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Mutations in the lipid A deacylase PagL which release the enzyme from its latency affect the ability of PagL to interact with lipopolysaccharide in *Salmonella enterica* serovar Typhimurium

Takayuki Manabe, Miyuki Kawano, Kiyoshi Kawasaki *

Faculty of Pharmaceutical Sciences, Doshisha Women's College, Kyotanabe, Kyoto 610-0395, Japan

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ABSTRACT

PagL, a lipid A deacylase, is unique in that it is latent in the outer membrane of *Salmonella enterica* serovar Typhimurium. Several point mutations in the extracellular loops of PagL, which do not affect its enzymatic activity, release it from this latency. Precipitation analysis revealed that latent wild-type PagL associated with lipopolysaccharide, but non-latent PagL mutants did not. In contrast, non-latent PagL mutants preferentially associated with some membrane proteins. Precipitation analysis using inactive PagL mutants demonstrated that membrane lipid A deacylation did not affect association. These results indicate that mutations in the lipid A deacylase PagL which relieve the enzyme from its latency affect the ability of PagL to interact with lipopolysaccharide.

1. Introduction

The outer membranes of pathogenic Gram-negative bacteria, including *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), function as barriers to harmful host-derived compounds, such as antimicrobial peptides and reactive oxygen species. This protection is afforded by strong lateral interactions between the lipid A portions of lipopolysaccharide (LPS), which forms the outer leaflet of the outer membrane with proteins (reviewed in [1]). In addition, lipid A induces host inflammatory responses, as it is recognized by the Toll-like receptor 4 (TLR4)-MD2 complex [2]. In response to signals from the host, *S.* Typhimurium covalently modifies its lipid A [3]. These lipid A modifications have been implicated in increasing resistance to cationic antimicrobial peptides and reducing recognition by the host TLR4-MD2 complex by changing the cell surface charge [4] and number of acyl chains [5], respectively.

A two-component regulatory system, PhoP–PhoQ, which is essential for *S.* Typhimurium pathogenesis [6,7], promotes the expression of genes involved in lipid A modification [3]. PhoQ is a sensor histidine kinase that responds to environmental signals, including mammalian tissues, which can be mimicked by magnesium-limitation [8]. In response to these signals, PhoQ phosphorylates PhoP, leading to the activation of *pagL* and *pagP*, which encode lipid A 3-*O*-deacylase and lipid A palmitoyltransferase, respectively [9,10]. Since 3-*O*-deacylation decreases the endotoxic activity of lipid A, it is thought to help *S.* Typhimurium evade immunosurveillance [5]. In addition to changes in gene expression, post-translational regulation of outer membrane enzymes is involved in modification of lipid A. For example, even when PagL was induced in *S.* Typhimurium, lipid A was not deacylated [9,11]; therefore, PagL is thought to be latent in the outer membrane [11]. Furthermore, PagL^{R43A} and PagL^{R135A} mutants were released from this latency [12], suggesting that Arg-43 and Arg-135, which are located in the extracellular loops of PagL, are involved in specific recognition that is essential for latency. In addition, the outer membrane enzyme PagP, which catalyzes lipid A palmitoylation, is latent in *Escherichia coli* [13]. Therefore, latency is thought to be a conserved characteristic of outer membrane enzymes involved in the lipid A modifications.

In this study, we found that PagL mutants that are released from latency, designated as non-latent PagL, lost their ability to associate with LPS. These results suggest that association with LPS in the outer membrane is crucial for PagL latency.

2. Materials and methods

2.1. Bacterial strains and growth conditions

CS283 (*phoN2 zxx*::*6251* Tn*10d*-Cam *pagL1*::Tn*phoA*) [6,11], a derivative of *S.* Typhimurium strain 14028s, was used. KCS040 (CS283 *pmrA*::Tn*10d*) [11] was used as *pmrA*-null *S.* Typhimurium. N-minimal medium supplemented with 10 μM $MgCl_2$ was used for

Abbreviations: LPS, lipopolysaccharides; *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; TLR4, Toll-like receptor 4; PBS, phosphate-buffered saline.

* Corresponding author. Fax: +81 774 65 8585.
E-mail address: kkawasak@dwc.doshisha.ac.jp (K. Kawasaki).

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cultivation [12]. Ampicillin (50 μg/ml) was used for strains transformed with plasmids. Overnight pre-cultures (~10 ml) were diluted 1:10 or 1:30 with fresh medium and then grown at 37 °C for 24 h, and were used for further analysis.

2.2. Plasmid constructs

His_{x6}-tagged $PagL^{S165A}$ and $PagL^{R43A\ S165A}$ mutants were generated by PCR-based overlap extension. Plasmids pWKS30-*pagL*-His_6 [11] and pWKS30-*pagL*^{R43A}-His_6 [12] were used as templates, and T3 (aattaaccctcactaaaggg), T7 (taatacgactcactataggg), S165A-FW (cggcatttcgcgaatggatca), and S165A-REV (tgatccattcgcgaaatgccg) were used as primers. The products were cloned into the EcoRI/BamHI sites of pWKS30, and the inserts were verified by DNA sequencing. Construction of other pWKS30-based plasmids encoding His_{x6}-tagged PagL mutants were described previously [12].

2.3. Mass spectrometric analysis of lipid A

Lipid A was prepared as described previously using Tri-reagent, and analyzed by a Voyager-DE STR mass spectrometer using 5-chloro-2 mercaptobenzothiazole as a matrix [14].

2.4. Preparation of membrane lysate and precipitation of PagL

All steps were carried out at 4 °C or on ice, and protease inhibitor cocktail (Complete™, Roche) was added to the buffers outlined below. The bacteria collected from 150 ml culture were suspended in 1 ml of phosphate-buffered saline (PBS) [137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM Na_2HPO_4 (pH 7.4)]. Membranes, prepared as described previously [12], were solubilized in 1.5 ml of extraction buffer [1% n-dodecyl-β-D-maltoside, 637 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM Na_2HPO_4 (pH 7.4) and 10 mM imidazole] with rotation for 1 h. The solubilized membranes were cleared by centrifugation at 100,000*g* for 30 min. 1.4 ml of cleared membrane lysate was mixed with 20 μl of TALON polyhistidine-affinity resin (Clontech) with rotation for 2 h. The resin was then washed sequentially with extraction buffer and wash buffer [0.5% n-dodecyl-β-D-maltoside, 637 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM Na_2HPO_4 (pH 7.4) and 20 mM imidazole]. Finally, the proteins were eluted from the resin with 50 μl of elution buffer [1% n-dodecyl-β-D-maltoside, 637 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM Na_2HPO_4 (pH 7.4) and 250 mM imidazole].

2.5. Biotin-labeling of cell-surface proteins and precipitation of His_{x6}-tagged PagL

All steps were carried out at 4 °C or on ice unless otherwise indicated, and Complete™ was added to the buffers outlined below. Cells were suspended in 10 ml of PBS at OD_{600} = 1.0, and incubated at room temperature for 1 h after addition of 3 mg of Sulfo-NHS-LC-Biotin (Pierce). The cells were next washed with PBS containing 100 mM glycine, and membrane lysates were prepared as described above, using 500 μl of extraction buffer. The lysate (450 μl) was mixed with 10 μl of TALON resin, and the resin was washed sequentially with extraction buffer and wash buffer. Finally, proteins were eluted with 50 μl of elution buffer.

2.6. Preparation of outer membrane proteins

Outer membrane proteins were prepared by differential solubilization with N-Lauroylsarcosine [15]. All steps were carried out at 4 °C or on ice unless otherwise indicated, and Complete™ was added to the buffers outlined below. Bacterial membranes prepared from 50 ml of culture as described above were suspended in 300 μl of 20 mM Tris/HCl (pH 7.5) containing 1% sodium N-Lauroylsarcosine, and incubated for 30 min at room temperature. The N-Lauroylsarcosine-insoluble outer membrane was collected from 150 μl of membrane fraction by centrifugation at 45,000*g* for 90 min, and then suspended in 150 μl of PBS.

2.7. Preparation of antibodies against PmrC

A fragment of the *S.* Typhimurium *pmrC* gene, encoding the 319 C-terminal amino acid residues, was amplified by PCR using primers KK148 (ccacatatgctgaaaggcgatcgc) and KK149 (gccggatcctcattcgcttagtctcct). The PCR product was cloned into the NdeI/BamH I sites of pET15b (Novagen). The purification of recombinant PmrC antigen, and the affinity purification of antibodies from rabbit serum was performed as described previously [14].

2.8. SDS–polyacrylamide gel electrophoresis and Western-blotting

Samples separated by SDS–polyacrylamide gel electrophoresis were stained with Coomassie blue, a silver-staining kit (Daiichi Pure Chemicals, Tokyo, Japan), or SYPRO Ruby gel stain (BIO-RAD).

For Western-blot analysis, samples separated by SDS–polyacrylamide gel electrophoresis were electroblotted onto nitrocellulose or PVDF (BIO-RAD) for the detection of proteins and LPS [16], respectively. The membranes were probed with the indicated primary antibody and secondary antibody linked to horseradish peroxidase. Alternatively, avidin-conjugated horseradish peroxidase was used for the detection of biotin-labeled proteins.

3. Results and discussion

3.1. Mutations that release PagL from latency affect the ability of PagL to co-precipitate LPS

Point mutations in the extracellular loops of PagL, such as R43A and R135A, release PagL from latency, suggesting that some specific recognition is involved in latency (Fig. 1) [12]. Since the outer leaflet of the outer membrane is occupied exclusively by LPS and proteins, we analyzed the association of PagL with LPS. PagL was precipitated from membrane lysates prepared from *S.* Typhimurium transformed with expression constructs encoding His_{x6}-tagged PagL, and the precipitates were analyzed. LPS co-precipitated with

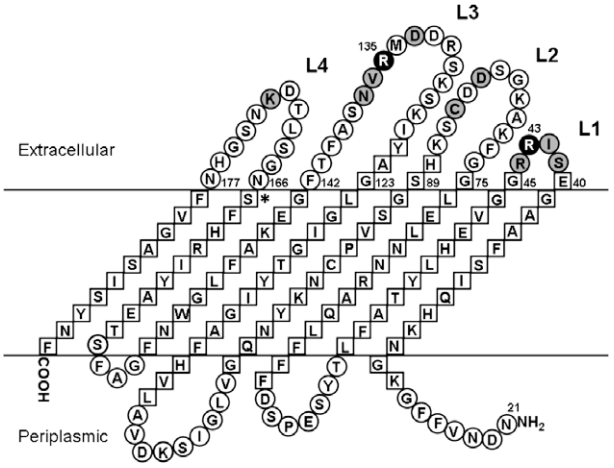

Fig. 1. Amino acid residues involved in PagL latency. A topology model of *S.* Typhimurium PagL [12] is shown. Residues that severely or mildly affect latency when replaced with alanine [12] are indicated with black or gray background, respectively. Ser-165, which is essential for deacylase activity, is indicated with an asterisk.