

# Sequence Diversity of FliC Protein from *Enterobacteriaceae* Family to Introducing a Promising Epitope-Delivery Platform

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## ABSTRACT

**Introduction:** Flagellin (FliC) is an essential and universal subunit of flagella in motile bacteria. Analysis of the sequence diversity of the FliC protein in the *Enterobacteriaceae* family may provide insight into the pathogenic strategy of these bacteria in the context of interaction with pathogen-associated molecular patterns (PAMPs). In addition, the efficacy of FliC as an adjuvant or a component of the multi-epitope vaccine has been demonstrated. **Methods:** We analyzed 392 full-length and non-redundant FliC proteins from the *Enterobacteriaceae* family. In the first step, isoelectric pH, molecular weight (kDa), and amino acid composition of FliC protein sequences were calculated using the ProtParam program. Next, the tertiary structure of the FliC proteins and its interaction with zebrafish TLR-5 was performed. Sequence alignments were performed using ClustalW software and the curricular phylogenetic tree was depicted using MEGA-7 software and iTOL web server. Finally, we evaluated the interaction of FliC proteins of *Enterobacteriaceae* family with TLR-5 to find the strongest docking. **Results:** Physicochemical properties and multiple sequence alignments revealed that FliC has a unique characterization in each genus. However, D1 as the binding domain site associated with TLR-5 exhibited high sequence conservation and the FliC protein of *S. enterica* subsp. *enterica* had the strongest interaction with TLR-5. **Conclusions:** FliC protein of *S. enterica* subsp. *enterica* can be consider as a promising epitope-delivery platform. In addition, phylogenetic analysis revealed that FliC may be acceptable marker for distinguishing genera in the family *Enterobacteriaceae*.

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## INTRODUCTION

Flagella are filamentous organelles with essential roles in bacterial physiology, such as motility and chemotaxis [1]. Intensive studies have shown that more than 50 genes are complicated in the assembly and function of flagella [2, 3]. Flagellin (FliC) is an essential and universal subunit of flagella in motile bacteria. The flagellin subunit, encoded by the *fliC* gene, has a tubular structure at the distal end of flagella [4]. The atomic model of flagellin as a component of the 11 proto-filaments was constructed from electron micrographs [5]. The 3D structure of the FliC protein showed that this protein typically has four main domains, consisting of D0, D1, D2 and D3. The D0 and D1 domains are required to mediate flagellar polymerization. They have conserved segments in the N and C terminals of FliC and are considered to be the filament core. The inner domain (D0) consists of ~30 N-terminal and ~30 C-terminal residues forming an  $\alpha$ -helical coiled-coil structure. The outer domain (D1) consists of three  $\alpha$ -helices, designated

ND1a, ND1b and CD1, two  $\beta$ -turns, a  $\beta$ -hairpin and a long chain extending along the three  $\alpha$ -helices [6]. Crystal structure of the N-terminal fragment of zebrafish TLR-5 in complex with *Salmonella* flagellin revealed that the D1 domain interacts with TLR-5 as a pathogen-associated molecular pattern (PAMPs) [7].

The D2 and D3 domains are highly variable segments consisting mainly of  $\beta$ -strands projecting from the central core of this protein. These domains would be exposed to the solution and could play an essential role in immunogenicity. Studies have shown that motility-mediated flagella contribute as virulence factors to infectious bacteria [8]. Mutational approaches showed that EHEC and EPEC *fliC* deletion mutants significantly impaired adherence and micro-colony formation on bovine intestinal tissues compared with parental wild-type strains [9]. An increasing number of investigations have

demonstrated the efficacy of FliC as an adjuvant for vaccine development [10, 11].

Moreover, its ability to induce pro-inflammatory cytokines has confirmed that flagellin has good potential for multi-epitope vaccine design [12]. Based on this background, this study aimed to investigate flagellin sequence conservation and diversity in light of recently determined FliC structures among *Enterobacteriaceae* family and introducing a potent self-adjuvanting protein carrier. We focused on the family *Enterobacteriaceae*, which are predominantly motile, except for the genera *Shigella* and *Klebsiella* spp.

## MATERIALS AND METHODS

### Sequence Retrieval

The sequences of FliC proteins were obtained from the UniProt database (<https://www.uniprot.org/>) [13] using the following keywords "bacterial flagellin" or "FliC" in *Enterobacteriaceae* family and was saved in FASTA format for further investigations.

### Evaluation of Physicochemical Properties of FliC

ProtParam (<https://web.expasy.org/protparam/>) is a program that calculates physicochemical properties for a protein contained in Swiss-Prot or TrEMBL, as well as for a user-entered protein sequence [14]. In this step, isoelectric pH, molecular weight (kDa), and amino acid composition were obtained from this database.

### Multiple Sequence Analysis and Phylogenic Tree Drawn

All of the alignments were done using ClustalW software [16]. The phylogenetic tree was created with MEGA-7 software [17] using the neighbor-joining method for any species (BLOSUM64 matrix, Bootstrap= 500). The curricular tree was depicted by the iTOL web server (<https://itol.embl.de/>) [18].

### Docking of FliC Proteins with TLR-5

To measure the interaction of FliC proteins of *Enterobacteriaceae* family with TLR5 the pyDockWEB (<https://life.bsc.es/pid/pydockweb>) was used. The PyDockWEB returns the best rigid-body docking orientations generated by FTDock and evaluated by pyDock scoring function, which includes electrostatics, desolvation energy and limited van der Waals contribution [19]. It should be mentioned that before this experiment the 3D structure prediction of FliC proteins was conducted using Robetta (<https://rosetta.bakerlab.org/>).

## RESULTS

### Sequence Retrieval

As a result, a total number of 1023 FliC FASTA sequences were retrieved. First, truncated or unrelated sequences were excluded. Finally, 329 non-redundant full-length sequences were selected. These sequences included ten groups of species, including *S. enterica* subsp. *enterica* serovars, *Escherichia coli*, *Yersinia enterocolitica*, *Proteus mirabilis*, *Citrobacter freundii*, *Edwardsiella tarda*, *Morganella morganii*, *Providencia* spp., *Serratia* spp., and *Enterobacter cloacae*. The distribution of sequences in each group has been presented in Table 1.

### Evaluation of Physicochemical Properties of FliC Proteins

The data obtained from the ProtParam program showed that the isoelectric pH of FliC proteins from the *Enterobacteriaceae* family ranged from 3.96 to 8.32 and their molecular weight ranged from 29 to 68 kDa (Table 2). The amino acid composition of FliC protein is shown in Fig. 1. The distribution of amino acid sequences of all FliC proteins from the *Enterobacteriaceae* family showed that Alanine (Ala), Threonine (Thr) and Serine (Ser) are common amino acids while there is no Cysteine (Cys) residue in these proteins. These results indicated that these proteins are free of disulfide bonds.

**Table 1.** Retrieving of FliC protein in *Enterobacteriaceae* family extracted from UniProt database.

Bacteria	Total entry	Full-length entry	Non-redundant entry
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovars	511	331	236
<i>Escherichia coli</i>	449	376	107
<i>Yersinia enterocolitica</i>	30	22	18
<i>Proteus mirabilis</i>	8	8	6
<i>Citrobacter freundii</i>	2	2	2
<i>Edwardsiella tarda</i>	4	4	4
<i>Morganella morganii</i>	5	5	5
<i>Providencia</i> spp.	3	3	3
<i>Serratia</i> spp.	9	9	9
<i>Enterobacter cloacae</i>	2	2	2
Total	1023	762	392

**Table 2.** Data on isoelectric pH and molecular weight (kDa) of FliC proteins in the family *Enterobacteriaceae*.

Bacteria	Isoelectric pH	Molecular weight (kDa)
<i>S. enterica</i> subsp. <i>enterica</i> serovars	4.47 - 4.85	34 - 61
<i>E. coli</i>	4.24 - 4.85	36 - 68
<i>Y. enterocolitica</i>	4.70 - 5.11	38 - 39
<i>P. mirabilis</i>	4.78 - 8.32	39
<i>C. freundii</i>	4.75 - 4.83	47
<i>E. tarda</i>	4.76 - 5.05	37 - 44
<i>M. morganii</i>	4.68 - 5.39	38 - 41
<i>Providencia</i> spp.	4.68 - 4.95	39 - 41
<i>Serratia</i> spp.	4.63 - 4.82	37 - 44
<i>E. cloacae</i>	3.96 - 5.50	29

### The Interaction of FliC Protein with TLR-5

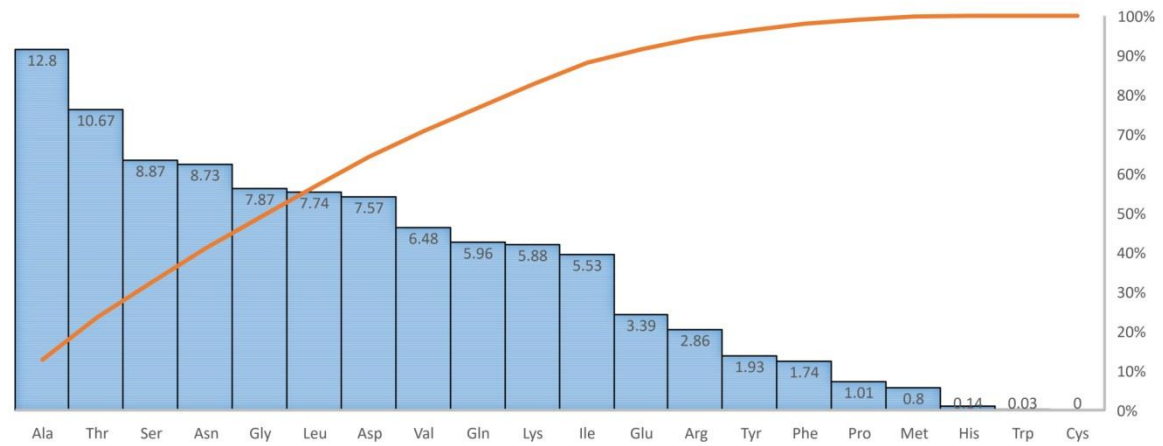
The interaction of FliC protein of with TLR-5 of *Danio rerio* (Zebrafish) have shown in Fig. 2. It was confirmed that TLR5 is known to specifically recognize flagellin [20].

It seems that Asn277 of TLR-5 has a central role in this interaction. On the other hand, Arg90, Gln97 and Asn440 are key residues in FliC. See Fig. 2. In addition, the multiple sequence alignments and amino acids of the D1 domain involved in this interaction from *S. enterica* subsp. *enterica* serovars have been shown in Fig. 3.

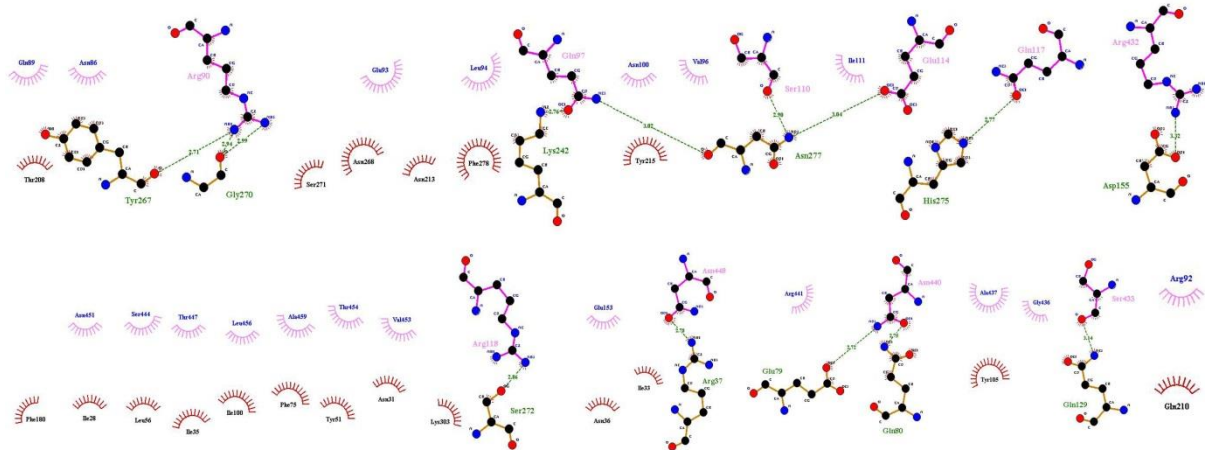
The FliC protein consists of different parts, such as the D1, the D2, C-terminal and the N-terminal domains. According to the obtained results, the amino acids of the D1 domain in *S. enterica* subsp. *enterica* serovars were involved in TLR-5 binding. In Fig. 3, the red amino acids interact electrostatically with TLR-5, while the yellow amino acids participate in Van

der Waals bonding. The positions of Arg90, Gln97 (R and Q amino acids, left hand), and Asn440 (N amino acid, right hand)

are shown in red. Q97 and N440 are conserved, while there is an amino acid substitution in R90 (R90S).



**Fig. 1.** Amino acid sequence distribution among 392 FliC proteins extracted from the *Enterobacteriaceae* family using the ProtParam program. According to obtained results Ala, Thr, and Ser were the most common amino acids. The orange curve shows the cumulative frequency of amino acid composition in FliC protein.



**Fig. 2.** The LIGPLOT representation of FliC- TLR-5 interactions. The pink amino acids of FliC interact with the green amino acids of TLR-5 in terms of electrostatic interaction. In addition, several amino acids (pink and red fluffy amino acids) are involved in Van der Waals binding. It seems that Asn277 of TLR-5 has a central role in this interaction. On the other hand, Arg90, Gln97 and Asn440 are key residues in FliC.

N-terminal of D1		C-terminal of D1	
3v47.C	.....SRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
11o1.A	.....NIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
1UCU.A	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
3AS5.A	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
spjP061.78	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfD3JX55	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfD3JXT1	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfD3JK12	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfE7C146	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfE7CZ12	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO53991	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO53993	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO54863	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO54864	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO56999	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO66PR1	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO66P55	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V25	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V268	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO66V217	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V214	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V216	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2K0	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2K2	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2K6	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2M1	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2M3	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2M5	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2N1	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2N9	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2P2	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2R7	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2U0	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO66V2U4	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLASIDALSALKVDVAVSGLGAIQRFRFSATTLNGVTNLSARSRIE
trfO66V2Y2	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfO66V2Z2	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfR9K1V5	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfR7RH9K	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE
trfR7RN44	DDAAGQAIANRFANIKGLTQASRNANDGISIAQTTEGALNEINNLQVRELSVATNGTNSDSDUK	ODIDRLRLNEIDRVSGOTFNGVKVLAODNTLTIQVANDGETTIDLO	NPLKIDDAALAOVDTLRDLGAVORRFRFSATTLNGVTNLSARSRIE

**Fig. 3.** Multiple sequence alignment of D1 in *S. enterica* subsp. *enterica* serovars involved in TLR-5 binding. The red amino acids interact with TLR-5 electrostatically, while the yellow amino acids participate in Van der Waals binding. The green vertical line separates the N-terminal and C-terminal ends of the D1 domain. The positions of Arg90, Gln97 (R- and Q-amino acids, left), and Asn440 (N-amino acid, right) are shown in red. Q97 and N440 are conserved, while there is an amino acid substitution in R90 (R90S).

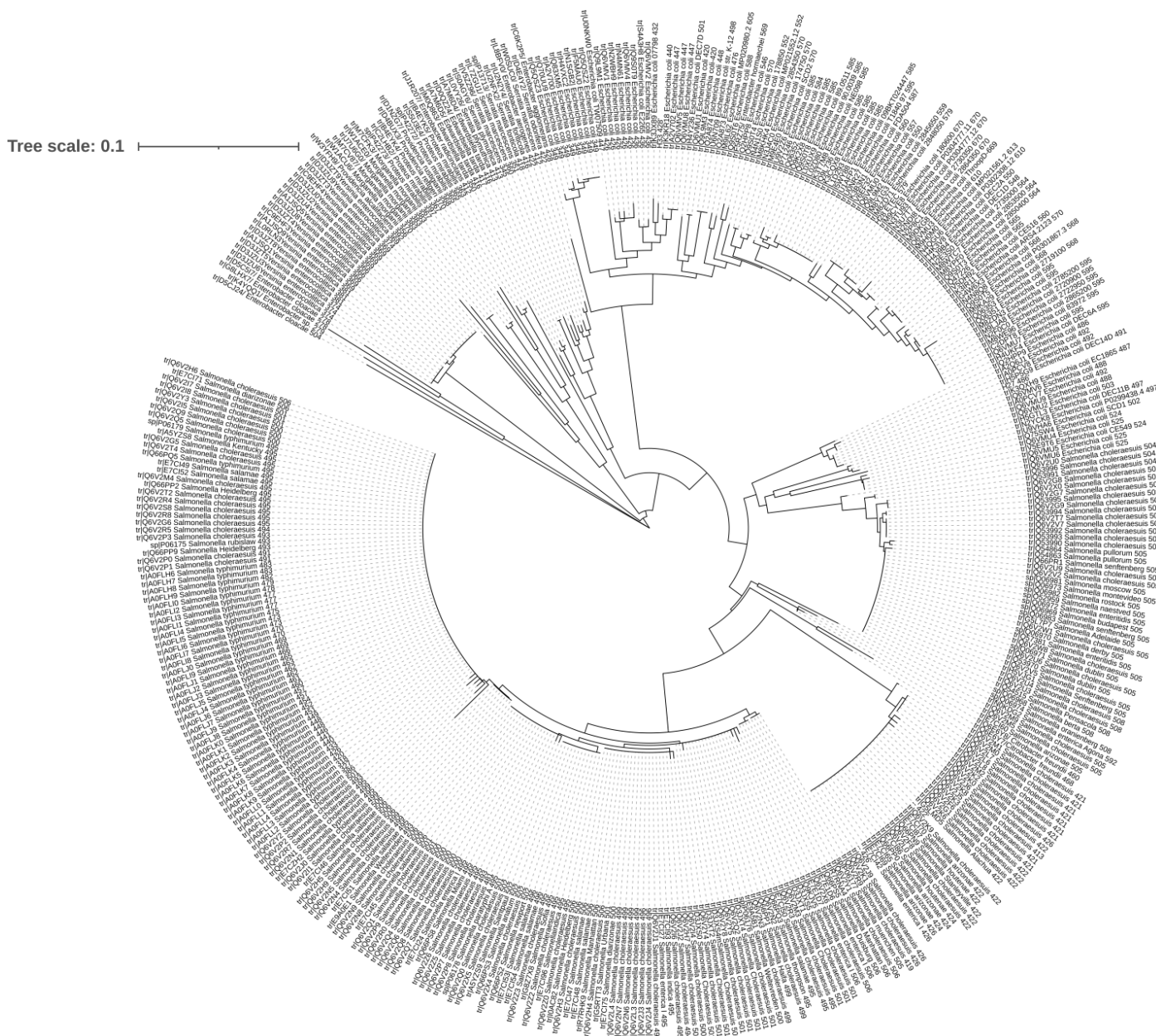
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[ DOI: 10.52547/vaccines.7.2.85 ]

**Multiple Sequence Analysis and the Phylogenic Tree of FliC**

Multiple sequence alignment revealed that there are two conserved (D0 and D1 domains) and hypervariable regions (middle part of FliC proteins). The FliC of *S. enterica* subsp. *enterica* serovars represented high heterogeneity of sequence diversity. The multiple sequence alignment of 392 FliC proteins and NEWICK file were deposited in supplemental data. In this part, the obtained results from the neighbor-joining phylogenetic tree showed that the sequence of FliC can be used

to distinguish the *Enterobacteriaceae* family. Our data indicated that there are three major clades of *S. enterica* subsp. *enterica* serovars compared to one clade of *E. coli*. The distance matrix showed that the FliC proteins of *E. coli* are more homogeneous compared to *S. enterica*. Neighbour-joining phylogenetic tree showed that several FliC types from the genus *Escherichia* have merged into *S. enterica*. See Fig. 4. In addition, the FliC of *Y. enterocolitica* has low diversity of sequence variation compared to other *Enterobacteriaceae* families. See Fig. 4.



**Fig. 4.** Neighbour-joining phylogenetic tree of 392 non-redundant full-length FliC proteins extracted from *Enterobacteriaceae* family. This tree shows there are three major clades of *S. enterica* subsp. *enterica* serovars compared to one clade of *E. coli*. The distance matrix showed that the FliC proteins of *E. coli* are more homogeneous compared to *S. enterica*. It seems that several FliC types from the genus *Escherichia* have merged into *S. enterica*.

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### Interaction of FliC Protein of Enterobacteriaceae with TLR-5

The total interaction of FliC proteins with TLR-5 are as follows: *M. morganii* (-23.555), *Y. enterocolitica* (-27.772), *E. coli* (-22.639), *S. enterica* subsp. *enterica* (-29.774),

*P. mirabilis* (-22.968), *E. tarda* (-24.099), and *E. cloacae* (-23.703). According to obtained results, it seems that the FliC protein of *S. enterica* subsp. *enterica* had the most stable interaction with TLR-5 and could be considered as the putative vaccine candidate. The full results of this part was shown in Table 3.

**Table 3.** The interaction of FliC proteins of Enterobacteriaceae family with TLR-5

Bacterium	Electrostatic interaction	Desolvation	Van der Waals forces	Total
<i>M. morganii</i>	-21.420	-3.779	16.444	-23.555
<i>Y. enterocolitica</i>	-20.762	-11.731	47.208	-27.772
<i>E. coli</i>	-25.132	5.837	-33.441	-22.639
<i>S. enterica</i> subsp. <i>enterica</i>	-16.207	-16.194	26.275	-29.774
<i>P. mirabilis</i>	-19.084	-2.801	-10.835	-22.968
<i>E. tarda</i>	-25.302	-1.661	28.641	-24.099
<i>E. cloacae</i>	-7.243	-20.158	36.976	-23.703

## DISCUSSION

The term *Enterobacteriaceae* was first proposed in 1936 [21]. This bacterial family is one of the most common causes of severe nosocomial and community-acquired bacterial infections in humans. Antibiotic resistance of these bacteria has become a growing problem in healthcare systems [22].

The flagellum is a whip-like appendage that allows bacteria to move. Flagellin is a component protein of the flagellum. Previously, flagellin was thought to be only a virulence factor that aids in host cell adhesion and invasion. However, it was discovered that it is a potent immune activator that affects both the innate and adaptive arms of immunity during microbial infections [23, 24]. Several genes are involved in flagella expression; however, FliC encodes the flagellin subunits that make up the bulk of a flagellum's structure [25]. In addition, FliC has specific roles in bacterial virulence. For example, in Enteropathogenic *E. coli* (EPEC), FliC induces the release of IL-8 in the T84 cell lineage [26]. In addition, Yang He and colleagues confirmed that FliC plays diverse roles in flagellar function, bacterial growth, protein secretion by TTSS, and virulence in *E. tarda* [27]. On the other hand, Horstmann et al. showed that post-translational methylation of flagellin facilitates adhesion to host cell surfaces and contributes to efficient intestinal colonization and host infection [28].

The efficacy of FliC as an adjuvant has been demonstrated in a growing body of research. An *in silico* study of FliCs from *S. typhimorium*, *P. aeruginosa* and *E. coli* and their use as adjuvants showed that FliC from *S. typhimorium* has a higher quality of physicochemical properties and a higher affinity for TLR-5 than the other FliCs, making its use more desirable as an adjuvant [29]. A study conducted with *S. typhimurium* and *E. coli* flagellin protein showed that purified recombinant FliC proteins can be used for DNA vaccine studies, therapeutic and multi-epitope platform purposes [30]. TLR-5 has three structural domains: extracellular leucine-rich repeats, a transmembrane domain, and a TIR domain. TLR-5 uses a group of relatively conserved amino acid residues located on both the N- and C-terminal domains of flagellin to recognize it [24]. Our data revealed some key residues of FliC protein associated with TLR-5 interaction had high conservation. On the other hand, molecular docking of FliC with TLR-5 showed that *S. enterica* subsp. *enterica* and *E. coli* had the strongest and weakest interactions with TLR-5, respectively. However, the scores of

these interactions in *Enterobacteriaceae* have narrow range (from -22.639 to -29.774).

Comparison of the flagellin amino acid sequence of many bacterial species revealed the unique domain structure of the protein [31]. The N- and C-terminal parts of the molecule responsible for secretion and polymerization are conserved between species, while the central region where the antigenic part exposed on the surface of the flagellar filaments is produced varies greatly within and between species [32]. A study by Reid *et al.*, on the sequencing of the fliC gene in pathogenic strains of *E. coli* with different H antigens, showed that the N- and C-terminal regions of the alleles are largely conserved, while the central region is more ethical and very diverse [33]. Sequencing of fliC genes related to flagellar antigens H25 and H28 revealed the genetic heterogeneity of fliC H25 and fliC H28 gene sequences in *E. coli*. Based on the allelic discrimination of these fliC genes, it is easy to develop a real-time PCR test to identify EHEC O145: H25 and O145: H28 [34]. In this study, the distance matrix showed a narrow difference between FliC proteins in *E. coli*. Potentially, PCR-sequencing or real-time PCR could be applied to detect pathotypes of this bacterium based on FliC sequence variation. However, further analysis needs to be performed.

The flagellar genes including *fliC* and *fliB*, which encode the Salmonella phase 1 and phase 2 antigens, respectively, are considered the Salmonella serotype-determining genes and contribute to the pathogenesis of Salmonella. Sequence diversity of flagellar genes suggested that there were two distinct evolutionary patterns of Salmonella flagellin gene clusters [35]. Mortimer et al. proposed a DNA-sequence based approach to serotyping of *Salmonella enterica*. It seems that sequencing and characterization of fliC can perform a molecular serotyping method [36]. The FliC protein shows great diversity in the middle of this protein among *S. enterica* subsp. *enterica* serovars, as revealed by our multiple sequencing analysis. Indeed, this bacterium exploits this property for its pathogenicity and outwits the immune system [35]. Moreover, *S. enterica* serovars were shown to reduce the expression of FliC below the level required to trigger TLR-5 to minimize identification by innate host defenses [37]. Although resistance to innate immunity is critical for the early survival of

*Salmonella*, bacterial mechanisms that prevent recognition by adaptive immunity can significantly promote survival [38].

## CONCLUSION

In conclusion, FliC protein of *S. enterica* subsp. *enterica* could be considered as a promising epitope-delivery platform. It had the strongest interaction with TLR-5. It is clear that the higher affinity for TLR-5, making FliC use more desirable as an adjuvant. Moreover, the results showed that FliC is an acceptable marker for distinguishing genera in the *Enterobacteriaceae* family. However, these phylogenetic relationships inferred based on FliC sequences should be treated with caution.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

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