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Nonconservation of TLR5 activation site in *Edwardsiella tarda* flagellin decreases expression of interleukin-1β and NF-κB genes in Japanese flounder, *Paralichthys olivaceus*

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ABSTRACT

Flagellin is the subunit protein that composes bacterial flagella and is recognized by toll-like receptor 5 (TLR5) as a ligand. Flagellin protein (*e.g.*, FliC and FlaA) contains the D1, D2, and D3 domains; the D1 domain is important for recognition by TLR5 for activation of the innate immune system. In teleosts, there are two types of TLR5, the membrane form (TLR5M) and soluble form (TLR5S), the latter of which is not present in mammals. In this study, the potential of flagellin from *Edwardsiella tarda* (EtFliC) to induce inflammation-related genes interleukin (IL)-1β and NF-κB-p65 through TLR5S in Japanese flounder (*Paralichthys olivaceus*) was elucidated. A transient overexpression system was developed in flounder natural embryonic (HINAE) cells using constructs encoding two flagellin genes derived from *E. tarda* (pEtFliC) and *Escherichia coli* (pEcoFliC) and the flounder TLR5S gene (pPoTLR5S). Expression of inflammation-related genes in EtFliC- and PoTLR5S-overexpressing HINAE cells was significantly lower than in EcoFliC- and PoTLR5S-overexpressing cells. To clarify the difference between EtFliC and EcoFliC potency, the amino acid sequence of EtFliC was compared with that of other bacterial flagellin. The 91st arginine residue, known as the mammalian TLR5 activation site, was conserved in the flagellin of *E. coli* and other bacteria but not in EtFliC. To reveal the importance of the 91st arginine residue in FliC, a pEtFliC construct in which the 91st asparagine was mutated to arginine (pEtFliC_N91R) was generated. Expression of the IL-1β and NF-κB-p65 genes in the HINAE cells co-transfected with pEtFliC_N91R and pPoTLR5S was significantly higher than that in cells co-transfected with pEtFliC and pPoTLR5S. The results suggested that the 91st arginine residue of bacterial flagellin is involved in inflammatory response through TLR5S in teleosts. Thus, EtFliC improved by site-directed mutagenesis could be an effective adjuvant against *E. tarda* infection in Japanese flounder.

1. Introduction

Edwardsiella tarda is a Gram-negative bacterium and the causative agent of edwardsiellosis, which causes serious damage in cultivated Japanese flounder (also known as bastard halibut, *Paralichthys olivaceus*). This pathogen is known as an intracellular bacterium [1,2], and countermeasures against this bacterium are still insufficient. Therefore, the development of an adjuvant that enhances the effect of the vaccine and eliminates the pathogen before intracellular parasitization is needed.

Flagellin, which is important for the motility and pathogenicity of many bacteria, composes flagella fibers along with other bacterial flagellar constituent proteins [3]. As a virulence factor, flagellin is known to promote the invasion of intracellular bacteria in host cells [4,5]. In host defense against bacterial invasion, flagellin is recognized by particular pattern recognition receptors known as toll-like receptors (TLRs) as pathogen-associated molecular patterns (PAMPs) [6]. TLRs typically consist of an extracellular leucine-rich repeat (LRR) domain, transmembrane domain, and intracellular toll/interleukin-1 receptor (TIR) domain [7]; the extracellular LRR domain in particular is important for recognition of ligand [8,9]. In mammals, all TLRs have a transmembrane and TIR domain [10]. However, the soluble form of TLR5 (TLR5S), which contains only the extracellular LRR domain, exists in some teleosts [11–16]. In mammals, flagellin binding to the

Abbreviations: 3D, three-dimensional; EcoFliC, *Escherichia coli* FliC; EtFliC, *Edwardsiella tarda* FliC; HINAE, hirame natural embryo; IL, interleukin; LRR, leucine-rich repeat; NF-κB, nuclear factor-κB; PCR, polymerase chain reaction; PoTLR5, Japanese flounder TLR5; qPCR, quantitative real-time PCR; SEMs, standard errors of the mean; TIR, toll/interleukin-1 receptor; TLR, toll-like receptor; TLR5M, membrane TLR5; TLR5S, soluble TLR5

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extracellular LRR domain of TLR5 results in activation of the myeloid differentiation primary response gene 88 (MyD88) signaling pathway and nuclear factor-κB (NF-κB) [6]. It was also demonstrated that TLR5S promotes flagellin-mediated NF-κB activity in teleosts [11,17].

Flagellin is composed of four domains known as D0, D1, D2, and D3 based on the conformation of the protein. The D0 and D1 domains are highly conserved among bacterial species and formed by an α-helix structure in the N- and C-terminal regions, whereas the D2 and D3 domains are highly diverse [18,19]. The D1 domain is important for recognition by TLR5 and activation of the innate immune response [20–23]. In particular, the 90th arginine residue in flagellin derived from *Salmonella typhimurium* is critical for induction of TLR5 expression in mouse [8]. The 89th arginine residue in flagellin from *Bacillus subtilis* binds to the 9th LRR of the membrane form of TLR5 (TLR5M) in zebrafish (*Danio rerio*) [9,24], whereby NF-κB is most strongly activated. In addition, in human and cattle, the amount of secreted CXCL8 downstream of the TLR5 signaling pathway varies depending on the type of bacteria from which the flagellin is derived [25]. However, the activation site of the flagellin derived from fish with bacterial infection has not yet been studied, and the pathogen recognition mechanism in fish is unknown. In this study, to understand the potential of *E. tarda* flagellin to induce inflammation-related genes mediated by Japanese flounder TLR5S, we conducted transient overexpression experiments in the Japanese flounder embryonic cell line with DNA constructs encoding FliC composing an *E. tarda* flagellin and TLR5S. Because TLR5S is a pattern-recognition receptor important for activation of innate immunity, this study may provide insight into the use of *E. tarda* FliC (EtFliC) in the prevention of edwardsiellosis.

2. Materials and methods

2.1. Bacterial strains and sequence analysis

Edwardsiella tarda OA-3 isolated from diseased Japanese flounder was grown in brain-heart infusion (BHI) medium (Becton, Dickinson and Company, USA) at 25 °C. *Escherichia coli* DH5α was cultured in Luria-Bertani (LB) broth at 37 °C.

The coding regions of the EtFliC and *E. coli* FliC (EcoFliC) genes were amplified from genomic DNA extracted from *E. tarda* OA-3 and *E. coli* DH5α by polymerase chain reaction (PCR) using KAPA HiFi HotStart Ready Mix (NIPPON Genetics Co, Ltd, Japan). The primers, which were designed based on the nucleotide sequences of *E. tarda* TX01 and *E. coli* K-12 (GenBank accession nos. AB375858 and NC_000913, respectively), are shown in Table 1. PCR was performed for 35 cycles with the following conditions: 5 min at 95 °C, 20 s at 98 °C, 15 s at 56 °C, 1.5 min at 72 °C, and 5 min at 72 °C. The PCR products were subcloned into pGEM-T Easy vector (Promega, USA), and the nucleotide sequences of the cloned genes in the vector plasmids (pGEM_EtFliC and pGEM_EcoFliC) were confirmed by DNA sequencing. A domain search was performed with the conserved domain search program of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The amino acid sequences were aligned with BioEdit v7 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and the phylogenetic analysis was performed using MEGA7 (http://www.megasoftware.net/). The accession numbers of all amino acid sequences analyzed in this study are indicated in Supplementary Table 1. The three-dimensional (3D) structure of the flagellin protein was predicted by SWISS-MODEL (https://swissmodel.expasy.org/).

2.2. Plasmid DNA construction

The coding regions of Japanese flounder TLR5S (PoTLR5S) and PoTLR5M cDNA were obtained by PCR of Japanese flounder PBLs cDNA using KAPA HiFi HotStart Ready Mix and the primers shown in Table 1. PCR was performed for 40 cycles with the following conditions: 5 min at 95 °C, 20 s at 98 °C, 15 s at 56 °C, 2.5 min at 72 °C, and 5 min at 72 °C. The PCR product was subcloned into the pGEM-T Easy vector, and the nucleotide sequences of the cloned genes were confirmed by DNA sequencing.

Plasmid mutated at N91 to R91 in EtFliC (pEtFliC_N91R) was constructed with the PrimeSTAR® Mutagenesis Basal Kit (Takara, Japan) using the primers shown in Table 1. PCR was performed for 30 cycles with the following conditions: 10 s at 98 °C, 15 s at 55 °C, and 10 s at 72 °C. The pGEM plasmid constructs including pGEM_EtFliC, pGEM_EcoFliC, pGEM_PoTLR5S, and pGEM_EtFliC_N91R were used as DNA templates for the following expression vector constructs. The open reading frames (ORFs) of PoTLR5S, EtFliC, and EcoFliC were amplified by PCR, following which the PCR products were digested with a set of two restriction enzymes (*i.e.*, *Eco*RI/*Xho*I or *Eco*RI/*Not*I) and ligated into pcDNA4-HisMaxA vector (Promega). The plasmid DNA including pEtFliC, pEcoFliC, pPoTLR5S, and pEtFliC_N91R was purified using the PureLink™ HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific, USA) for transfection.

2.3. Cell line and transfection

Hirame natural embryo (HINAE) cells derived from a Japanese flounder embryo [26] were maintained in Leibovitz's L-15 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin/streptomycin (Thermo Fisher Scientific). Prior to transfection, HINAE cells were precultured in 24-well plates (5×10^5 cells/well) at 20 °C. The HINAE cells were washed with phosphate-buffered saline (PBS) and cultured with Opti-MEM (Gibco, USA). Transfection was performed with Lipofectamine 3000 reagent (Invitrogen, USA) according to the manufacturer's instructions. Equimolar amounts of flagellin gene expression vector (0.71 M of pcDNA4-Empty, pEtFliC, pEcoFliC, or pEtFliC_N91R) and PoTLR5 expression vector (0.71 M of pcDNA4-Empty, pPoTLR5S, or PoTLR5M), 1.5 μl of Lipofectamine 3000 reagent, and 1 μl of P3000 reagent were mixed with 50 μl of Opti-MEM. The DNA mixtures were transfected into HINAE cells in 450 μl of Opti-MEM and then incubated at 20 °C for 4 h. After the incubation, the Opti-MEM replaced to the cultured medium and cultured at 20 °C for 24 h.

2.4. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR)

For gene expression analysis, total RNA was extracted with RNAiso Plus (Takara) according to the manufacturer's instructions. All RNA samples were quantitatively and qualitatively measured with the ND-1000 NanoDrop spectrometer (Thermo Fisher Scientific). The extracted total RNA was used for cDNA synthesis *via* reverse transcription using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO Co., LTD., Japan).

Quantitative real-time PCR (qPCR) was performed in duplicate using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, USA) and the primers shown in Table 1. Each 15 μl reaction contained 3 μl of diluted cDNA template, 7.5 μl of Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (2×), and 1.5 μl of forward and reverse primers (5 μM). Thermocycling was conducted for 45 cycles with the following conditions: 3 min at 95 °C, 15 s at 95 °C, and 45 s at 60 °C. The comparative threshold cycle (CT) method ($2^{-\Delta\Delta CT}$ method) [27] was used to analyze the relative expression level of the target mRNAs, with the flounder β-actin gene as an internal reference. All primer sets used in the current study are listed in Table 1. Values are presented as the mean ± standard errors of the mean (SEMs) of four individual experiments. The statistical *p*-values were calculated by Student's *t*-test.

Table 1
Oligonucleotide sequences used in this study

Name	Primer #	Sequences (5' - 3')	Mer
Primers for cloning into pGEM-T Easy vector:			
EtFliC-F1	P#900	ATGGCACAAGTAATTAATACCA	22
EtFliC-R1	P#901	TTAACGCAGCAGAGACAG	18
EcoFliC-F1	P#1090	GGATAACGAATCATGGCACA	20
EcoFliC-R1	P#1091	TTAACCCTGCAGCAGAGAC	19
PoTLR5S-F2	P#930	ATGTGGAGGCTGTTTCTCCA	20
PoTLR5S-R3	P#933	TTACTCCTGTGTGACTTGAGC	21
PoTLR5M-F2	P#921	ATGTGGACACTGGCCCCT	18
PoTLR5M-R3	P#924	TCACATGGCAACAGCTCTG	19
Primers for cloning into pcDNA4-HisMaxA vector:			
EtFliC-EcoRI-F1	P#947	TGAATTCATGGCACAAGTAATTAATACCA	28
EtFliC-XhoI-R2	P#1045	GTACTCGAGTTAACGCAGCAGAGACAG	27
EcoFliC-EcoRI-F1	P#1112	GCCGAATTCGATAACGAATCATGGCACA	28
EcoFliC-XhoI-R1	P#1113	ATTCTCGAGTTAACCCTGCAGCAGAGAC	28
PoTLR5S-EcoRI-F1	P#934	GCTGAATTCTGTGGAGGCTGTTTCTCCAG	29
PoTLR5S-NotI-R2	P#1044	CTTGCGGCCGCTTACTCCTGTGTGACTTGA	30
PoTLR5M-EcoRI-F2	P#1055	CATGAATTCCAGCATGTGGACACTGGC	27
PoTLR5M-XhoI-R2	P#1056	ATACTCGAGTCCATGGCAACAGCTCTG	28
Primers for site-directed mutagenesis:			
EtFliC-mut-F1	P#1129	CTGCAGCGTATCCGTCGTACCGTACAG	27
EtFliC-mut-R2	P#1159	ACGGATACGCTGCAGGTTGTCGTTGAC	27
Primers for qPCR analyses:			
PoIL-1β_rt_F3	P#1174	CCTCCTCTCCACTGACTACCA	21
PoIL-1β_rt_R3	P#1175	CTCCACATCTGGCTCACGTT	20
PoNF-κB-p65_rt_F1	P#814	AAGCACAGCACGGAGAACAGT	21
PoNF-κB-p65_rt_R1	P#815	GCAACTTGCGCGTTCATAGA	20
PoTLR5S_rt_F1	P#1143	ATCTGTGTGTTCCTGCAGATG	21
PoTLR5S_rt_R1	P#1144	TGGAGTTAATCTCCCCGATG	20
EtFliC_rt_F1	P#1141	GCTGAACGAAGTCAACGACA	20
EtFliC_rt_R1	P#1142	AGTCGGTCTGCTGGGAGATA	20
EcoFliC_rt_F1	P#1139	TTGGCCTTGATGGTTTTAGC	20
EcoFliC_rt_R1	P#1140	AGTGGCTGCTTCCGTAGAAA	20
Poβ-actin_rt_F1	P#1147	CTGGACTTCGAGCAGGAGAT	20
Poβ-actin_rt_R1	P#1148	TTCCACAGGACTCCATACCG	20

3. Results

3.1. Induction of inflammation-related genes by overexpression of PoTLR5S and EtFliC in HINAE cells

First, to confirm whether EtFliC and EcoFliC induce inflammation-related gene expression *via* PoTLR5M, whose structure is similar to that of mammalian TLR5, expression of inflammation-related genes in HINAE cells co-transfected with EtFliC, EcoFliC, and PoTLR5M was analyzed by qPCR (Fig. 1A and B). The expression of the interleukin (IL)-1β and NF-κB-p65 genes in both EtFliC- and EcoFliC-overexpressing HINAE cells was higher than in those transfected with empty vector. Moreover, the expression of the IL-1β gene in both FliC- and PoTLR5M-overexpressing cells was significantly higher than in FliC-overexpressing cells. Furthermore, the expression levels of the IL-1β and NF-κB-p65 genes in EcoFliC-overexpressing cells were significantly higher than those in EtFliC-overexpressing cells (Fig. 1A and B).

In EtFliC- and/or PoTLR5S-overexpressing cells, expression of the IL-1β and NF-κB-p65 genes also increased compared with that in EcoFliC- and/or PoTLR5S-overexpressing cells (Fig. 1C and D). The EtFliC- or EcoFliC-expressing cells tended to induce IL-1β and NF-κB-p65 gene expression (Fig. 1C and D). Moreover, the expression level of the IL-1β and p65 genes in EcoFliC- and PoTLR5S-overexpressing cells was significantly higher than in EtFliC- and PoTLR5S-overexpressing cells (Fig. 1C and D). Furthermore, in cells overexpressing PoTLR5S only, gene expression of IL-1β significantly increased (Fig. 1C). The overexpression of all genes (*i.e.*, PoTLR5M, PoTLR5S, EtFliC, and EcoFliC) was confirmed in the transfected HINAE cells (Supplementary Fig. 1).

3.2. Characterization of E. tarda FliC

The FliC gene derived from *E. tarda* OA-3 (GenBank accession number LC427229) contains a 1251-bp coding region encoding 417 amino acid residues. The deduced amino acid sequence contains N- and C-terminal helical regions of the D1 domain (located at positions 45–140 and 330–369) (Fig. 2A). Alignment of EtFliC and other known bacterial flagellin amino acid sequences revealed that the N- and C-terminal helical regions and some TLR5 activation sites were highly conserved (Fig. 2A). Twenty-six among 31 amino acids identified as important residues for flagellin recognition [9] were highly conserved in EtFliC (highlighted in black, Fig. 2A). However, R91, which is the most important residue for flagellin-TLR5S binding sites to activate immune responses, was not conserved. The full-length EtFliC amino acid sequence shared 30–51.6% identity with the flagellin of other bacteria, and the N- and C- terminal helical regions had higher identities of 49.1–78.7% (Supplementary Table 2). In the phylogenetic tree, EtFliC clustered within the cradle of the FliC group, including *Salmonella* FliC, *Escherichia* FliC, and *Yersinia* FliC (Fig. 2B). To compare the 3D structures of the EtFliC, EtFliC_N91R, and EcoFliC D1 domains, the 3D structures were predicted based on *Salmonella* flagellin [18,28–30]. The protein structures were similar (Fig. 3B–D), but the structures of the D2 and D3 domains in EtFliC were different from those of EcoFliC (Fig. 3B and D).

3.3. Contribution of E. tarda flagellin R91 to IL-1β gene induction

To understand the potential role of the 91st residue in FliC, N91 to R91 of pEtFliC was mutated in pEtFliC_N91R by site-directed mutagenesis (Fig. 3A). The constructs were transfected into HINAE cells, and the response of IL-1β and NF-κB-p65 gene expression was evaluated. The overexpression of all genes (*i.e.*, PoTLR5S, EtFliC, and

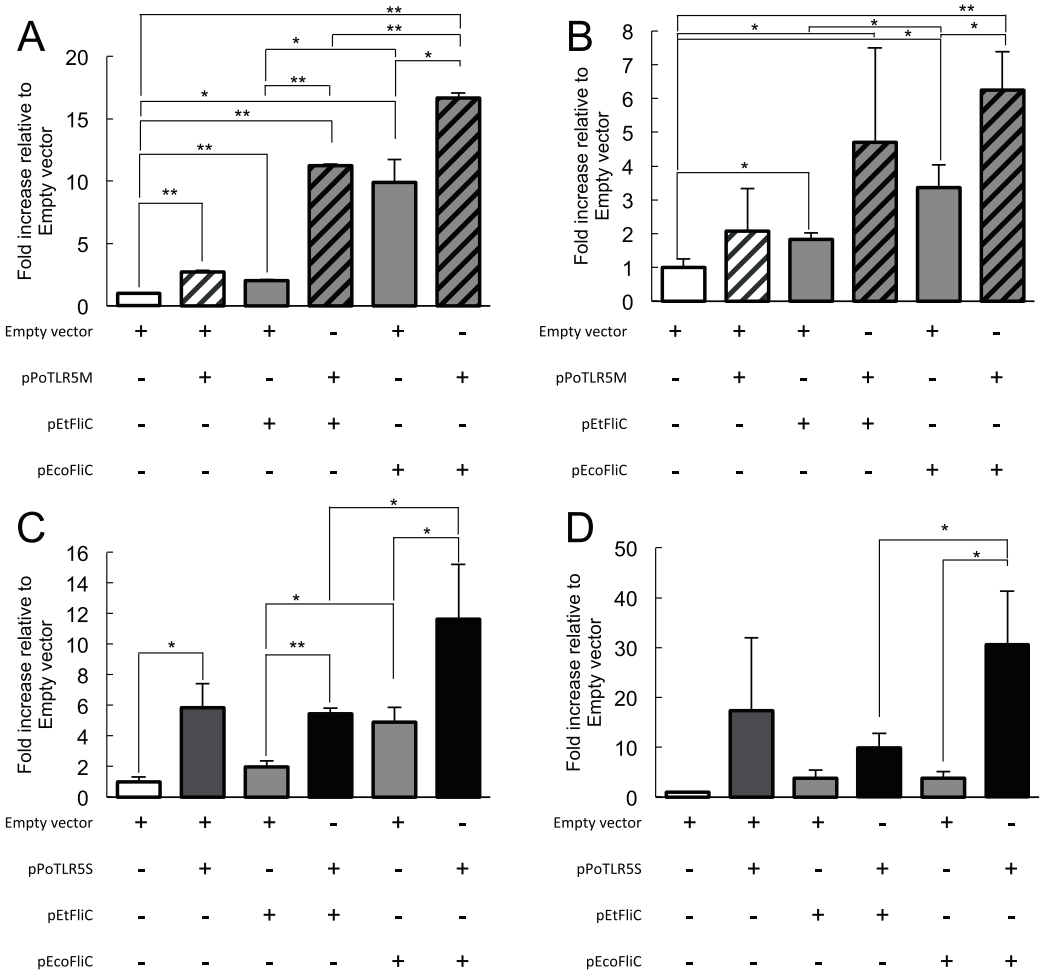

Fig. 1. Expression of inflammation-related genes (IL-1β and NF-κB-p65) in HINAE cells transfected with expression vectors of two flagellins (EtFliC and EcoFliC) and two types of TLR5 (PoTLR5M and PoTLR5S). The expression of the IL-1β (A and C) and NF-κB-p65 (B and D) genes in HINAE cells transfected with pEtFliC, pEcoFliC, and pPoTLR5M (A and B) or pPoTLR5S (C and D). Expression levels of all genes were normalized with β-actin and shown as fold increase values relative to the empty vector control. Error bars indicate mean ± SEMs (n = 4, *p < 0.05, **p < 0.01).

EtFliC_N91R) was confirmed in the transfected HINAE cells (Supplementary Fig. 2). The expression levels of the IL-1β and NF-κB-p65 genes in EtFliC_N91R-overexpressing cells were significantly higher than in EtFliC-overexpressing cells (Fig. 4A and B). Moreover, the expression level of the NF-κB-p65 gene was significantly increased in the EtFliC_N91R- and PoTLR5S-overexpressing cells compared with that in EtFliC- and PoTLR5S-overexpressing cells (Fig. 4B). The expression of the IL-1β gene in EtFliC_N91R- and PoTLR5S-overexpressing cells was also increased compared with that in EtFliC- and PoTLR5S-overexpressing cells (Fig. 4A).

4. Discussion

In this study, we focused on the potential of *E. tarda* FliC to activate innate immune response *via* TLR5 in Japanese flounder. In fish, there are two types of TLR5: TLR5S and TLR5M. TLR5M is similar to mammalian TLR5, containing an extracellular LRR domain, transmembrane region, and TIR domain [11,12,14]. However, Cyprinidae harbor two types of TLR5M known as TLR5a and TLR5b [31]. In mammals, both gram-negative and -positive bacterial flagellins activate and are recognized by TLR5M and activate the NF-κB signaling pathway to induce production of inflammatory cytokines [6,32]. The results in Fig. 1A and B showed that EtFliC and EcoFliC induced IL-1β and NF-κB-p65 through Japanese flounder TLR5M. In a previous study, *Salmonella enterica* flagellin stimulation strongly induced TLR5S gene expression in Japanese flounder liver cells, and the expression of the IL-1β and IL-6 genes was subsequently induced in the flounder liver cells and PBLs [12]. Moreover, the rainbow trout TLR5S promotes flagellin-mediated NF-κB activity [11,17]. The results in Fig. 1C and D demonstrated that both TLR5S and bacterial flagellin are required to induce IL-1β and NF-κB-p65 gene expression. Therefore, it was considered that Japanese flounder TLR5S enhanced inflammation mediated by induction of pro-inflammatory cytokine expression.

In EtFliC, the D1 domain, which is important for recognition by TLR5, is highly conserved. The EtFliC D1 domain showed higher identities of 49–79% with those of other bacteria (Supplementary Table 2), and the 3D structure of the EtFliC D1 domain predicted by SWISS-MODEL was quite similar to that of EcoFliC (Fig. 3B–D). However, the amino acid residue at the TLR5 activation site was N91 instead of R91 as in other bacteria (Figs. 2A and 3A). Moreover, the expression of the IL-1β and NF-κB-p65 genes in EtFliC- and PoTLR5S-overexpressing HINAE cells was lower than in EcoFliC- and PoTLR5S-overepxressing cells (Fig. 1C and D). It was previously demonstrated that R89 in H7 flagellin derived from *E. coli* O175, which corresponds to N91 of EtFliC, is important for motility of *E. coli* O175 [33]. In addition, the amino acid residues R90, Q97, and E114 in H7 flagellin and those of R90, Q97 and E114 in *Salmonella* flagellin (*i.e.*, those of R91, Q98 and E116 in EtFliC) play a key role in effective flagellin-TLR5 binding and signaling in zebrafish and mammals [9,25]. Furthermore, the three amino acid residues in *B. subtilis* flagellin were defined as a hot spot for

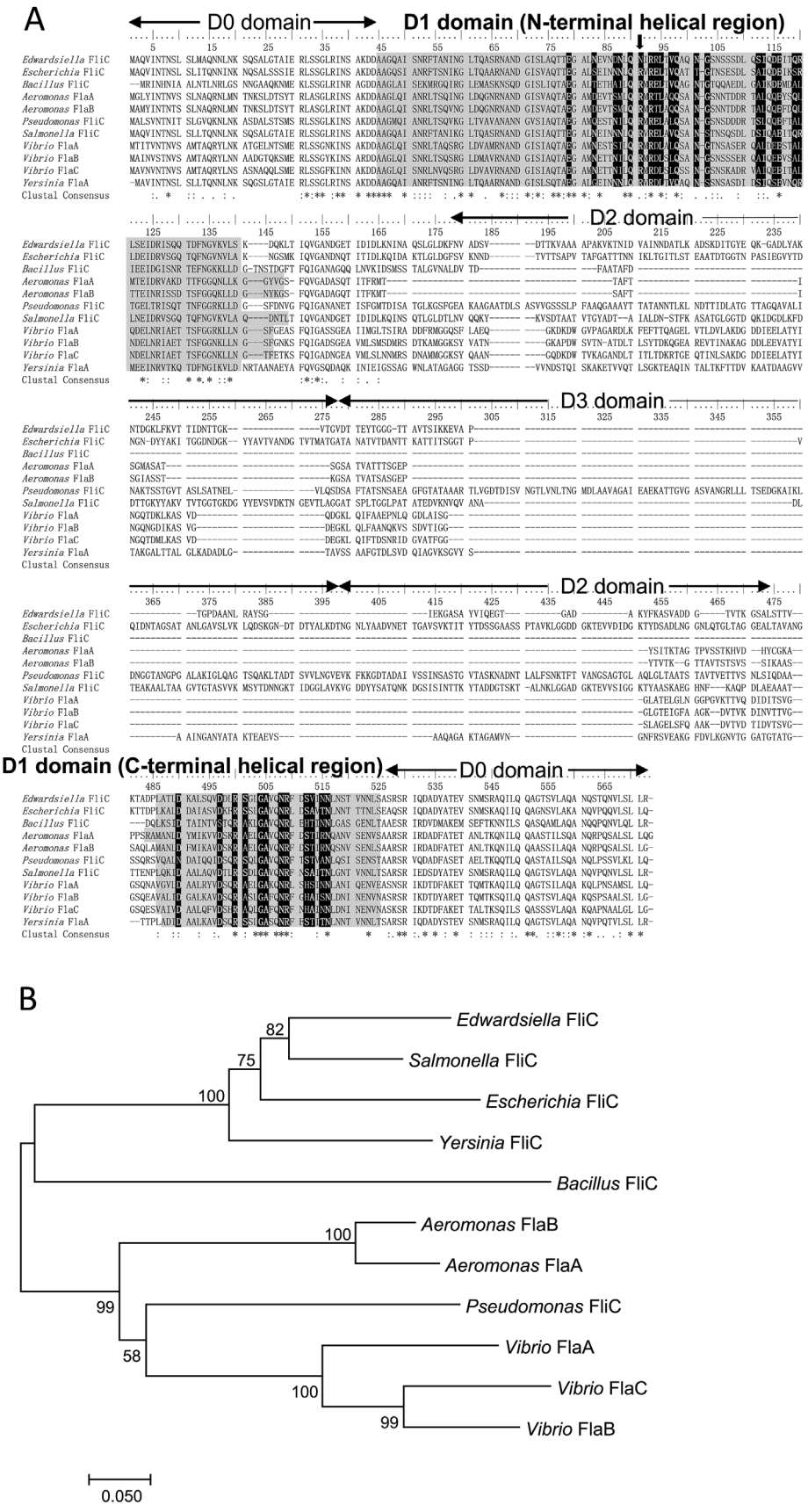

Fig. 2. Comparison of the deduced amino acid sequences of flagellin from *E. tarda* and other known bacterial species. (A) Multiple sequence alignment of EtFliC and other bacterial flagellins. The D1 domains that consist of N- and C-terminal α-helix regions are highlighted in gray, and the amino acids bound to TLR5 are highlighted in black [9]. The arrow shows the position of the 91st arginine residue related to TLR5 activation. (B) Phylogenetic relationship of EtFliC with flagellin of other bacterial species.

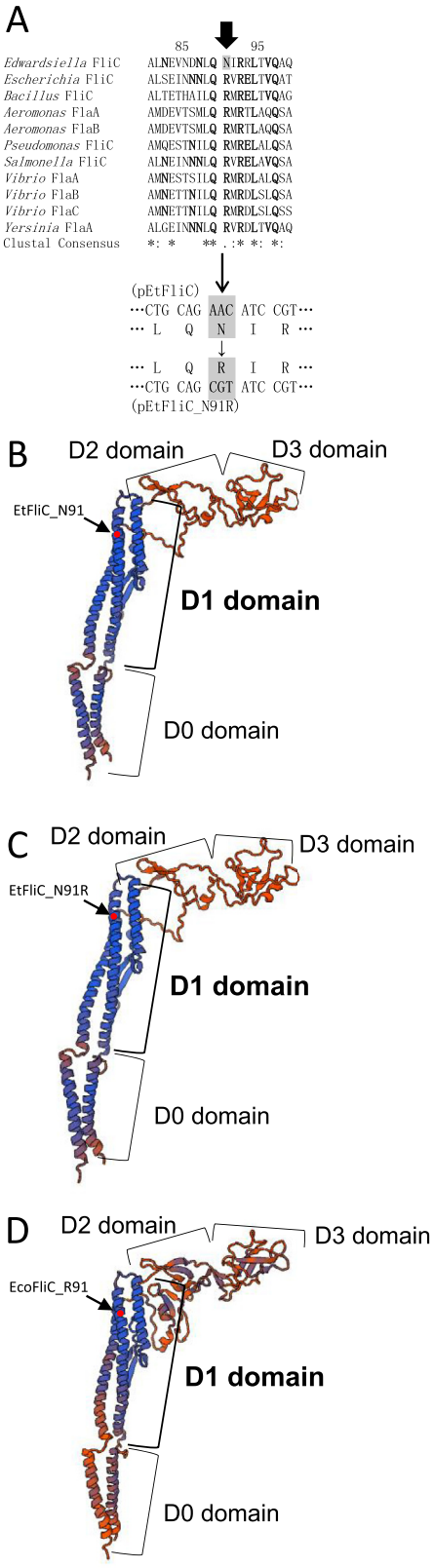

Fig. 3. The predicted three-dimensional structure of flagellin protein. (A) The amino acids bound to TLR5 are indicated in bold letters, and the arrow shows the position of the 91st arginine residue (R91) related to TLR5 activation. Based on pGEM_EtFliC plasmid, the triplet codon of "AAC" encoding asparagine (N91) in EtFliC was mutated to "CGT" encoding arginine (R91). The structures of EtFliC (B), EtFliC_N91R (C), and EcoFliC (D) were predicted by SWISS-MODEL.

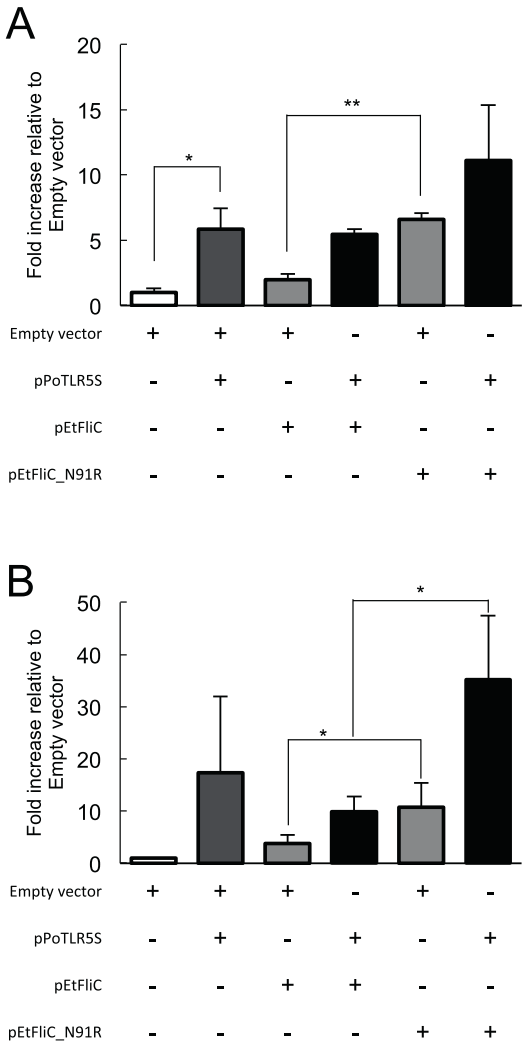

Fig. 4. Expression of inflammation-related genes in HINAE cells transfected with the expression vectors of EtFliC, EtFliC_N91R, and PoTLR5S. The expression of the IL-1β (A) and NF-κB-p65 (B) genes in HINAE cells transfected with pEtFliC, pEcoFliC, and/or pPoTLR5S. Expression levels of all genes were normalized with β-actin and shown as fold increase values relative to the empty vector control. Error bars indicate mean ± SEMs (n = 4, *p < 0.05, **p < 0.01).

TLR5 activation [24]. Moreover, these three hot spot residues are not conserved in *Campylobacter jejuni*, *Helicobacter pylori*, and *Bartonella bacilliformis* flagellins, which are known as TLR5 non-activators [24,34–36]. In EtFliC, the residues of Q98 and E116 were conserved, whereas R91 was not conserved (Figs. 2A and 3A), and mutation experiments using pEtFliC_N91R expression vector demonstrated that the expression level of both the IL-1β and NF-κB-p65 genes increased in EtFliC_N91R-overexpressing HINAE cells compared with that in cells overexpressing wild-type EtFliC (Fig. 4). Therefore, the results suggested that the 91st residue in EtFliC is important for induction of IL-1β and NF-κB-p65 gene expression *via* PoTLR5S. This may explain why the potential of EtFliC to activate the innate immune response *via* TLR5 in Japanese flounder is lower than that of EcoFliC.

In mammals, flagellin is known as an activator involved in innate and adaptive immunity and has been demonstrated to be an effective adjuvant [37,38]. EtFliC could function as both a ligand of TLR5 and an antigen in fish [39,40]; however, recombinant EtFliC showed no apparent immunoprotectivity [39]. The results of this study suggested that EtFliC improved by site-directed mutagenesis could be an effective

adjuvant against *E. tarda* infection, considering *in vivo* further experiments in Japanese founder next.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.024.

References

[1] S.H.M. Ling, X.H. Wang, L. Xie, T.M. Lim, K.Y. Leung, Use of green fluorescent protein (GFP) to study the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models, Microbiology 146 (1) (2000) 7–19 https://doi.org/10.1099/00221287-146-1-7.

[2] R.P. Srinivasa, T. Lim, K. Leung, Opsonized virulent *Edwardsiella tarda* strains are able to adhere to and survive and replicate within fish phagocytes but fail to stimulate reactive oxygen intermediates, Infect. Immun. 69 (2001) 5689–5697 https://doi.org/10.1128/IAI.69.9.5689-5697.2001.

[3] K. Yonekura, S. Maki-Yonekura, K. Namba, Growth mechanism of the bacterial flagellar filament, Res. Microbiol. 153 (4) (2002) 191–197 https://doi.org/10.1016/S0923-2508(02)01308-6.

[4] Y.P. Tan, Q. Lin, X.H. Wang, S. Joshi, C.L. Hew, K.Y. Leung, Comparative proteomic analysis of extracellular proteins of *Edwardsiella tarda*, Infect. Immun. 70 (11) (2002) 6475–6480 https://doi.org/10.1128/IAI.70.11.6475-6480.2002.

[5] H.C. Ramos, M. Rumbo, J.C. Sirard, Bacterial flagellins: Mediators of pathogenicity and host immune responses in mucosa, Trends Microbiol. 12 (11) (2004) 509–517 https://doi.org/10.1016/j.tim.2004.09.002.

[6] F. Hayashi, K.D. Smith, A. Ozinsky, T.R. Hawn, E.C. Yi, D.R. Goodlett, J.K. Eng, S. Akira, D.M. Underhill, A. Aderem, The innate immune response to bacterial flagellin is mediated by Toll- like receptor 5, Nature 410 (6832) (2001) 1099–1103 https://doi.org/10.1038/35074106.

[7] S. Akira, Toll-like Receptor Signaling, J. Biol. Chem. 278 (40) (2003) 38105–38108 https://doi.org/10.1074/jbc.R300028200.

[8] E. Andersen-Nissen, K.D. Smith, R. Bonneau, R.K. Strong, A. Aderem, A conserved surface on Toll-like receptor 5 recognizes bacterial flagellin, J. Exp. Med. 204 (2) (2007) 393–403 https://doi.org/10.1084/jem.20061400.

[9] S.-I. Yoon, O. Kurnasov, V. Natarajan, M. Hong, A.V. Gudkov, A.L. Osterman, I.A. Wilson, Structural basis of TLR5-flagellin recognition and signaling, Science 335 (6070) (2012) 859–864 https://doi.org/10.1126/science.1215584.

[10] L.A.J. O'Neill, C.A. Dinarello, The IL-1 receptor/toll-like receptor superfamily: Crucial receptors for inflammation and host defense, Immunol. Today 21 (2000) 206–209 https://doi.org/10.1016/S0167-5699(00)01611-X.

[11] T. Tsujita, H. Tsukada, M. Nakao, H. Oshiumi, M. Matsumoto, T. Seya, Sensing bacterial flagellin by membrane and soluble orthologs of toll-like receptor 5 in rainbow trout (*Onchorhynchus mikiss*), J. Biol. Chem. 279 (47) (2004) 48588–48597 https://doi.org/10.1074/jbc.M407634200.

[12] S.D. Hwang, T. Asahi, H. Kondo, I. Hirono, T. Aoki, Molecular cloning and expression study on Toll-like receptor 5 paralogs in Japanese flounder, *Paralichthys olivaceus*, Fish Shellfish Immunol. 29 (4) (2010) 630–638 https://doi.org/10.1016/j.fsi.2010.06.011.

[13] J.Y. Moon, B.H. Nam, H.J. Kong, Y.O. Kim, W.J. Kim, B.S. Kim, K.K. Kim, S.J. Lee, Maximal transcriptional activation of piscine soluble Toll-like receptor 5 by the NF-κB subunit p65 and flagellin, Fish Shellfish Immunol. 31 (6) (2011) 881–886 https://doi.org/10.1016/j.fsi.2011.08.002.

[14] I. Muñoz, M.P. Sepulcre, J. Meseguer, V. Mulero, Molecular cloning, phylogenetic analysis and functional characterization of soluble Toll-like receptor 5 in gilthead seabream, *Sparus aurata*, Fish Shellfish Immunol. 35 (1) (2013) 36–45 https://doi.org/10.1016/j.fsi.2013.03.374.

[15] F. Liu, B. Su, Q. Fu, M. Shang, C. Gao, F. Tan, C. Li, Identification, characterization and expression analysis of TLR5 in the mucosal tissues of turbot (*Scophthalmus maximus* L.) following bacterial challenge, Fish Shellfish Immunol. 68 (2017) 272–279 https://doi.org/10.1016/j.fsi.2017.07.021.

[16] P.K. Jayaramu, G. Tripathi, A.P. Kumar, J. Keezhedath, M.K. Pathan, P.P. Kurcheti, Studies on expression pattern of toll-like receptor 5 (TLR5) in *Edwardsiella tarda* infected *Pangasianodon hypophthalmus*, Fish Shellfish Immunol. 63 (2017) 68–73 https://doi.org/10.1016/j.fsi.2017.01.041.

[17] T. Tsujita, A. Ishii, H. Tsukada, M. Matsumoto, F.S. Che, T. Seya, Fish soluble Toll-like receptor (TLR)5 amplifies human TLR5 response *via* physical binding to flagellin, Vaccine 24 (12) (2006) 2193–2199 https://doi.org/10.1016/j.vaccine.2005.11.003.

[18] S. Maki-Yonekura, K. Yonekura, K. Namba, Conformational change of flagellin for polymorphic supercoiling of the flagellar filament, Nat. Struct. Mol. Biol. 17 (4) (2010) 417–422 https://doi.org/10.1038/nsmb.1774.

[19] N.A. Serratore, C.B. Anderson, G.B. Frost, T.G. Hoang, S.J. Underwood, P.M. Gemmel, M.A. Hardy, C.J. Douglas, Integrating metal-catalyzed C-H and C-O functionalization to achieve sterically controlled regioselectivity in arene acylation, J. Am. Chem. Soc. 140 (31) (2018) 10025–10033 https://doi.org/10.1021/jacs.8b06476.

[20] K.G.K. Murthy, A. Deb, S. Goonesekera, C. Szabó, A.L. Salzman, Identification of conserved domains in *Salmonella muenchen* flagellin that are essential for its ability to activate TLR5 and to induce an inflammatory response *in vitro*, J. Biol. Chem. 279 (7) (2004) 5667–5675 https://doi.org/10.1074/jbc.M307759200.

[21] K.D. Smith, E. Andersen-Nissen, F. Hayashi, K. Strobe, M.A. Bergman, S.L.R. Barrett, B.T. Cookson, A. Aderem, Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility, Nat. Immunol. 4 (12) (2003) 1247–1253 https://doi.org/10.1038/ni1011.

[22] R. González-Stegmaier, F. Guzmán, F. Albericio, F. Villarroel-Espíndola, A. Romero, V. Mulero, L. Mercado, A synthetic peptide derived from the D1 domain of flagellin induced the expression of proinflammatory cytokines in fish macrophages, Fish Shellfish Immunol. 47 (1) (2015) 239–244 https://doi.org/10.1016/j.fsi.2015.09.016.

[23] R. González-Stegmaier, A. Romero, A. Estepa, J. Montero, V. Mulero, L. Mercado, Effects of recombinant flagellin B and its ND1 domain from *Vibrio anguillarum* on macrophages from gilthead seabream (*Sparus aurata* L.) and rainbow trout (*Oncorhynchus mykiss*, W.), Fish Shellfish Immunol. 42 (1) (2015) 144–152 https://doi.org/10.1016/j.fsi.2014.10.034.

[24] W.S. Song, Y.J. Jeon, B. Namgung, M. Hong, S.I. Yoon, A conserved TLR5 binding and activation hot spot on flagellin, Sci. Rep. 7 (2017) 1–11 https://doi.org/10.1038/srep40878.

[25] A. Tahoun, K. Jensen, Y. Corripio-Miyar, S. McAteer, D.G.E. Smith, T.N. McNeilly, D.L. Gally, E.J. Glass, Host species adaptation of TLR5 signalling and flagellin recognition, Sci. Rep. 7 (2017) 1–13 https://doi.org/10.1038/s41598-017-17935-5.

[26] H. Kasai, M. Yoshimizu, Establishment of Japanese flounder embryo cell lines, Bull. Fish. Sci. Hokkaido Univ. 52 (2001) 67–70.

[27] T.D. Schmittgen, K.J. Livak, Analyzing real-time PCR data by the comparative CT method, Nat. Protoc. 3 (6) (2008) 1101–1108 https://doi.org/10.1038/nprot.2008.73.

[28] F.A. Samatey, K. Imada, S. Nagashima, F. Vonderviszt, T. Kumasaka, M. Yamamoto, K. Namba, Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling, Nature 410 (6826) (2001) 331–337 https://doi.org/10.1038/35066504.

[29] K. Yonekura, S. Maki-Yonekura, K. Namba, Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy, Nature 424 (6949) (2003) 643–650 https://doi.org/10.1038/nature01830.

[30] F. Wang, A.M. Burrage, S. Postel, R.E. Clark, A. Orlova, E.J. Sundberg, D.B. Kearns, E.H. Egelman, A structural model of flagellar filament switching across multiple bacterial species, Nat. Commun. 8 (2017) 960 https://doi.org/10.1038/s41467-017-01075-5.

[31] A.H. Meijer, S.F. Gabby Krens, I.A. Medina Rodriguez, S. He, W. Bitter, B. Ewa Snaar-Jagalska, H.P. Spaink, Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish, Mol. Immunol. 40 (11) (2004) 773–783 https://doi.org/10.1016/j.molimm.2003.10.003.

[32] S.B. Mizel, A.P. West, R.R. Hantgan, Identification of a sequence in human Toll-like receptor 5 required for the binding of gram-negative flagellin, J. Biol. Chem. 278 (26) (2003) 23624–23629 https://doi.org/10.1074/jbc.M303481200.

[33] A. Tahoun, K. Jensen, Y. Corripio-Miyar, S.P. McAteer, A. Corbishley, A. Mahajan, H. Brown, D. Frew, A. Aumeunier, D.G.E. Smith, T.N. McNeilly, E.J. Glass, D.L. Gally, Functional analysis of bovine TLR5 and association with IgA responses of cattle following systemic immunisation with H7 flagella, Vet. Res. 46 (2015) 1–15 https://doi.org/10.1186/s13567-014-0135-2.

[34] E. Andersen-Nissen, K.D. Smith, K.L. Strobe, S.L.R. Barrett, B.T. Cookson, S.M. Logan, A. Aderem, Evasion of Toll-like receptor 5 by flagellated bacteria, Proc. Natl. Acad. Sci. U.S.A. 102 (26) (2005) 9247–9252 https://doi.org/10.1073/pnas.0502040102.

[35] L. Zhang, Z. Pan, X. Kang, Y. Yang, H. Kang, N. Zhang, J.M. Rosati, X. Jiao, Amino acids 89-96 of *Salmonella typhimurium* flagellin represent the major domain responsible for TLR5-independent adjuvanticity in the humoral immune response, Cell. Mol. Immunol. 12 (5) (2015) 625–632 https://doi.org/10.1038/cmi.2014.76.

[36] J.H. Kim, B. Namgung, Y.J. Jeon, W.S. Song, J. Lee, S.G. Kang, S.I. Yoon, *Helicobacter pylori* flagellin: TLR5 evasion and fusion-based conversion into a TLR5 agonist, Biochem. Biophys. Res. Commun. 505 (2018) 3–9 https://doi.org/10.1016/j.bbrc.2018.09.179.

[37] A. Pashine, N.M. Valiante, J.B. Ulmer, Targeting the innate immune response with improved vaccine adjuvants, Nat. Med. 11 (2005) S63–S68 https://doi.org/10.1038/nm1210.

[38] S.B. Mizel, J.T. Bates, Flagellin as an adjuvant: Cellular mechanisms and potential, J. Immunol. 185 (10) (2010) 5677–5682 https://doi.org/10.4049/jimmunol.1002156.

[39] X.-D. Jiao, M. Zhang, Y.-h. Hu, L. Sun, Construction and evaluation of DNA vaccines encoding *Edwardsiella tarda* antigens, Vaccine 27 (38) (2009) 5195–5202 https://doi.org/10.1016/j.vaccine.2009.06.071.

[40] X.-D. Jiao, S. Cheng, Y.-h. Hu, L. Sun, Comparative study of the effects of aluminum adjuvants and Freund's incomplete adjuvant on the immune response to an *Edwardsiella tarda* major antigen, Vaccine 28 (7) (2010) 1832–1837 https://doi.org/10.1016/j.vaccine.2009.11.083.