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Salmonella enterica serovar Typhimurium lipopolysaccharide deacylation enhances its intracellular growth within macrophages

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ABSTRACT

Modification of lipid A is essential for bacterial adaptation to its host. *Salmonella* Typhimurium LpxR potentially detoxifies lipid A by 3′-*O*-deacylation; however, the involvement of deacylation in its adaptation remains unclear. LpxR-dependent 3′-*O*-deacylation was observed in the stationary phase. When macrophages were infected with stationary phase bacteria, the intracellular growth of the *lpxR*-null strain was lower than that of the wild-type strain. Furthermore, the expression level of inducible nitric oxide synthase was higher in the cells infected with the *lpxR*-null strain than in the cells infected with the wild-type strain. These results indicate that lipid A 3′-*O*-deacylation is beneficial for intracellular growth.

1. Introduction

Lipopolysaccharides (LPS) are a major constituent of the outer leaflet of the outer membrane of Gram-negative bacteria, including *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) [1]. The presence of lipid A, a membrane anchor portion of LPS, is essential for the outer membrane to be able to act as an effective permeability barrier that protects against harmful compounds in the environment [1]. Furthermore, lipid A is responsible for the endotoxic activities of LPS [2]. In macrophages, when the precise structure of lipid A, such as the number of acyl chains, is recognized by the Toll-like receptor (TLR) 4-MD2 complex, inflammatory responses are initiated [3,4].

Gram-negative pathogenic bacteria change their surface structure, including lipid A, in response to environmental signals [5]. Such remodeling is essential for bacterial adaptation to its host. In *S.* Typhimurium, most lipid A modification enzymes are regulated by a two-component regulatory system, PhoP/PhoQ, which is required for virulence in mice [6,7] and survival in macrophages [6–8]. Lipid A modifications are involved in bacterial virulence: aminoarabinose-modification of lipid A increases bacterial resistance to antimicrobial peptide [9], and lipid A palmitoylation and/or 3-*O*-deacylation reduces recognition by host immune surveillance via TLR4-MD2 [10].

Three lipid A modification enzymes in *S.* Typhimurium, PagP, PagL, and LpxR, are localized in the outer membrane and change the number of acyl chains in lipid A: PagP catalyzes palmitoylation, PagL catalyzes 3-*O*-deacylation, and LpxR catalyzes 3′-*O*-deacylation (Fig. 1) [11–13]. Since 3′-*O*-deacylation removes two acyl chains of lipid A, LpxR is thought to reduce the biological activity of lipid A [13]. Unlike PagP and PagL, LpxR is not regulated by PhoP/PhoQ [13]. LpxR-dependent lipid A deacylation is not usually observed in living cells under standard culture conditions, although 3′-*O*-deacylase activity is observed in isolated membranes [13]. Since the overexpression of LpxR in *S.* Typhimurium induces lipid A 3′-*O*-deacylation [13], lipid A might be deacylated in *S.* Typhimurium under optimal conditions for inducing LpxR. In such specific conditions, LpxR-dependent lipid A deacylation might result in lower or altered immunological responses, possibly aiding the bacterium in establishing a prolonged infection.

In this study, we found the conditions in which LpxR-dependent lipid A deacylation was observed, and examined the functions

Abbreviations: LPS, lipopolysaccharides; *S.* Typhimurium, *Salmonella enterica* serovar Typhimurium; TLR, Toll-like receptor; DMEM, Dulbecco's modified Eagle's medium; OD, optical density; PBS, phosphate-buffered saline; moi, multiplicity of infection; CFU, colony-forming unit; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; MS, mass spectrometry; iNOS, inducible nitric oxide synthase

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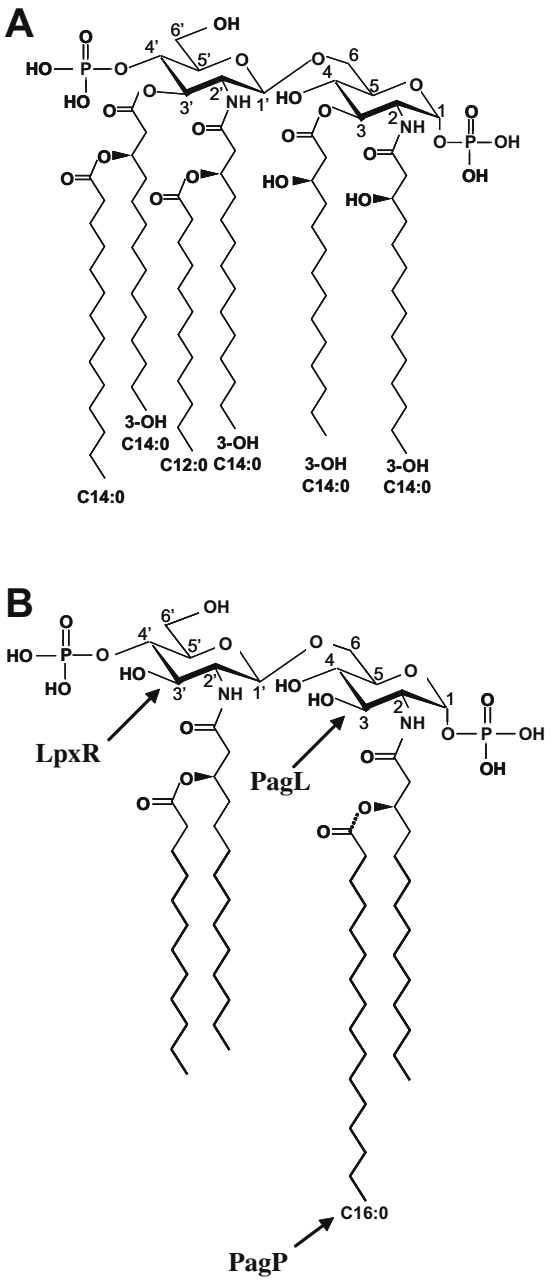

Fig. 1. Lipid A modifications in the outer membrane. The structures of unmodified lipid A (A) and lipid A modified by outer-membrane enzymes (B) are shown. The incorporation of the palmitate chain is catalyzed by PagP [11]. PagL catalyzes the removal of the *R*-3-hydroxymyristoyl chain at the 3-position [12]. LpxR catalyzes the removal of the 3′-*O*-acyloxyacyl moiety at the 3′-position [13].

of LpxR during intracellular replication which is essential for *S.* Typhimurium virulence [14].

2. Materials and methods

2.1. Culture of mammalian cells

RAW264.7 and J774.1 cells were routinely cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), under a 5% CO_2 atmosphere at 100% humidity and 37 °C. Penicillin and streptomycin were omitted for the analysis of intracellular bacterial growth.

2.2. Bacterial strains and growth conditions

S. Typhimurium strain 14028s (ATCC) was used as the wild-type strain. The non-polar *lpxR*-deletion mutant ΔlpxR was constructed by the established method [15] using primers MK10 (5′-tcagaagaagaaggtgatgtctccgttgatataagagtgtaggctggagctgcttc-3′) and MK11 (5′-gtgaacaaatacagctattgcgcaacgatgattgcccatatgaatatcctccttag-3′), which are designed to delete 888 bp of the 960 bp LpxR-coding region.

S. Typhimurium was grown at 37 °C with vigorous shaking in LB medium. The full-growth pre-culture was diluted in 50 ml of fresh medium at an optical density $(OD)_{600}$ of ~0.05 in a 200 ml flask and then grown at 37 °C with vigorous shaking. After cultivation, the cells were used for further analysis. Ampicillin (20 μg/ml) was used for strains transformed with the low copy vector pWKS30 [16] or its derivatives.

2.3. Plasmid construction

S. Typhimurium *lpxR* and the flanking 200 bp upstream regions amplified by PCR were cloned into the *Bam*H I/*Hind* III sites of pWKS30, and the resulting construct was named pLpxR. The sequences amplified by PCR were confirmed by sequencing.

2.4. Preparation of anti-LpxR antibodies

The *lpxR*-coding region was amplified by PCR using the primers MK4 (5′-gggaattccatatgagtagccttgctatttcag-3′) and MK5 (5′-gcggatcctcagaagaagaaggtg-3′). The PCR product was cloned into the *Nde* I/*Bam*H I sites of pET15b (Novagen), and the resulting construct, which was designed to produce recombinant LpxR protein carrying the His_6 epitope instead of the 23 amino acid residues at the N terminus, was introduced into *Escherichia coli* BL21(DE3). The recombinant protein, which was purified with Ni-NTA Agarose (Qiagen), was used to immunize a rabbit. Antibodies against LpxR were purified [17] using the recombinant LpxR.

2.5. Lipid A preparation for mass spectrometry (MS)

Lipid A was prepared from 10 ml of bacterial culture with Tri-reagent (Molecular Research Center) as described previously [18,19].

2.6. Gentamicin-protection assay with RAW264.7 cells

The assay was performed as described previously [8,20], with slight modifications. Cells (1×10^5 cells/well) were allowed to adhere to a 24-well for 48 h. After 48 h, the number of RAW264.7 cells were estimated to reach approximately 4×10^5 cells/well. *S.* Typhimurium grown for 24 h were washed with phosphate-buffered saline (PBS) and opsonized with 20% mouse serum in DMEM at 37 °C for 30 min. The mouse serum was used without heat-inactivation. Then, 4×10^6 of the opsonized bacteria were added to a well, to make a 10:1 multiplicity of infection (moi). To synchronize the incorporation of bacteria, the 24-well dish was centrifuged at 200×*g* for 5 min. Following cultivation for 30 min, the cells were washed with PBS and then cultivated with 100 μg/ml gentamicin to kill the extracellular bacteria. After 2 h cultivation, the cells were washed again and then cultivated with 10 μg/ml gentamicin. After 22 h cultivation with 10 μg/ml gentamicin, the cells were lysed with 1% Triton-X100 in PBS at 37 °C for 5 min, and then the colony-forming units (CFU) in the lysate were counted.

2.7. Gentamicin-protection assay with J774.1 cells

The assay was performed as described previously [21]. Briefly, *S.* Typhimurium opsonized as described above was mixed