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Original Article

In silico Studies for Comparison of FliCs from *Salmonella typhimorium, Pseudomonas aeruginosa,* and *Escherichia coli* and their Implication as an Adjuvant

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An increasing number of studies have demonstrated the effectiveness of flagellin (FliC) as an adjuvant. In this study, FliC(s) from *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Escherichia coli* were compared. *In silico* studies were designed to determine physicochemical parameters of FliC from different bacteria. The *fliC* gene corresponding to best model was amplified, cloned and expressed into pET28a vector. Bioactivity of the protein was determined by measurement of the interleukin 8 in cell culture after interaction of FliC with TLR-5. According to *in silico* studies, FliC of *S. typhimorium* has a higher quality of physiochemical properties, and more affinity to TLR-5 than the other FliCs. SDS-PAGE showed an approximately 54 kDa band, and Western blot confirmed the presence of recombinant protein. IL-8 was expressed significantly in cell culture under *S. typhimorium* FliC induction. Considering side effects, and costs of conventional adjuvants, FliC can be introduced as a potent natural adjuvant in future studies. *In silico* studies can save time and cost, and screen potent proteins.

Keywords: *Salmonella typhimurium*, FliC, adjuvant

Flagellin was characterized by the phase variation phenomenon, in which the controlled expression of several flagellin-encoding genes results in the expression of alternate flagellar antigens, initially called phase 1 (H1) and phase 2 (H2) flagellins, but presently known as FliC and FljB flagellins, respectively (1). FliC, the structural subunit of flagellar filaments, contribute both to the virulence pathogenicity, and inflammatory respnses activation in mammalian hosts (2).

The important function of flagellin (FliC) in bacterial motility, presents it as an ideal candidate for innate immune recognition (3).

Adjuvants are agents which help to increase, and appropriately orient immune responses (4). Vaccine adjuvants are administered together with different vaccines, and work to enhance and sufficiently skew antigen-specific adaptive immune responses. There are three main mechanisms associated with different vaccine adjuvants: recruit-tment of innate immune cells (such as antigen-presenting dendritic cells), proper presentation of the antigen to these cells (the ‘depot effect’), and their activation. These mechanisms are broadly based on the stimulation of innate immunity, which in turn activates the adaptive immune response to clear the pathogen (5).

Few purified proteins have auto adjuvant activity, with other proteins such as FliC, tetanus toxoid, and pertussis toxin. The capacity of FliC to

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be both a target antigen of the immune response, and a natural adjuvant is well reported (1).

Toll-like receptor 5 (TLR5) is a pattern-recognition receptor, and FliC is its pathogen-associated molecular pattern (PAMP), which stimulates host defense in a variety of organisms, including plants, insects, and mammals. This adjuvant property of FliC has been exploited by fusing polypeptides to FliC to render them antigenic. FliC is a unique PAMP because it harbors an antigenic hypervariable region, and a conserved domain which is involved in TLR5-dependent systemic, mucosal pro-inflammatory, and adjuvant activities (6).

FliCs have been implicated in the production of different cytokines such as IL-8, tumor necrosis factor (TNF), and IL-1 in mammalian cells. Different authors have reported that FliCs are potent systemic and mucosal adjuvants that elicit humoral, and cellular responses to both the FliCs themselves, and co-administered antigens (6). These TLR agonists not only stimulate "broadly specific" pro-inflammatory immune responses, but also increase the adaptive immune response to defined antigens. Thus, they may be considered as adjuvants (7). Moreover, in contrast to other vaccine adjuvants, such as complete Freund's adjuvant (CFA), FliC may exert strong adjuvant effects following administration through mucosal routes (8).

The FliC from *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Escherichia coli* are the known paradigms for studies on flagellum structure-function, immunity, and TLR-5 signaling (adjuvant activities) (9) so that several reports have investigated the adjuvant effects of FliC from mentioned bacteria (10-13).

In this study, physicochemical characterizations of FliC from *S. typhimorium*, *P. aeruginosa*, and *E. coli*, and their interaction with TLR-5 were analyzed. According to *in silico* studies, the best model of FliC from mentioned bacteria was selected, then amplified, cloned, expressed and purified, and finally its bioactivity was determined.

Materials and methods

Protein modeling

Nucleotide sequences of *fliC* of *S. typhimorium*, *E. coli*, and *P. aeruginosa* were obtained from NCBI database (Genbank accession no. WP_000079805.1, JX847136, and WP_003120600.1, respectively).

Iterative Threading ASSEmbly Refinement (I-TASSER) server, a hierarchical modeling approach based on multiple threading alignments for modeling of protein, was used which produces 3-dimensional (3D) models along with their confidence score (C-score). According to I-TASSER criteria, the higher C-score shows the better quality. The quality, and reliability of modeled structure were validated and evaluated using RAMPAGE (14) and ProSa web (15). Some properties of the primary structure such as estimated half-life, aliphatic index, molecular weight, theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY), instability index, and amino acid composition were determined by the ExPASy ProtParam online tool (16).

Protein–protein interaction studies

The 3D structure of human TLR-5 was obtained from RCSB Protein Data Bank (PDB: 3J0A). Docking of the fusion protein with TLR-5 was performed using Hex docking server. Total interaction free energies were calculated based on shape and electrostatics parameters.

Bacterial strains

According to bioinformatics studies, the strains was selected, and obtained from Pasteur institute of Iran. The bacterial strain was cultured, and confirmed by biochemical tests after 18-24 h.

Amplification of *fliC*

DNA extraction was done by phenol and chloroform method. *fliC* amplification was performed as described previously (17). The primers were designed to introduce *Nco*I site at the 5´-terminus and *Hind*III site at the 3'-terminus of the recombinant gene (Table 1).

PCR condition was as follows: an initial dena-

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Table 1. Primers used to amplify FliC of *Salmonella enterica* serovar Typhimurium

Primer	Sequence
fliC F	5'- CAT GCC ATG GCG ATG GCA CAA GTC ATT AAT-3'
fliC R	5'- CCC AAG CTT ACG CAG TAA AGA GAG GAC-3'

turation for 5 min at 94 °C, followed by 30 cycles consisting of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 72 °C, with a final step at 72 °C for 5 min.

The amplified gene was digested by *Nco*I and *Hind*III restriction enzymes, then inserted into the pTZ57R vector, and the selected recombinant plasmid was subjected to sequencing (MWG service).

Cloning and expression of FliC protein

The gene cloned in the pTZ57R vector was used as the source of DNA. PCR was performed using the *Pfu* DNA polymerase (Fermentas, USA). The amplified products were cleaned up, digested, and cloned into pET28a expression vector (Novagen, USA). pET28a has the T7 promoter with histidine Tag to generate recombinant protein with 6-His at the C-terminal end. pET28a-*fliC* was transformed into competent *E. coli* BL21 (DE3). Cloning was confirmed by different ways such as gel electrophoresis of the vector, colony PCR, enzyme double digestion, and sequencing, respectively. *E. coli* BL21 (DE3) cells containing pET28a-*fliC* were grown overnight in Luria Bertani broth (LB) medium containing kanamycin (50 µg/ml) at 37 °C. Then, expression of the cloned gene was induced by different concentrations (0.1-1 mM final) of IPTG (Isopropyl β-D-1-Thiogalactopyranoside). After incubation for different times (2, 3, 4, 7, 12, and 24 h), bacteria were harvested by centrifugation at 4 °C, and stored at – 80 °C till further study.

SDS-PAGE analysis

The expressed protein was subjected to 15% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

Western blot

The loaded samples in the SDS-PAGE gel were transferred into nitrocellulose membrane (Schleicher and Schuell, Germany) using a liquid transfer system (Bio-Rad, USA). The nitrocellulose membrane was blocked by skimmed milk in PBST (PBS 1% + 0.1% Tween 20), and then was washed three times by PBST. The membranes were incubated with the conjugated His-tag antibody (Abcam, USA) for 1.5 h at room temperature. At the end, the blot was developed using substrate (Diaminobenzidine (DAB)-H_2O_2 solution).

Protein purification

After Expression, the protein was purified using Ni-NTA column (Qiagen) according to the manufacturer's instructions, and Reichelt et al.'s procedure (18) with some modifications. Lipopolysaccharide (LPS) contamination of purified protein was cleaned up by Triton X-114 (Sigma) (18), and the level of LPS contamination was assayed by the Limulus Amebocyte method. The concentration of protein was measured by NanoDrop and Bradford assay.

Biological activity

The HT-29 cell line (the human colorectal adenocarcinoma epithelial cell that expresses TLR5) was prepared from the Cell Bank, Pasteur Institute of Iran. The biological activity of FliC protein was evaluated based on the induction of IL-8. Cells were cultured in 24-well plates (Greiner, Germany) at a density of 5×10^4 cells/well in 1 ml of fresh Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, UK), and 1% penicillin-streptomycin antibiotics (Gibco, UK). After an overnight incubation, the cells were incubated for 5 h with 5 µg/ml of sterilized FliC protein (19). Supernatants of cell culture were collected and finally, IL-8 expression was assessed by the enzyme-linked immunosorbent (ELISA) test (R&D systems, USA).

Statistical analysis

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The one-way ANOVA, Student's t test was used to compare the differences between the mean values of the cell culture groups using SPSS software. $P < 0.05$ was considered as significant.

Results

Protein modeling

Sequence and composition of proteins were obtained by the ExPASy ProtParam online tool. I-TASSER server was used for 3D structure, and modeling of proteins. I-TASSER server showed C-scores of +1.23, +0.22, and +1.01 for FliC of *S. typhimorium*, *E. coli*, and *S. aeruginosa*, respectively. The Ramachandran plot of modeled structures showed that more than 98% of FliC residues of all strains fell within the allowed regions. Validation of 3D structures with ProSa-web revealed that Z-score value of FliC of *S. typhimorium* (-9.55) was closer to the range of native conformations of crystalized structures than other models (*E. coli*: -5.62, *S. aeruginosa*: -4.28), indicating the ideal quality of this model. Other physiochemical properties of the FliC(s) of different bacteria are shown in table 2.

Docking analysis

According to total free energy, FliC of *S. typhimrium* showed the best interaction tendency to TLR-5 with -222.23 kJ/mol free energy while *E. coli* and *S. aeruginosa* free energies were –178.86, and -98.3, respectively (Table 3).

According to *in silico* results, FliC of *S. typhimorium* was selected for cloning, expression, and purification.

Bacterial strains

The bacterial strains were cultured, and biochemical tests confirmed *S. typhimurium* after 18-24 h.

PCR amplification

The DNA of standard strain ATCC 14028 was used for amplification of *fliC* gene, which showed 1488 bp segment (Fig. 1). Sequencing confirmed that the gene was correctly constructed.

Cloning and expression

After amplification, the gene was cloned into pET28a vector system, and transformed into *E. coli* BL21 (DE3). Cloning and transformation of *fliC* gene were confirmed by colony PCR, digestion by *Nco*I and *Hind*III restriction enzymes, and sequencing (Fig. 1). Expression of *fliC* was optimized by parameters such as different concentrations of IPTG, incubation temperature, and time. Optimum expression was obtained with 0.5 mM IPTG, and 4 h incubation time at 37 °C. The level of expression was analyzed by SDS-PAGE, and confirmed by Western blot (Fig. 2). The analysis of SDS-PAGE and Western blot of the proteins revealed a specific band with approximately 54 kDa molecular weight.

Protein purification

After protein purification, SDS-PAGE analyzes revealed that a high concentration of purified protein. We obtained more than 3.56 mg from 1 liter bacterial culture by modified purifycation (Fig. 2). The level of LPS in the protein was ≤ 0.01 EU/ml.

TLR5 activity

FliC induced a significantly higher IL-8 response (880 pg/ml) than untreated cells (30 pg/ml) ($P < 0.001$) (Fig. 3).

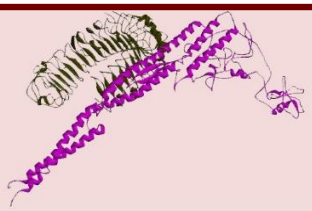
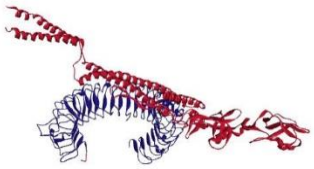
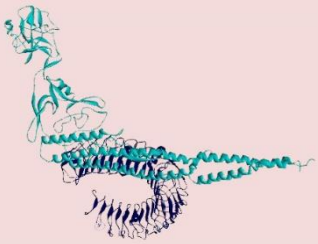
Table 2. Chemo- physical parameters of FliC proteins

Bacteria	Estimated half-life (h)***	Instability index*	Aliphatic index	GRAVY**	Isoelectric point (pI)	Molecular weight (Da)
S. typhimorium	30	24.98	89.86	- 0.395 (hydrophil)	4.79	51611.70
E. coli	10	21.02	81.33	- 0.295	4.54	58760.23
P. aeruginosa	10	16.18	88.1	- 0.093	5.12	50209.26

*: According to ExPASy ProtParam online tool criteria these proteins are classified as stable. **: Grand average of hydropathicity (GRAVY), smaller value shows protein is more hydrophilic. ***: Half- life time shows if the protein is stable in host cell or not. The lower half-life time results in faster disintegration of protein in host cell during cloning, and expression process.

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Table 3. Schematic figure and free energy result upon FliC interaction with TLR-5

FliC of different bacteria	Schematic figure of interaction	Free energy of interaction
Pseudomonas aeruginosa		– 178.86 kJ/mol
Salmonella typhimorium		-222.23kJ/mol
Escherichia coli		-98.3 kJ/mol

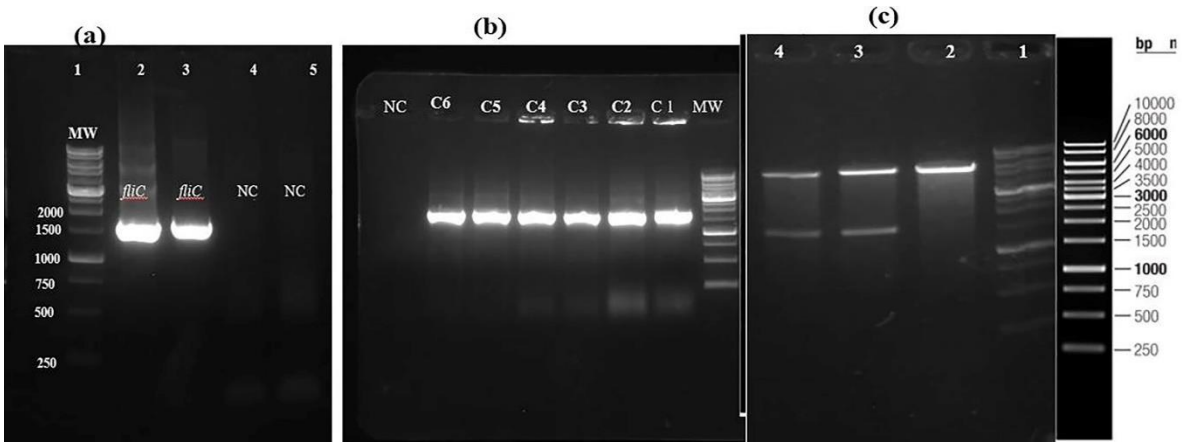


Figure 1. Agarose gel electrophoresis. (a): PCR amplification, lane 1: molecular weight marker; lanes 2 and 3: fliC gene; lanes 4 and 5: negative control. (b): Colony PCR, NC: negative control; lanes C1-C6: fliC gene (1488 bp); MW: molecular weight marker. (c): Digestion of gene cloned into pET28a vector by NcoI-HindIII restriction enzymes: molecular weight marker; lane 2: digested pET28a; lanes 3 and 4: digested pET28a-fliC.

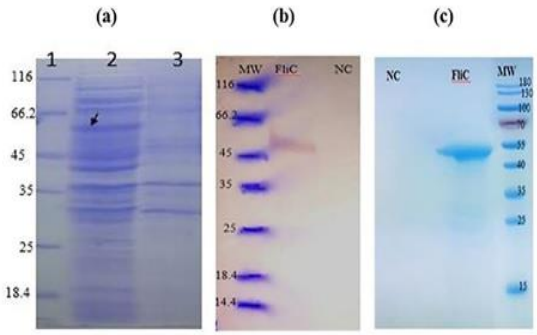


Figure 2 SDS-PAGE analysis. (a): Total cell lysate of *E. coli* BL21 (DE3) containing pET28a and pET28a-*FLIC*, lane 1: molecular weight marker; lane 2: FliC induced by 0.5 mM IPTG (arrow indicates the position of FliC); lane 3: negative control. **(b)**: Western bloting analysis, lane 1: molecular weight marker; lane 2: FliC; lane 3: negative control. **(c)**: Protein extraction, lane 1: negative control; lane 2: FliC (~ 54 kDa); lane 3: molecular weight marker.

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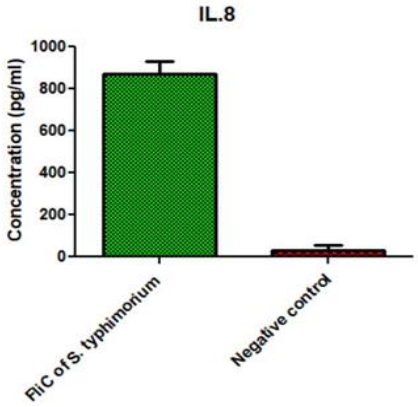


Figure 3. IL-8 expression by FliC in HT-29 cell culture. FliC induced TLR5 by producing more than 880 pg/ml IL-8.

Discussion

The discovery of mammalian TLRs, and their corresponding microbial agonists, has opened up new windows for the development of alternative adjuvants (10). FliC interaction with TLR5, results in expression of co-stimulatory molecules, and production of cytokines by cell like macrophages, and dendritic cells, leading to a more efficient activation of adaptive immune responses. Therefore it can be introduced through a novel fusion protein, as a natural adjuvant to increase its immunogenicity. However, it was not shown which bacteria has a FliC with the highest affinity to TLR5.

Chandrabali Ghose et al. tested the immuno-adjuvant properties of *Salmonella enterica* serovar Typhimurium FliC subunit D1 with toxin A and toxin B of *Clostridium difficile* as a recombinant fusion vaccine in mice. Their results showed that this fusion protein can induce rapid, high-level protection in a mouse model (20). Monica Teixeira et al. showed that *Salmonella enterica* serovar Typhimurium-derived FliC fused to CSP of *Plasmodium vivax* can activate the innate immune system (12).

In the present study, we determined the 3D structure, and modeled FliCs of *S. typhimorium*, *P. aeruginosa*, and *E. coli* which had been used previously as adjuvants. C-score depicts the quality of protein structure according to I-TASSER criteria. Accordingly, FliC of *Salmonella enterica* serovar Typhimurium has the best quality. Table 2 shows the chemo-physical parameters which are critical for synthetic chimeric proteins. According to ExPASy ProtParam online tool criteria, the index stability of protein shows how much the protein is resistant to protease enzymes, half-life shows how long the protein is stable in the host cell (less half-time leads to less gain proteins in purification, and less stability in *in vivo* circumstances), the hydrophilic index shows the quality of the protein, and high aliphatic index indicates that the protein may be stable in a wide range of temperature. FliC of *Salmonella enterica* serovar Typhimurium has more index stability, half-life, hydrophilicity, and aliphatic index than other FliCs (Table 2). Moreover, as shown in table 3, interaction of *Salmonella enterica* serovar Typhimurium FliC interaction with TLR-5 releases more free energy than the other FliC(s). Therefore, this protein has more affinity for TLR5 is more than other ones, but further animal studies are required to confirm our results.

The *fliC* gene of *Salmonella enterica* serovar Typhimurium was inserted into pET28a vector and was expressed by *E. coli* strain BL21 (DE3) as host in the form of inclusion bodies (insoluble form). His-tag, a small purification partner, has been designed into pET28a vector to facilitate purification procedures without affecting protein folding, and bioactivity. Therefore, in the present study, according to pET28a and *fliC* gene sequences, we designed one 6-His tag at the C-terminus end of the *fliC* coding sequence. We selected pET system because it is a very powerful system developed specially for the cloning, expression, and purification of recombinant proteins in *E. coli*, and also has been utilized to overexpress exogenous proteins for decades (21), although we examined other expression systems (results not shown).

FliC of *Salmonella enterica* serovar Typhimurium produced a significant amount of IL.8 in cell culture (880 pg/ml), showing that the protein has an ideal folding after expression, and purify-cation.

In conclusion, according to bioinformatics studies, FliC of *Salmonella enterica* serovar Typhi-

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murium has more affinity to TLR5 than FliC of other bacteria. Therefore, this protein can be used as a natural adjuvant with different fusion protein models.

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Conflict of interest

The authors declare that they have no competing interest.

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