



NOTE

Bacteriology

The effect of *rfaD* and *rfaF* of *Haemophilus parasuis* on lipooligosaccharide induced inflammation by NF- κ B/MAPKs signaling in porcine alveolar macrophages

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ABSTRACT. In *Haemophilus parasuis*, the *rfa* cluster has been identified as a virulence-associated factor that is involved in lipooligosaccharide (LOS) biosynthesis. In this study, we assessed the roles of *rfaD* and *rfaF* genes in *H. parasuis* SC096 on LOS-induced pro-inflammatory factors and the related signaling pathways in porcine alveolar macrophages (PAMs) by real-time PCR and western blotting. The results showed that the LOSs of both *rfaD* and *rfaF* mutants ($\Delta rfaD$ -LOS and $\Delta rfaF$ -LOS) significantly decreased the mRNA expression of pro-inflammatory factors (IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α) in PAMs compared with *H. parasuis* SC096 LOS (WT-LOS). Furthermore, in $\Delta rfaD$ -LOS- and $\Delta rfaF$ -LOS-treated cells, I κ B α degradation was significantly inhibited and levels of phospho-p65 and phospho-p38 were significantly reduced in PAMs. These findings suggested that the *rfaD* and *rfaF* genes mediated LOS induction of pro-inflammatory cytokines in PAMs by regulating the NF- κ B and MAPKs signaling pathways during *H. parasuis* infection.

KEY WORDS: *Haemophilus parasuis*, pro-inflammatory factor, *rfaD*, *rfaF*, signaling pathway

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Haemophilus parasuis is an important respiratory-tract pathogen of Glässer's disease in swine, which is characterized by pleuritis, pericarditis, peritonitis, pneumonia, arthritis, and meningitis [6]. Recent studies have demonstrated that *H. parasuis* infections in hosts could stimulate inflammatory cytokines released through the regulation of nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways [1, 2].

Lipopolysaccharides (LOSs) are a major component of the outer membranes of gram-negative bacteria. LOSs can be identified by cell-surface molecules such as TLR4 and MD-2 when cells were stimulated by LOS, then by the activation of a variety of transcription factors through regulation of the NF- κ B and MAPK signaling pathways and secretion of inflammatory cytokines [4]. *Haemophilus parasuis* LOS could induce pro-inflammatory responses and upregulated the expression of interleukin-1 α (IL-1 α), IL-1 β , IL-6 and IL-8 in host cells [11]. Nevertheless, it is uncertain which LOS residues participate in the process of pro-inflammatory responses. As virulence-associated factors, both *rfaD* and *rfaF* genes are important parts of the *rfa* cluster involved in LOS biosynthesis of *H. parasuis* [8, 10]. The $\Delta rfaD$ mutant showed impaired ability to adhere to and invade host cells [10]. Loss of the *rfaF* gene resulted in a severely truncated LOS structure and decreased abilities of serum resistance, adhesion, and invasion [8], which suggested that the full LOS structure influenced the ability of the bacteria to interact with the host cells. However, the roles of *rfaD* and *rfaF* genes in pro-inflammatory responses are still unknown on *H. parasuis* LOSs. In this study, we purified the LOS from $\Delta rfaD$ and $\Delta rfaF$ mutants to demonstrate the expression of pro-inflammatory factors and their related signaling pathways in porcine alveolar macrophages (PAMs).

H. parasuis SC096 and its *rfaD* and *rfaF* mutant strains were cultivated in liquid medium [8, 10]. LOSs were extracted using the hot-phenol method [5] and quantified using the anthrone-sulfuric acid method [11]. Compared to LOS from the *H. parasuis* SC096 strain (WT-LOS), the LOS from the *rfaD* mutant strain ($\Delta rfaD$ -LOS) migrated faster (Fig. S1) and exhibited the truncated LOS structure. PAMs (3D4/2 cell line from the American Type Culture Collection) were plated in 12-well microplates in RPMI 1640 medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10% (V/V) heat-inactivated fetal bovine serum and cultured with 5% CO₂ at 37°C for 24 hr, and stimulated with WT-LOS, $\Delta rfaD$ -LOS, and $\Delta rfaF$ -LOS at concentrations of 5 or 10 μ g/ml. Cell pellets and supernatants were collected at 6, 12 and 24 hr after incubation. Total RNA was extracted with TRIzol (Invitrogen) and cDNA was synthesized with PrimeScriptTM RT reagent Kit (TaKaRa, Dalian, China). Real-time PCR was performed with the

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Table 1. Sequences of the PCR primers used in this study

Primers	Nucleotide sequence (5'–3')	PCR product length (bp)
IL-1 β -F	ACCTGGACCTTGGTTCTCTG	83
IL-1 β -R	CATCTGCCTGATGCTCTTGT	
IL-1 α -F	GAAGAAGAGACGGTTGAG	109
IL-1 α -R	GCTGTATGTTGCTGATCT	
IL-6-F	AATCCAGACAAAGCCACCAC	79
IL-6-R	TCCACTCGTTCTGTGACTGC	
IL-8-F	TAGGACCAGAGCCAGGAAGA	92
IL-8-R	AGCAGGAAAAGTCCAAGAA	
TNF- α -F	CCACCAACGTTTTCTCACT	82
TNF- α -R	TTGATGGCAGAGAGGAGGTT	
RPL4-F	GCTCTATGGCACTTGGCGT	124
RPL4-R	GCGGAGGGCTCTTTGGAT	

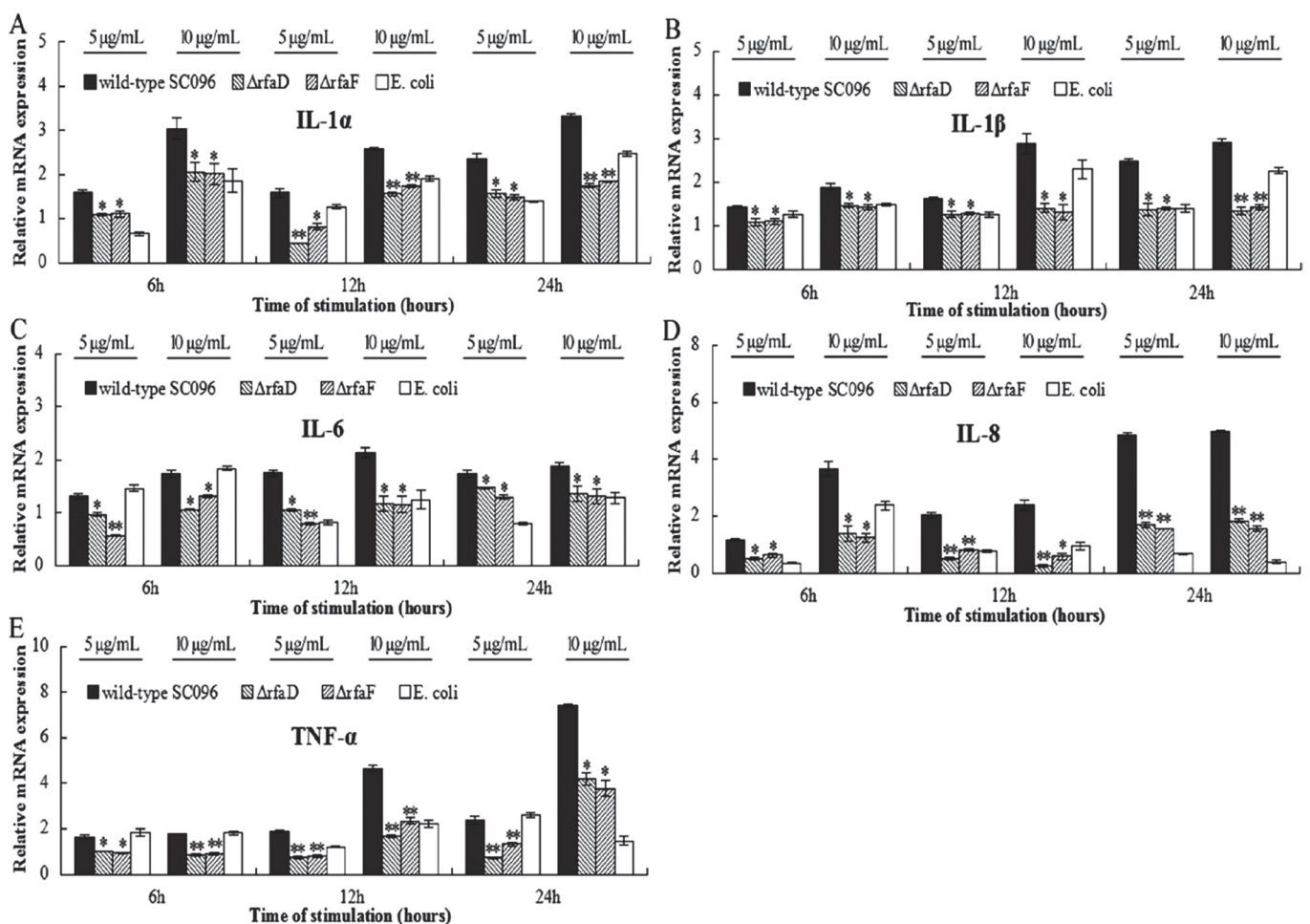


Fig. 1. The mRNA expression of pro-inflammatory cytokines in lipooligosaccharide (LOS)-stimulated porcine alveolar macrophages (PAMs). PAMs were stimulated with *ΔrfaD*-LOS, *ΔrfaF*-LOS and WT-LOS (5 and 10 μg/ml) for 6, 12 and 24 hr. The levels of IL-1 α (A), IL-1 β (B), IL-6 (C), IL-8 (D) and TNF- α (E) mRNAs were measured by qRT-PCR. The values presented are mean \pm SD of three independent experiments, and data were analyzed using one-way ANOVA. * P <0.05; ** P <0.001 compared to WT-LOS-treated PAMs.

primer pairs (Table 1) using an Applied Biosystems 7300 Real-time PCR System (ABI, Foster City, CA, U.S.A.). The data were analyzed using the $2^{-\Delta\Delta CT}$ method in triplicates for three independent experiments. A LPS preparation from *Escherichia coli* O111: B4 (Sigma Aldrich, St. Louis, MO, U.S.A.) was used as a positive control, and the unstimulated PAMs cells were used as a mock-stimulus. The ribosomal protein L4 (RPL4), stably expressed in PAMs, was used as a reference gene to normalize the results of gene expression detected by the real-time PCR assay [3].

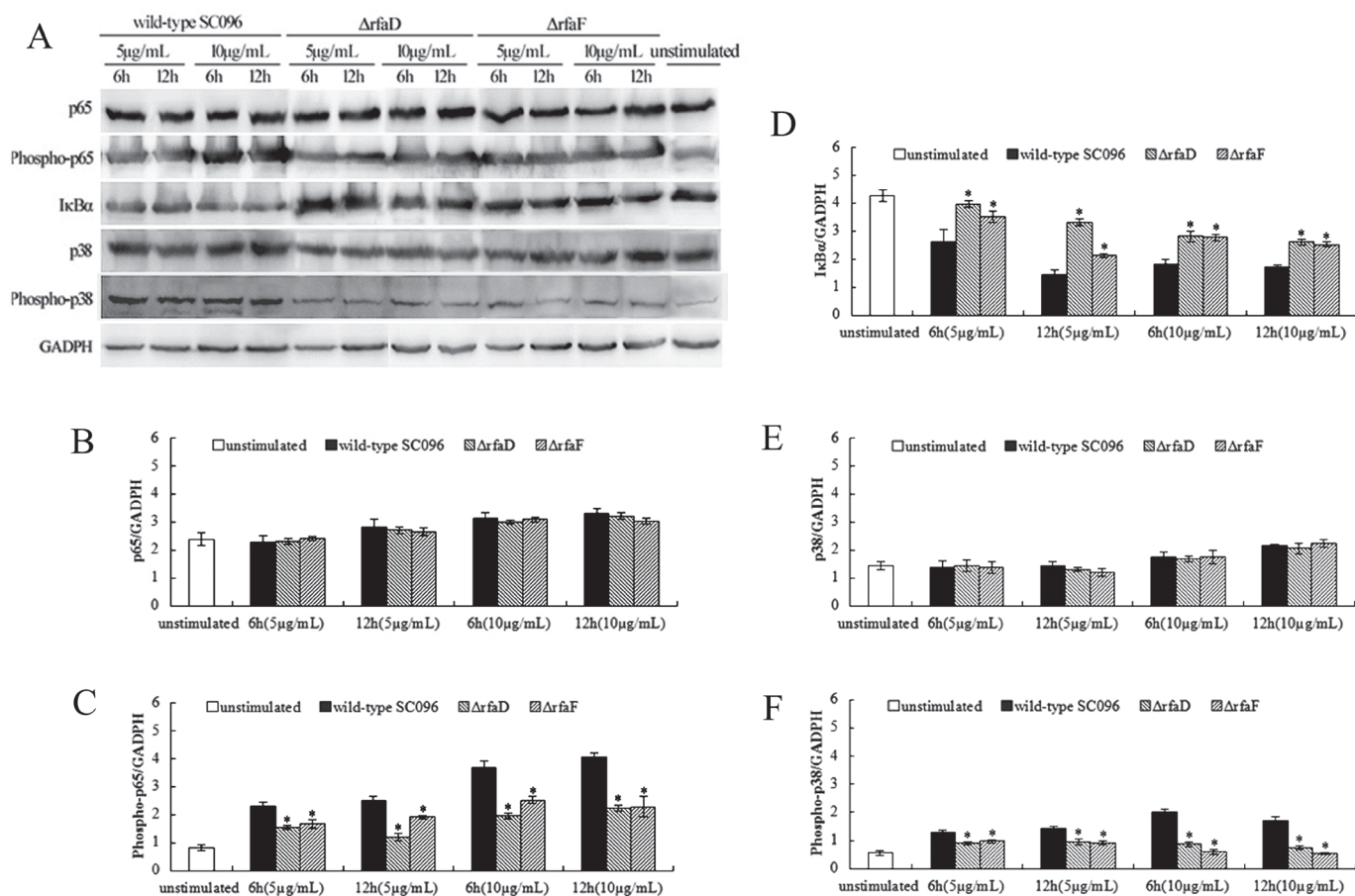


Fig. 2. Both *ΔrfaD*-lipooligosaccharide (*ΔrfaD*-LOS) and *ΔrfaF*-LOS resulted in a decrease in phosphorylation of p65 and p38 during NF- κ B and MAPK signaling in PAMs. PAMs were stimulated with *ΔrfaD*-LOS, *ΔrfaF*-LOS, and WT-LOS (5 and 10 μ g/ml) for 6 and 12 hr. Western blot analyses of p65, phospho-p65, I κ B α , p38 and phospho-p38 were performed (A). Western blot of the above-mentioned proteins and quantifications of p65 (B), phospho-p65 (C), I κ B α (D), p38 (E) and phospho-p38 (F). GAPDH was used as a loading control. Bar graphs show the relative protein expression quantified from three separate experiments. The values presented are mean \pm SD, and data were analyzed using one-way ANOVA. * P <0.05 compared to WT-LOS-treated PAMs.

In *Neisseria meningitidis*, both the *rfaD* and *rfaF* mutant strains downregulated expression of pro-inflammatory factors in mouse serum [7, 9]. However, the roles of *rfaD* and *rfaF* in the pathogenesis of *H. parasuis* LOS were largely unknown. In this study, compared to the WT-LOS, LOSs from both mutants have significantly poorer abilities to induce mRNAs of inflammatory cytokine in treated PAMs at 6, 12 and 24 hr (P <0.05), including IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α (Fig. 1). The results demonstrated that the loss of *rfaD* and *rfaF* genes in *H. parasuis* SC096 resulted in decreased LOS-mediated pro-inflammatory cytokine expressions in PAMs, which suggested that the truncated LOS structure might attenuate the inflammatory response during a *H. parasuis* infection.

To further investigate the signaling mechanisms underlying the induction of the inflammatory response, we measured the expression of NF- κ B and MAPK signaling molecules by western blotting, including NF- κ B p65, phospho-NF- κ B p65, I κ B α , p38 and phospho-p38. PAMs were treated with WT-LOS, *ΔrfaD*-LOS, and *ΔrfaF*-LOS (5 and 10 μ g/ml) for 6 and 12 hr. Western blot analysis was performed as described previously [2]. Anti-NF- κ B p65, anti-phospho-NF- κ B p65, anti-I κ B α and anti-GADPH monoclonal antibodies as well as anti-p38 and anti-phospho-p38 polyclonal antibodies were obtained from Cell Signaling Technology (CST, Danvers, MA, U.S.A.). The HRP-conjugated goat anti-mouse or goat anti-rabbit IgG were obtained from Abbkine (Redlands, CA, U.S.A.). Densitometry values of immunoblot signals were obtained from three separate experiments using FusionCapt Advance software (Vilber Lourmat, Eberhardzell, Germany).

The phosphorylation of p65 and p38 were noticeably decreased in a dose-dependent manner in both *ΔrfaD*-LOS- and *ΔrfaF*-LOS-treated PAMs compared to the WT-LOS-treated group (Fig. 2). Both *ΔrfaD*-LOS and *ΔrfaF*-LOS resulted in a higher concentration of I κ B α in a dose-dependent manner in stimulated PAMs compared to WT-LOS. Furthermore, an analysis of the densitometry values showed that the relative ratios of I κ B α /GADPH significantly increased (P <0.05), while the phosphorylation of p65/GADPH and p38/GADPH significantly decreased in both the *ΔrfaD*-LOS- and *ΔrfaF*-LOS-treated PAMs compared with the WT-LOS-treated group (P <0.05). Nevertheless, there was no obvious difference in the relative ratios of p65/GADPH

and p38/GAPDH. Therefore, the results suggested that the LOSs of *H. parasuis* participated in the activation of NF- κ B and MAPK signaling pathways during infection. Both $\Delta rfaD$ -LOS and $\Delta rfaF$ -LOS resulted in a decrease in phosphorylation of p65 and p38 in the NF- κ B and MAPK signaling pathways. Based on the above results, we postulated that the reduced ability to induce the inflammatory cytokines in $\Delta rfaD$ -LOS- and $\Delta rfaF$ -LOS-treated PAMs may be related to the reduction of p65 and p38 phosphorylation during NF- κ B and MAPK signaling.

In conclusion, both *rfaD* and *rfaF* mutants of the *H. parasuis* SC096 strain, with the truncated LOS structures, had a significant effect on LOS-induced pro-inflammatory factors in PAMs, such as IL-1 α , IL-1 β , IL-6, IL-8 and TNF- α . Also, LOSs of both mutants had decreased p65 and p38 phosphorylation. The above results indicated that both *rfaD* and *rfaF* genes mediated LOS induction of pro-inflammatory cytokines in PAMs by regulating the NF- κ B and MAPKs signaling pathways during *H. parasuis* infection, which suggested that the full LOS structure had a significant role on the inflammatory response in *H. parasuis*. Overall, this study focused on the LOS-induced inflammatory mechanism that will provide a theoretical basis for the pathogenic mechanism of *H. parasuis*.

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