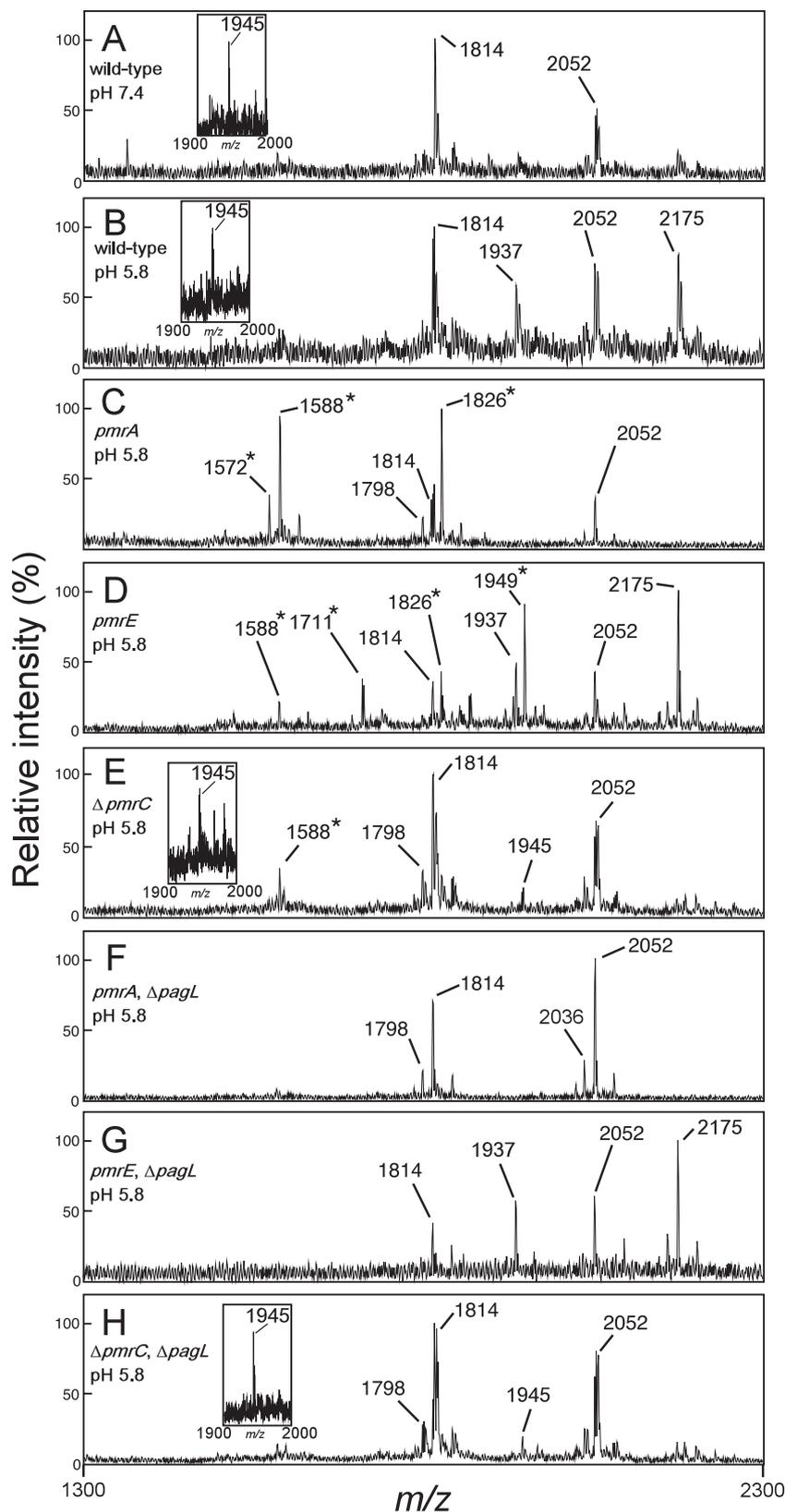


FIG. 2. MALDI-TOF mass spectrometry of lipid A purified from *S. enterica* serovar Typhimurium strains. The wild-type (ATCC 14028s), *pmrA* (JSG421), *pmrE* (KCS041), $\Delta pmrC$ (KCS180), $\Delta pagL$ (KCS216), *pmrA* $\Delta pagL$ (KCS208), *pmrE* $\Delta pagL$ (KCS209), and $\Delta pmrC$ $\Delta pagL$ (KCS210) strains were cultivated in growth medium at pH 7.4 (A) or 5.8 (B to H). The *m/z* values of lipid A species are shown, and those that represent deacylated lipid A species are denoted by asterisks. Insets in panels A, B, E, and H show results of MALDI-TOF mass spectrometry of lipid A using 2-5-dihydroxybenzoic acid matrices. The structural interpretations of lipid A species are summarized in Table 2.

and Wanner (5). A DNA fragment containing the kanamycin resistance cassette of pKD4 (5) was amplified by PCR with EX *Taq* DNA polymerase (TAKARA BIO). Primers KK85 (GCAAGGGCAACAAGCATCAGATCTCTTTTGCTGCGGGAGAAAGTATAAGAGTGTAGGCTGGAGCTGCTTC) and KK86 (TATGCCCTGAATTTTTATCCGTAAGTGATCCATTCGAGAAATGCCG GATACATATGAATATCCTCTTAG) were designed to delete 350 bp of the 558-bp *PagL*-coding DNA region. Primers KK71 and KK72, which were identical to primers 2635 and 2636 (27), respectively, were used for the generation of a nonpolar *pmrC*-deletion mutant ($\Delta pmrC$). The PCR products were introduced into *S. enterica* serovar Typhimurium strain 14028s carrying the plasmid pKD46 (5), which encodes Red recombinase. Generation of the $\Delta pagL::kan$ allele in the resulting strain, KCS202, and the $\Delta pmrC::kan$ allele in KCS178 was confirmed by PCR analysis. Kanamycin resistance cassettes were eliminated from the $\Delta pagL::kan$ and $\Delta pmrC::kan$ strains by using the plasmid pCP20 (5), and the elimination was confirmed by PCR analysis.

Construction of *PmrC* expression plasmid. The *pmrC*-coding region was amplified from genomic DNA of strain 14028s by PCR with *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). The primers used were KK33 (GATTG GATCCGTCGCGTTTGTGTATTGCATCTGG) and KK35 (ATCATCCCAT GGTAATGGACGCATCAACATGTTAAAGCG). The amplified DNA fragment was cloned into *Nco*I and *Hind*III sites of pBAD24 (20) under the control of the arabinose *P_{BAD}* promoter, and the resulting expression construct was named pKK28. The insert in the plasmid construct was verified by sequencing.

Preparation of lipid A. The lipid A used for mass spectrometry was purified as described previously (43). In brief, cells collected from 25 ml of culture were resuspended in 500 μ l of Tri-reagent (Molecular Research Center Inc., Cincinnati, OH). After incubation for 30 min at room temperature, 100 μ l of chloroform was added. After 15 min, the mixture was centrifuged, and the aqueous phase was recovered. LPS was extracted three times by the addition of 500 μ l of water to the organic phase, and the aqueous phase containing LPS was dried up with a vacuum concentrator. Five hundred microliters of 10 mM sodium acetate buffer (pH 4.5) containing 1% sodium dodecyl sulfate (SDS) was added to the dried LPS, and then the LPS was hydrolyzed to remove sugar chains from lipid A by incubation at 100°C for 1 h (35) followed by drying. The dried lipid A was washed once with 0.02 N HCl in 95% ethanol, and three times with 95% ethanol. The washed lipid A was dried up with a vacuum concentrator and then used for mass spectrometric analysis.

Alternatively, the lipid A used for mass spectrometry (insets in Fig. 2A, B, E, and H) was prepared from LPS as described previously (4). In brief, LPS purified from 25 ml of cell culture by using a LPS extraction kit (iNtRON Biotechnologies Inc., Seongnam-Si, Korea) was hydrolyzed for 3 h at 95°C in 150 μ l of 100 mM sodium acetate buffer (pH 4.5). Then, 600 μ l of a chloroform/methanol mixture (1:2, vol/vol), 200 μ l of chloroform, and 100 μ l of phosphate-buffered saline were added in succession, and the lipid A fraction (chloroform phase) was dried under a stream of nitrogen gas.

Mass spectrometry. Dried lipid A was dissolved in 20 mg/ml 5-chloro-2-mercaptobenzothiazole matrices in chloroform/methanol (1:1, vol/vol). Alternatively, it was dissolved in chloroform/methanol (1:2, vol/vol) and then mixed with 2,5-dihydroxybenzoic acid matrices (77 mg/ml in methanol) at a ratio of 1:1 (insets in Fig. 2A, B, E, and H). The mixtures were allowed to dry at room temperature on the sample plate prior to analysis. Spectra were obtained in the negative reflection mode using a matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) Voyager-DE STR mass spectrometer (Applied Biosystems Japan, Tokyo, Japan). Each spectrum was the average of 200 shots. Structural interpretations of lipid A species detected by mass spectrometry in this study are summarized in Table 2.

Anti-*PagL* antibodies. The His₆-tagged recombinant *PagL* protein was used as an antigen with which to produce polyclonal antibody. The recombinant protein was prepared as follows. The *pagL*-coding region was amplified from the plasmid pWLP21 (39) with *Pfu* Turbo DNA polymerase. The primers used for PCR were KK14 (GATTGGATCCGTCGCGTTTGTGTATTGCATCTGG) and KK15 (CGCGGATCCTCAGAAATTATACTAATTGA). A *PagL* expression plasmid was designed to express a partial *PagL* protein, which lacks 18 amino acid residues at the N terminus. The amplified DNA fragment was cloned into *Nde*I and *Bam*HI sites of pET15b (Novagen, Darmstadt, Germany), which was designed to produce a recombinant protein carrying a His₆ epitope at the N terminus. The resulting *PagL* expression construct pKK7 was introduced into *E. coli* strain BL21(DE3). The transformant grown in Luria-Bertani medium containing ampicillin (100 μ g/ml) was cultivated for 4 h in the presence of 1 mM isopropyl β -D-thiogalactoside to induce the expression of the recombinant *PagL* protein. The His₆-tagged recombinant *PagL* protein was purified from *E. coli* lysate with Ni-nitrilotriacetic agarose (QIAGEN) in accordance with the manufacturer's instructions. Immunization of a rabbit and preparation of serum were

TABLE 2. Structural interpretations of lipid A species detected by mass spectrometry in this study

<i>m/z</i>	Lipid A modifications ^a
1572	Deacylation
1588	Deacylation and hydroxylation
1668	Deacylation and hydroxylation (pyrophosphate lipid A) ^b
1711	Deacylation, hydroxylation, and phosphoethanolamine attachment
1798	(Unmodified)
1810	Deacylation and palmitoylation
1814	Hydroxylation
1826	Deacylation, palmitoylation, and hydroxylation
1894	Hydroxylation (pyrophosphate lipid A) ^b
1921	Phosphoethanolamine attachment
1937	Hydroxylation and phosphoethanolamine attachment
1945	Aminoarabinose attachment and hydroxylation
1949	Deacylation, palmitoylation, hydroxylation, and phosphoethanolamine attachment
2036	Palmitoylation
2052	Palmitoylation and hydroxylation
2132	Hydroxylation and palmitoylation (pyrophosphate lipid A) ^b
2159	Palmitoylation and phosphoethanolamine attachment
2175	Palmitoylation, hydroxylation, and phosphoethanolamine attachment

^a Interpretations were based on previous papers (17, 24, 39, 44).

^b Pyrophosphate lipid A has a pyrophosphate residue instead of a monophosphate residue at position 1 of the disaccharide glucosamine of lipid A. How the synthesis of pyrophosphate lipid A is regulated is not known (44).

performed by Charles River Japan (Yokohama, Japan). The antibody against *PagL* protein was affinity purified as described previously (25) using the purified recombinant *PagL* protein.

SDS-polyacrylamide gel electrophoresis and Western blotting. Proteins were fractionated by SDS-12.5% polyacrylamide gel electrophoresis under reducing conditions (26). Proteins separated on the gel were stained with Coomassie blue. For the Western blot analysis, proteins separated on the gel were electroblotted onto a nitrocellulose membrane in 25 mM Tris–192 mM glycine–0.02% SDS–20% methanol at 22 V/cm for 60 min. The blot was then incubated with affinity-purified anti-*PagL* antibodies and subsequently with anti-rabbit immunoglobulin G linked to horseradish peroxidase. Cross-reactive proteins were detected with ECL Western blotting detection reagents (GE Healthcare Bio-Sciences, Piscataway, NJ).

Polymyxin B killing assay. Harvested cells from 5 ml of culture were washed two times with 1 ml of Luria-Bertani medium and then suspended with Luria-Bertani medium at an optical density at 600 nm of 0.12. The bacterial suspension (475 μ l) was mixed with 25 μ l of water or a polymyxin B solution and stood at 37°C. After 1 h, the cells were diluted 1:10,000 with Luria-Bertani medium and the diluted suspension (50 μ l) was plated on a Luria-Bertani agar plate to determine the number of CFU. The dilution eliminated the killing effect of polymyxin B on the plates (data not shown). Percent survival was calculated as follows: (CFU of polymyxin B-treated culture/CFU of water-treated culture) \times 100.

RESULTS

***PagL*-dependent lipid A deacylation was observed in *Salmonella pmrA* and *pmrE* mutant strains grown in mild acid medium but not in the wild-type strain.** Previously, Kawasaki et al. showed that *PagL*-dependent 3-O-deacylation of lipid A was clearly observed in mutant strains of *S. enterica* serovar Typhimurium deficient in aminoarabinose-modified lipid A, including the *pmrA*, *pmrE*, and *pmrF* strains, when grown in defined minimal magnesium-limited neutral medium (pH 7.4), but not in the wild-type strain (24). We examined whether or not aminoarabinose-dependent latency of *PagL* is also observed in *S. enterica* grown in defined minimal magnesium-limited mild acid medium (pH 5.8), which is often used to mimic host microenvironments, such as phagolysosomes of

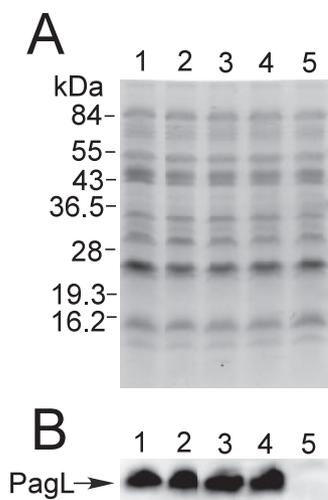


FIG. 3. Expression levels of PagL protein were similar among *Salmonella* wild-type, *pmrA*, *pmrE*, and $\Delta pmrC$ strains grown in the mild acid medium. Ten-microgram samples of membrane proteins prepared from strains cultivated in mild acid medium (pH 5.8) containing 10 μ M $MgCl_2$ were subjected to SDS-polyacrylamide gel (12.5%) electrophoresis and analyzed with staining (A) or by Western blotting (B). Lanes: 1, wild-type strain; 2, *pmrA* strain (JSG421); 3, *pmrE* strain (KCS041); 4, $\Delta pmrC$ strain (KCS180); 5, $\Delta pagL$ strain (KCS216).

macrophages (1, 10, 12, 27, 38, 42). In a MALDI-TOF mass spectrometric analysis, deacylated species were not detected in the lipid A prepared from the wild-type strain grown in the mild acid medium or in the neutral medium (Fig. 2A and B). In addition, significant amounts of lipid A species modified with phosphoethanolamine (m/z 1937 and 2175) were detected in the wild-type strain cultivated in the mild acid medium but not in that cultivated in the neutral medium (Fig. 2A and B). These observations were consistent with the finding that the wild-type *Salmonella* strain modifies its lipid A with phosphoethanolamine in defined minimal magnesium-limited mild acid medium (pH 5.8) (27). Aminoarabinose-modified lipid A (m/z 1945) was clearly detected in the wild-type strain (insets of Fig. 2A and B) but not in the *pmrA* and *pmrE* mutant strains (data not shown). On the other hand, deacylated lipid A species (m/z 1572, 1588, 1711, 1826, and 1949) were detected in the *pmrA* and *pmrE* mutant strains cultivated in the mild acid medium (Fig. 2C and D) as well as in the neutral medium (data not shown) (24). 3-O-deacylated lipid A species were not observed in the nonpolar *pagL* deletion ($\Delta pagL$) mutant strains (*pmrA* $\Delta pagL$ and *pmrE* $\Delta pagL$) cultivated in the mild acid medium (Fig. 2F and G), indicating that the deacylation observed in the *pmrA* and *pmrE* mutants is dependent on PagL. Furthermore, PagL protein levels were similar among the wild-type, *pmrA*, and *pmrE* strains grown in the mild acid medium (Fig. 3). These results indicate that PagL is latent in *S. enterica* strains grown in the mild acid medium as well as in the neutral medium.

PagL mutant strains are more susceptible to polymyxin B than the parental strains. In *S. enterica*, *pmrA*-*pmrB*-dependent attachment of aminoarabinose to lipid A, which decreases the net charge of LPS and the electrostatic repulsion between neighboring LPS molecules, is responsible for the resistance to the cationic antimicrobial peptide polymyxin B (15, 31). It is

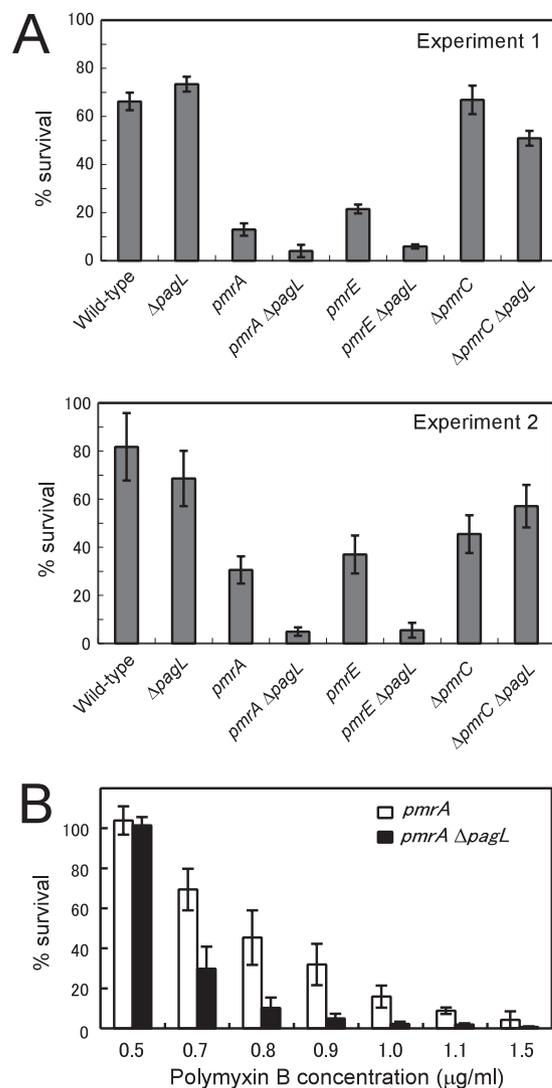


FIG. 4. The *pagL* mutants were more susceptible to polymyxin B than were the parental mutants. (A) The wild-type (14028s), $\Delta pagL$ (KCS216), *pmrA* (JSG421), *pmrA* $\Delta pagL$ (KCS208), *pmrE* (KCS041), *pmrE* $\Delta pagL$ (KCS209), $\Delta pmrC$ (KCS180), and $\Delta pmrC$ $\Delta pagL$ (KCS210) strains grown in the growth medium (pH 5.8) were treated with 1 μ g/ml of polymyxin B. Percent survival was determined as described in Materials and Methods. The results shown are for two independent sets of experiments (experiments 1 and 2). (B) The *pmrA* and *pmrA* $\Delta pagL$ mutant strains cultivated in the growth medium (pH 5.8) were treated with the indicated concentrations of polymyxin B. Percent survival is the average of triplicate measurements, and error bars indicate standard deviations.

plausible that the release of PagL from latency observed in the *pmrA* and *pmrE* mutant strains, which lack aminoarabinose-modified lipid A, increases the resistance of salmonellae to polymyxin B. Mutant strains *pmrA* $\Delta pagL$ and *pmrE* $\Delta pagL$ grown in the mild acid medium were more susceptible to polymyxin B (1.0 μ g/ml) than were the parental *pmrA* and *pmrE* strains, respectively, suggesting that PagL-dependent deacylation of lipid A in the *pmrA* and *pmrE* mutant strains increased resistance to polymyxin B (Fig. 4A). To confirm the involvement of the PagL-dependent deacylation in the resistance, the *pmrA* and *pmrA* $\Delta pagL$ mutant strains grown in the mild acid

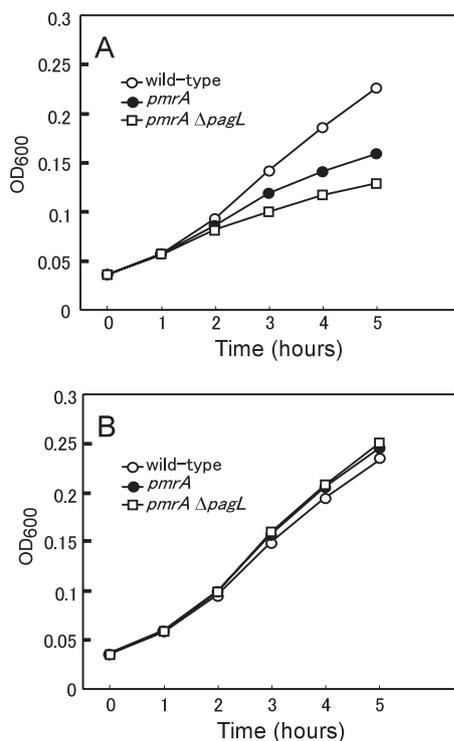


FIG. 5. Growth of *S. enterica* serovar Typhimurium strains in the presence of polymyxin B. The wild-type (14028s), *pmrA* (JSG421), and *pmrA* Δ*pagL* (KCS208) strains cultivated in the growth medium (pH 5.8) as described in Materials and Methods were diluted 1:10 with fresh growth medium (pH 5.8). Then, the cells were grown at 37°C in the presence (A) or absence (B) of 1 µg/ml of polymyxin B. The results shown are representative of at least two independent experiments. OD₆₀₀, optical density at 600 nm.

medium were treated with various concentrations of polymyxin B. As shown in Fig. 4B, the *pmrA* strain was more resistant to polymyxin B than was *pmrA* Δ*pagL*. These results suggest that the PagL-dependent deacylation of lipid A in the *pmrA* strain increased resistance to polymyxin B. Mutant strains *pmrA* and *pmrE* were more susceptible to polymyxin B than was the wild-type strain (Fig. 4A), and these observations are consistent with the previous finding that *pmrA* and *pmrE* are essential for developing resistance to polymyxin B (15).

To further confirm that the PagL-dependent deacylation of lipid A increases resistance to polymyxin B in the mutant strains, the wild-type, *pmrA* mutant, and *pmrA* Δ*pagL* mutant strains were grown in the presence or absence of 1 µg/ml of polymyxin B. In the presence of the polymyxin B, the growth rate of the *pmrA* Δ*pagL* strain was lower than that of the *pmrA* strain, which was in turn lower than that of the wild-type strain (Fig. 5A). In contrast, the *pmrA* Δ*pagL* and *pmrA* strains had a rate of growth similar to that of the wild-type strain in the absence of polymyxin B (Fig. 5B), indicating that the *pmrA* Δ*pagL* strain is more susceptible to polymyxin B than is the parental *pmrA* strain. These results, taken together, indicate that PagL-dependent deacylation of lipid A in the *pmrA* and *pmrE* strains increases their resistance to the cationic antimicrobial peptide polymyxin B.

Introduction of the PagL expression construct into a *pagL* mutant increased polymyxin B resistance. We introduced a

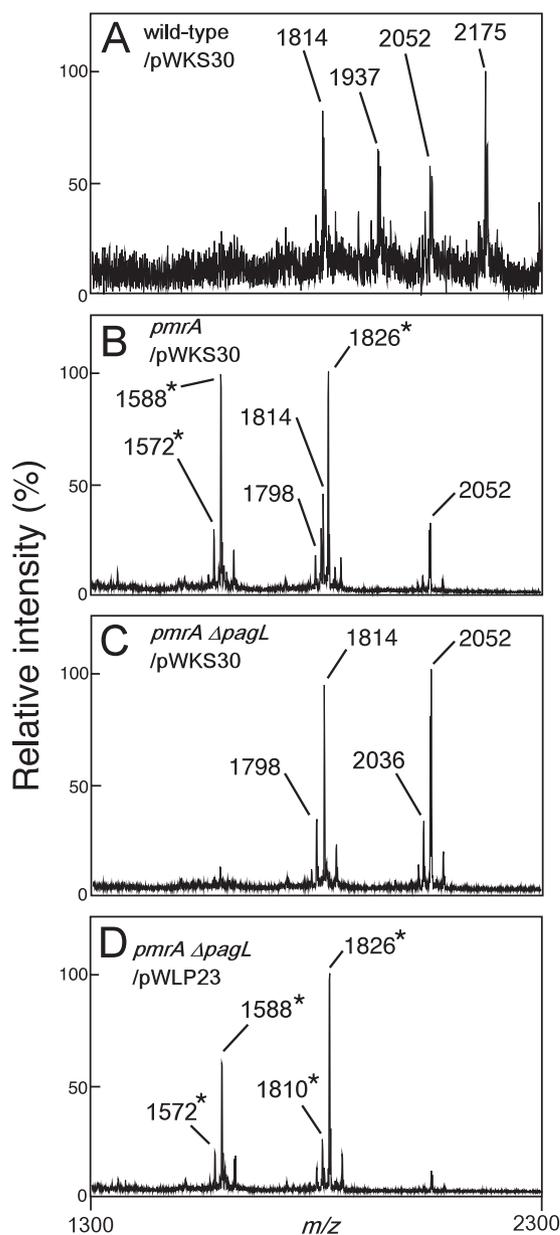


FIG. 6. MALDI-TOF mass spectrometry of lipid A purified from *pagL*-null mutant strains transformed with a PagL expression plasmid. Wild-type (ATCC 14028s), *pmrA* (JSG421), and *pmrA* Δ*pagL* (KCS208) strains transformed with a PagL expression construct pWLP23 or the control vector pWKS30 were grown in the growth medium (pH 5.8), and their lipid A was analyzed with a MALDI-TOF mass spectrometer. The *m/z* values of lipid A species are shown, and those that represent deacylated lipid A species are denoted by asterisks. The structural interpretations of lipid A species are summarized in Table 2.

PagL expression plasmid construct into the *pmrA* Δ*pagL* strain in order to examine whether or not expression of PagL in the *pmrA* Δ*pagL* mutant increased the resistance to polymyxin B. At first, we examined lipid A species prepared from *pmrA* Δ*pagL* transformed with pWLP23, a derivative of the low-copy-number vector pWKS30 containing the *pagL* gene. MALDI-TOF mass spectrometry revealed that almost all lipid A molecules (*m/z* 1572, 1588, 1810, and 1826) were 3-O-deac-

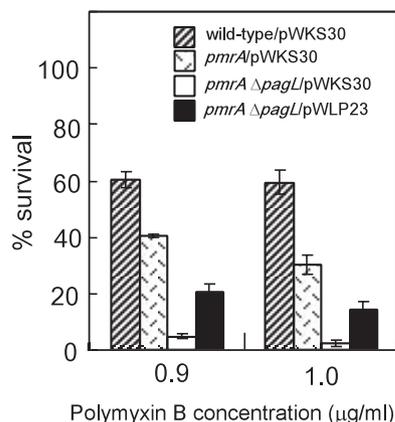


FIG. 7. Introduction of PagL expression plasmids increased the resistance of the *pagL*-null mutant strain to polymyxin B. Wild-type (ATCC 14028s), *pmrA* (JSG421), and *pmrA* Δ*pagL* (KCS208) strains transformed with the control vector (pWKS30) or PagL expression construct (pWLP23) were cultivated in the growth medium (pH 5.8) and then treated with 0.9 or 1.0 µg/ml of polymyxin B. Percent survival is the average of triplicate measurements, and the error bars indicate standard deviations.

ylated in *pmrA* Δ*pagL*/pWLP23 (Fig. 6D). In contrast, non-deacylated lipid A species (*m/z* 1798, 1814, and 2052) as well as deacylated lipid A species were apparently observed in the *pmrA* strain transformed with the control vector pWKS30 (*pmrA*/pWKS30) (Fig. 6B), indicating that the level of deacylated lipid A molecules was higher in *pmrA* Δ*pagL*/pWLP23 than in *pmrA*/pWKS30. Compared with *pmrA* Δ*pagL*/pWKS30, *pmrA* Δ*pagL*/pWLP23 was resistant to polymyxin B treatment (Fig. 7), indicating that the deacylation increased the resistance. It is noteworthy that *pmrA* Δ*pagL*/pWLP23 was more susceptible to polymyxin B treatment than was *pmrA*/pWKS30 (Fig. 7), indicating that the susceptibility of the *pmrA* Δ*pagL* strain was not complemented completely on introduction of the PagL expression plasmid construct pWLP23. Since the level of deacylated lipid A was lower in *pmrA*/pWKS30 than in *pmrA* Δ*pagL*/pWLP23 as described above, it appears that not only the existence of deacylated lipid A molecules in the outer membrane but also the balance of modified lipid A species or the amount of deacylated lipid A might be important for the susceptibility.

Modification of lipid A with phosphoethanolamine is partly involved in the latency of PagL. The attachment of phosphoethanolamine to lipid A, a modification mediated by the PmrA-PmrB-activated gene product PmrC, partly contributes to the *pmrA*-dependent resistance to polymyxin B of *S. enterica* serovar Typhimurium (27). Interestingly, a 3-O-deacylated lipid A species (*m/z* 1588) was observed in the *pmrC* mutant cultivated in the mild acid medium (Fig. 2E), suggesting that this modification of lipid A is partly involved in the latency of PagL. Although the effect of *pmrC*'s deletion on the resistance to polymyxin B was not observed (Fig. 4A), the lack of phosphoethanolamine-modified lipid A might be enough to release PagL from latency in the membrane. To confirm the involvement of the modification of lipid A with phosphoethanolamine in the latency of PagL, a PmrC expression plasmid construct was introduced into the *pmrE* and *pmrA* mutants. Lipid A was modified in the mutants transformed with PmrC expression plasmid, and no 3-O-deacylation was ob-

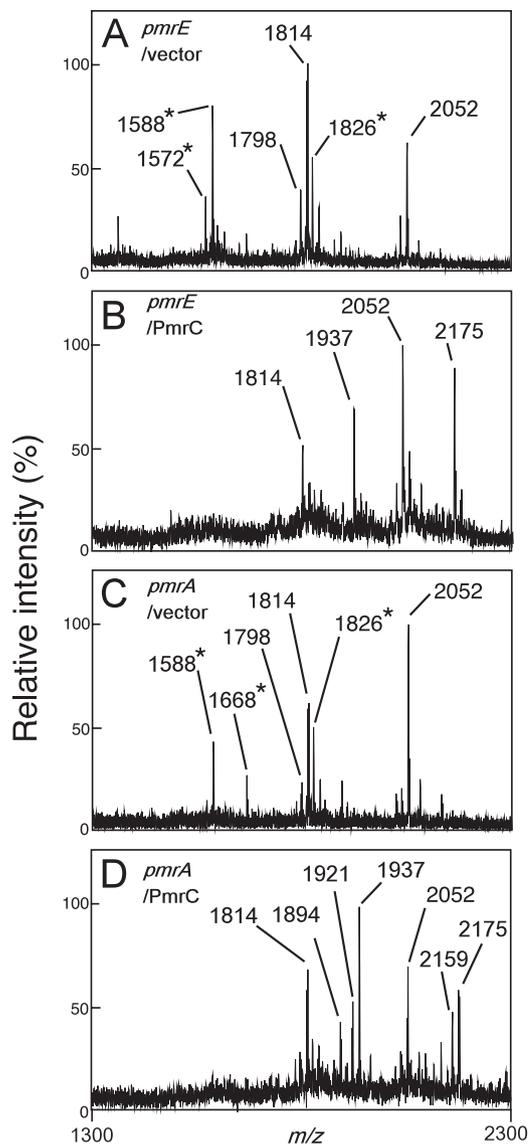


FIG. 8. The attachment of phosphoethanolamine inhibited the deacylation of lipid A. The *pmrE* (KCS041) or *pmrA* (JSG421) strain transformed with pBAD24 (control vector) or pKK28 (PmrC) was cultivated in growth medium (pH 7.4) containing 0.2% (wt/vol) arabinose to induce PmrC expression under the control of the P_{BAD} promoter, and then its lipid A was analyzed with a MALDI-TOF mass spectrometer. The *m/z* values of lipid A species are shown, and those that represent deacylated lipid A species are denoted by asterisks. The structural interpretations of lipid A species are summarized in Table 2.

served (Fig. 8). These results, taken together, indicate that the attachment of phosphoethanolamine as well as aminoarabinose to lipid A is involved in the latency of PagL.

DISCUSSION

Previously, Kawasaki et al. demonstrated that the *Salmonella* lipid A deacylase PagL is dormant in the outer membrane and that the modification of lipid A with aminoarabinose is involved in the latency (24), although its biological significance was not apparent. In this paper, we showed that the release of

PagL from latency compensates for the loss of the aminoarabinose-dependent resistance to polymyxin B.

Gram-negative pathogenic bacteria such as *S. enterica* serovar Typhimurium adapt to host microenvironments through changes at the cellular surface, including changes to LPS. The PmrA-PmrB-regulated attachment of aminoarabinose to lipid A alters the net charge at the surface, which reduces electrostatic repulsion between neighboring LPS molecules. These changes may help *S. enterica* to adapt to host microenvironments because they increase resistance to cationic antimicrobial peptides, including polymyxin B, although the machinery responsible for the resistance remains to be elucidated. In this study, we demonstrated that PagL-dependent lipid A deacylation increases polymyxin B resistance in mutants lacking aminoarabinose-modified lipid A. In addition, introduction of a PagL expression construct into the *pmrA* Δ *pagL* strain increased resistance to polymyxin B. These results, taken together, indicate that lipid A deacylation is involved in polymyxin B resistance under specific conditions. However, the *pmrA* Δ *pagL* strain transformed with the PagL expression construct was more susceptible to polymyxin B than the *pmrA* strain transformed with the control vector. We speculated that the introduction of a PagL expression construct induced deacylation of lipid A, but the quality of the deacylation was not controlled; the quantity of deacylated lipid A molecules and the balance of lipid A modifications in the bacterial membrane were not well controlled by the transformation, although we used the *pagL* promoter region and a low-copy-number vector for the expression construct. The uncontrolled deacylation might not be sufficient for complementation. The deacylation of lipid A helps salmonellae to resist antimicrobial peptides including polymyxin B under certain conditions, but the molecular machinery that increases the resistance is complex and remains to be elucidated.

We demonstrated that a lack of aminoarabinose-dependent resistance to polymyxin B is compensated for by the induction of lipid A 3-O-deacylation. These observations suggest that bacterial lipid A modifications were flexible: a defect in a specific type of modification was functionally compensated by another type of modification. Similar flexibility has been reported previously. Zhou et al. showed that phosphoethanolamine-modified lipid A species are much less abundant than aminoarabinose-modified ones in wild-type *S. enterica* serovar Typhimurium (45). In contrast, phosphoethanolamine-modified lipid A species accumulate at high levels in *pmrA*-constitutive strains that harbor a null mutation in either *pmrE* or *pmrF*, which is essential for the modification of lipid A with aminoarabinose (45). Since the modification of lipid A with phosphoethanolamine as well as aminoarabinose is involved in *pmrA*-regulated resistance to polymyxin B (15, 27), induction of the phosphoethanolamine-type modification might be beneficial for salmonellae that lack aminoarabinose-modified lipid A to increase their resistance to cationic antimicrobial peptides including polymyxin B. Although the molecular machinery underlying this regulation remains to be elucidated, *Salmonella* lipid A-modifying enzymes might directly sense the conditions and then regulate their activity in order to adapt to their environment.

Recently, Tommassen and coworkers reported the crystal structure of *P. aeruginosa* PagL and identified the active site of

PagL (11, 36). They speculated that PagL is active as a single molecule and that dimerization might silence it (36). Changes in the outer membrane, including the modification of lipid A with aminoarabinose, might affect the structure of the outer membrane protein PagL and regulate its activity. Recently, a lipid A 3'-O-deacylase, LpxR, was identified in *S. enterica* serovar Typhimurium (34). Interestingly, LpxR is similar to PagL in that it is an outer membrane lipase and latent; LpxR deacylates lipid A in vitro, but 3'-O-deacylated lipid A species have not been reported for this organism (34). Elucidation of the latency of an outer membrane enzyme might provide new insight into bacterial membrane remodeling, which is very important for pathogenic bacterial adaptation to host tissues.

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REFERENCES

- Alpuche Aranda, C. M., J. A. Swanson, W. P. Loomis, and S. I. Miller. 1992. Salmonella typhimurium activates virulence gene transcription within acidified macrophage phagosomes. *Proc. Natl. Acad. Sci. USA* **89**:10079–10083.
- Belden, W. J., and S. I. Miller. 1994. Further characterization of the PhoP regulon: identification of new PhoP-activated virulence loci. *Infect. Immun.* **62**:5095–5101.
- Bishop, R. E., H. S. Gibbons, T. Guina, M. S. Trent, S. I. Miller, and C. R. Raetz. 2000. Transfer of palmitate from phospholipids to lipid A in outer membranes of gram-negative bacteria. *EMBO J.* **19**:5071–5080.
- Brett, P. J., M. N. Burtnick, D. S. Snyder, J. G. Shannon, P. Azadi, and F. C. Gherardini. 2007. Burkholderia mallei expresses a unique lipopolysaccharide mixture that is a potent activator of human Toll-like receptor 4 complexes. *Mol. Microbiol.* **63**:379–390.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- Davis, R. W., D. Botstein, and J. R. Roth. 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
- Ernst, R. K., T. Guina, and S. I. Miller. 2001. Salmonella typhimurium outer membrane remodeling: role in resistance to host innate immunity. *Microbes Infect.* **3**:1327–1334.
- Ernst, R. K., E. C. Yi, L. Guo, K. B. Lim, J. L. Burns, M. Hackett, and S. I. Miller. 1999. Specific lipopolysaccharide found in cystic fibrosis airway Pseudomonas aeruginosa. *Science* **286**:1561–1565.
- Fields, P. L., E. A. Groisman, and F. Heffron. 1989. A Salmonella locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243**:1059–1062.
- Garcia Vescovi, E., F. C. Soncini, and E. A. Groisman. 1996. Mg²⁺ as an extracellular signal: environmental regulation of Salmonella virulence. *Cell* **84**:165–174.
- Geurtsen, J., L. Steeghs, J. T. Hove, P. van der Ley, and J. Tommassen. 2005. Dissemination of lipid A deacylases (pagL) among gram-negative bacteria: identification of active-site histidine and serine residues. *J. Biol. Chem.* **280**:8248–8259.
- Gibbons, H. S., S. R. Kalb, R. J. Cotter, and C. R. Raetz. 2005. Role of Mg²⁺ and pH in the modification of Salmonella lipid A after endocytosis by macrophage tumour cells. *Mol. Microbiol.* **55**:425–440.
- Gibbons, H. S., S. Lin, R. J. Cotter, and C. R. Raetz. 2000. Oxygen requirement for the biosynthesis of the S-2-hydroxymyristate moiety in Salmonella typhimurium lipid A. Function of LpxO, a new Fe²⁺/alpha-ketoglutarate-dependent dioxygenase homologue. *J. Biol. Chem.* **275**:32940–32949.
- Groisman, E. A., E. Chiao, C. J. Lipps, and F. Heffron. 1989. Salmonella typhimurium phoP virulence gene is a transcriptional regulator. *Proc. Natl. Acad. Sci. USA* **86**:7077–7081.
- Gunn, J. S., K. B. Lim, J. Krueger, K. Kim, L. Guo, M. Hackett, and S. I. Miller. 1998. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. *Mol. Microbiol.* **27**:1171–1182.
- Gunn, J. S., and S. I. Miller. 1996. PhoP-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. *J. Bacteriol.* **178**:6857–6864.
- Gunn, J. S., S. S. Ryan, J. C. Van Velkinburgh, R. K. Ernst, and S. I. Miller. 2000. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance,

- and oral virulence of *Salmonella enterica* serovar Typhimurium. *Infect. Immun.* **68**:6139–6146.
18. Guo, L., K. B. Lim, J. S. Gunn, B. Bainbridge, R. P. Darveau, M. Hackett, and S. I. Miller. 1997. Regulation of lipid A modifications by *Salmonella* typhimurium virulence genes *phoP-phoQ*. *Science* **276**:250–253.
 19. Guo, L., K. B. Lim, C. M. Poduje, M. Daniel, J. S. Gunn, M. Hackett, and S. I. Miller. 1998. Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. *Cell* **95**:189–198.
 20. Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith. 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J. Bacteriol.* **177**:4121–4130.
 21. Kato, A., and E. A. Groisman. 2004. Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. *Genes Dev.* **18**:2302–2313.
 22. Kawahara, K., H. Tsukano, H. Watanabe, B. Lindner, and M. Matsuura. 2002. Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. *Infect. Immun.* **70**:4092–4098.
 23. Kawasaki, K., R. K. Ernst, and S. I. Miller. 2004. 3-O-deacylation of lipid A by PagL, a PhoP/PhoQ-regulated deacylase of *Salmonella typhimurium*, modulates signaling through Toll-like receptor 4. *J. Biol. Chem.* **279**:20044–20048.
 24. Kawasaki, K., R. K. Ernst, and S. I. Miller. 2005. Inhibition of *Salmonella enterica* serovar Typhimurium lipopolysaccharide deacylation by aminoarabinose membrane modification. *J. Bacteriol.* **187**:2448–2457.
 25. Kawasaki, K., O. Kuge, Y. Yamakawa, and M. Nishijima. 2001. Purification of phosphatidylglycerophosphate synthase from Chinese hamster ovary cells. *Biochem. J.* **354**:9–15.
 26. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
 27. Lee, H., F. F. Hsu, J. Turk, and E. A. Groisman. 2004. The *PmrA*-regulated *pmrC* gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J. Bacteriol.* **186**:4124–4133.
 28. Miller, S. I., A. M. Kukral, and J. J. Mekalanos. 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. *Proc. Natl. Acad. Sci. USA* **86**:5054–5058.
 29. Nelson, D. L., and E. P. Kennedy. 1971. Magnesium transport in *Escherichia coli*. Inhibition by cobaltous ion. *J. Biol. Chem.* **246**:3042–3049.
 30. Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **67**:593–656.
 31. Nummila, K., I. Kilpelainen, U. Zahringer, M. Vaara, and I. M. Helander. 1995. Lipopolysaccharides of polymyxin B-resistant mutants of *Escherichia coli* are extensively substituted by 2-aminoethyl pyrophosphate and contain aminoarabinose in lipid A. *Mol. Microbiol.* **16**:271–278.
 32. Paulus, H., and E. Gray. 1964. The Biosynthesis of polymyxin B by growing cultures of *Bacillus polymyxa*. *J. Biol. Chem.* **239**:865–871.
 33. Raetz, C. R., and C. Whitfield. 2002. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71**:635–700.
 34. Reynolds, C. M., A. A. Ribeiro, S. C. McGrath, R. J. Cotter, C. R. Raetz, and M. S. Trent. 2006. An outer membrane enzyme encoded by *Salmonella typhimurium* *lpxR* that removes the 3'-acyloxyacyl moiety of lipid A. *J. Biol. Chem.* **281**:21974–21987.
 35. Rosner, M. R., J. Tang, I. Barzilay, and H. G. Khorana. 1979. Structure of the lipopolysaccharide from an *Escherichia coli* heptose-less mutant. I. Chemical degradations and identification of products. *J. Biol. Chem.* **254**:5906–5917.
 36. Rutten, L., J. Geurtsen, W. Lambert, J. J. Smolenaers, A. M. Bonvin, A. de Haan, P. van der Ley, M. R. Egmond, P. Gros, and J. Tommassen. 2006. Crystal structure and catalytic mechanism of the LPS 3-O-deacylase PagL from *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. USA* **103**:7071–7076.
 37. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
 38. Soncini, F. C., and E. A. Groisman. 1996. Two-component regulatory systems can interact to process multiple environmental signals. *J. Bacteriol.* **178**:6796–6801.
 39. Trent, M. S., W. Pabich, C. R. Raetz, and S. I. Miller. 2001. A PhoP/PhoQ-induced lipase (PagL) that catalyzes 3-O-deacylation of lipid A precursors in membranes of *Salmonella typhimurium*. *J. Biol. Chem.* **276**:9083–9092.
 40. Vaara, M., and P. Viljanen. 1985. Binding of polymyxin B nonapeptide to gram-negative bacteria. *Antimicrob. Agents Chemother.* **27**:548–554.
 41. Wang, R. F., and S. R. Kushner. 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* **100**:195–199.
 42. Wosten, M. M., L. F. Kox, S. Chamnongpol, F. C. Soncini, and E. A. Groisman. 2000. A signal transduction system that responds to extracellular iron. *Cell* **103**:113–125.
 43. Yi, E. C., and M. Hackett. 2000. Rapid isolation method for lipopolysaccharide and lipid A from gram-negative bacteria. *Analyst* **125**:651–656.
 44. Zhou, Z., S. Lin, R. J. Cotter, and C. R. Raetz. 1999. Lipid A modifications characteristic of *Salmonella typhimurium* are induced by NH₄VO₃ in *Escherichia coli* K12. Detection of 4-amino-4-deoxy-L-arabinose, phosphoethanolamine and palmitate. *J. Biol. Chem.* **274**:18503–18514.
 45. Zhou, Z., A. A. Ribeiro, S. Lin, R. J. Cotter, S. I. Miller, and C. R. Raetz. 2001. Lipid A modifications in polymyxin-resistant *Salmonella typhimurium*: PMRA-dependent 4-amino-4-deoxy-L-arabinose, and phosphoethanolamine incorporation. *J. Biol. Chem.* **276**:43111–43121.