Erciyes University Journal of Institue Of Science and Technology

Research Article
10.65520/erciyesfen.1757982

Imprint:
Volume: 42(1)
Year: 2026
Page: 141-152

Selda Kılıç[a]
Betül Arı[b]
Orhan Erdoğan[c*]

[a] Master's Student, Atatürk University, kilicselda552@gmail.com
[b] PhD. Student, Atatürk University, betulari255@hotmail.com
[c] Prof., Atatürk University, oerdogan@atauni.edu.tr

* Corresponding Author

Received: 8/4/2025
Accepted: 3/9/2026

Citation:
Selda Kılıç, Betül Arı, Orhan Erdoğan, (2026). Cloning and Protein Production of the Antigenic FliC Gene of Salmonella enterica serovar typhimurium ATCC 14028 Strain. *Erciyes University Journal of Institue Of Science and Technology*, 42(1), 141-152. https://doi.org/10.65520/erciyesfen.*1757982*

Screened by iThenticate for Authors & Researchers

Except where otherwise noted, content in this article is licensed under a Creative Commons 4.0 International license. Icons by Font Awesome.

Cloning and Protein Production of the Antigenic FliC Gene of Salmonella enterica serovar typhimurium ATCC 14028 Strain

Abstract

Proteins are large and complex biomolecules that perform a wide variety of biological functions in living organisms. These biomolecules play a critical role in many fields, including medicine, industry, food, the environment, and scientific research. Producing these proteins in their natural environment is both inefficient and extremely difficult. Using a molecular technique called recombinant DNA technology, proteins can be easily produced in appropriate quantities in an organism other than their natural source. The primary objective of this study was to clone the FliC gene from Salmonella enterica serovar typhimurium ATCC 14028 into the pET-SUMO vector for the first time and produce recombinant protein. For this purpose, the FliC gene obtained from S. typhimurium ATCC 14028 was inserted into the pET-SUMO vector, and recombinant protein production was carried out in Escherichia coli (E. coli) BL21(DE3) cells. The results showed that the culture induced with 1 mM IPTG provided the highest protein yield. The produced protein, approximately 70 kDa in size, was confirmed and purified using SDS-PAGE and nickel (Ni+2) affinity chromatography. In this study, the S. typhimurium FliC gene was successfully transferred to the pET-SUMO vector using the A-T cloning method for the first time, enabling the production of the targeted recombinant FliC protein.

Keywords: Cloning, FliC, Purification, Recombinant protein production, Salmonella typhimurium

Salmonella enterica serovar typhimurium ATCC 14028 Suşuna ait Antijenik FliC Geninin Klonlanması ve Protein Üretimi

Öz

Proteinler, canlı organizmalarda çok çeşitli biyolojik işlevleri yerine getiren büyük ve karmaşık biyomoleküllerdir.

Bu biyomoleküller tıp, endüstri, gıda, çevre ve bilimsel araştırma gibi birçok alanda kritik bir rol oynar. Bu proteinleri doğal ortamlarında üretmek hem verimsiz hem de son derece zordur. Rekombinant DNA teknolojisi adı verilen bir moleküler teknik kullanılarak, proteinler doğal kaynakları dışındaki bir organizmada uygun miktarlarda kolayca üretilebilir. Bu çalışmanın temel amacı, Salmonella enterica serovar typhimurium ATCC 14028'in FliC genini ilk kez pET-SUMO vektörüne aktarıldı ve rekombinant protein üretmektir. Bu amaçla, S. typhimurium ATCC 14028'den alınan FliC geni pET-SUMO vektörüne klonlandı ve rekombinant protein üretimi Escherichia coli (E. coli) BL21(DE3) hücrelerinde gerçekleştirildi. Sonuçlar, 1 mM IPTG ile indüklenen kültürün en yüksek protein verimini sağladığını gösterdi. Üretilen protein yaklaşık 70 kDa olup, SDS PAGE ve nikel (Ni+2) afinite kromatografisi ile doğrulanmış ve saflaştırılmıştır. Bu araştırmada, S. typhimurium FliC geninin A-T klonlama yöntemi ile ilk kez pET-SUMO vektörüne aktarılarak hedeflenen rekombinant

FliC proteinin üretimi başarıyla gerçekleştirilmiştir.

Anahtar kelimeler: FliC, Klonlama, Rekombinant protein üretimi, Saflaştırma, Salmonella typhimurium

❋ ❋ ❋

1. Introduction

Protein is an essential molecule for all organisms [1]. In the past, isolation of proteins from their natural sources was both inefficient and time-consuming. Today, with recombinant DNA technology, proteins can be produced safely, appropriately and in sufficient quantities in organisms other than their source [2]. These molecules, often referred to as recombinant proteins, serve as vital biopharmaceuticals that address functional deficiencies or physiological damage within an organism. This broad category encompasses a diverse range of therapeutic agents, including enzymes, growth factors, hormones, blood factors, thrombolytics, anticoagulants, vaccines, monoclonal antibodies, and various cytokines such as interferons and interleukins [3]. To produce recombinant proteins using recombinant DNA technology, an expression system must be used. Most of these proteins are produced using bacterial or eukaryotic expression systems. *Escherichia coli* (*E.coli*) is an intriguing bacterial expression system used for recombinant protein production [4]. *E. coli* is preferred because of its well-characterized genetics, versatile cloning tools, rapid growth in inexpensive media, and protein production of high quality and yield [5].

Purification is necessary to define the function, interaction with other proteins and structure of recombinant proteins [6]. Chromatographic methods are generally used to obtain proteins with high purity. Among chromatographic methods, purification is usually performed using affinity chromatography. In this method, the target protein is completely separated from other protein mixture and non-protein components in the medium [7].

Salmonella is a gram negative, rod shaped pathogen belongs to the *Enterobacteriaceae* family that lives in the intestines of humans and animals and can infect both humans and animals [8]. The genus *Salmonella* consists of two species, *Salmonella enterica* (*S.enterica*) and *Salmonella bongori* (*S. bongori*), distinguished by differences in 16S rRNA sequence analysis [9].

To date, more than 2,600 *Salmonella* serotypes have been identified [10]. *Salmonella enterica* serovar typhimurium *(S. typhimurium*), which usually causes self-limiting intestinal infection (gastroenteritis) in humans, is one of the dominant serotypes in many countries [11]. Salmonellosis is the term used to describe infections caused by the bacterium *S. typhimurium*. Symptoms of this infection include diarrhea, nausea, abdominal cramps, vomiting, and headache. These symptoms can be more severe in infants, children, the elderly, and individuals with weakened immune systems [12].

Flagella, which aid in both the pathogenicity and movement of bacteria, are present in numerous bacterial species, including Salmonella [13]. Flagella are a crucial virulence factor often associated with the early stages of infections. Flagellin, the primary structural protein component of flagella, facilitates bacterial movement [14]. *S. typhimurium* possesses two distinct flagellin proteins: FliC and FljB [15]. The FliC protein is a potent antigen that stimulates the innate immune response. Additionally, it is a powerful adjuvant that increases the effectiveness of vaccines [16].

The objective of this study was to clone the antigenic FliC gene of *Salmonella enterica* serovar typhimurium (*S. typhimurium*) ATCC 14028 into a SUMO vector and to produce recombinant FliC protein in the widely used *E. coli* BL21 (DE3) strain. As this FliC protein has a dual function as both antigen and adjuvant, it is believed that it could be used as a DNA vaccine candidate and thus protect humans from *Salmonella* infection.

2. Materials and Methods

2.1. Bacterial strains, plasmids, mediums and other reactives

E. coli One Shot Mach1™-T1R and *E. coli* BL21 (DE3) One Shot chemically competent strains from ThermoFisher Scientific (Invitrogen) were used for vector transformation and protein expression, respectively. The pET SUMO vector (Invitrogen) was used for TA cloning and protein expression. The Champion™ pET-SUMO vector is a prokaryotic expression system developed for high throughput production of recombinant proteins in *E. coli*. It contains a small ubiquitin-like modifier (SUMO; small ubiquitin-like modifier) tag fused to the N-end of the target protein. This tag acts as a molecular chaperone, increasing protein solubility, promoting correct folding and increasing overall expression efficiency. The SUMO tag is specifically cleaved by the SUMO protease, allowing the native target protein to be obtained without additional amino acid residues. These properties make it a widely preferred vector for the production of functional recombinant proteins [17]. All the other ingredients used in the study were sourced from Sigma-Aldrich Cheme, Fermentas, Merck, GeneDirex, Promega.

2.2. Construction of expression plasmids

For recombinant proteins production in a prokaryotic expression systems, coding sequences of the antigenic FliC gene from *Salmonella typhimurium* ATCC 14028 strain (GenBank: CP102669.1 , 2089078-2090565) were inserted firstly in the pET SUMO expression vector (Invitrogen). To provide a high-quality template for fliC amplification, genomic DNA was extracted from the Salmonella typhimurium ATCC 14028 strain using the "Wizard® Genomic DNA Purification Kit" (Promega, Madison, WI, USA) according to the manufacturer's instructions. Briefly, the procedure involved cell lysis, RNase treatment, protein precipitation, and DNA isopropanol precipitation. The integrity of the isolated genomic DNA was verified by 1% (w/v) agarose gel electrophoresis. The purified DNA was stored at -20°C for use as a template for fliC gene amplification. The *FliC* gene coding sequences was replicated through PCR utilizing the forward primer 5'-ATGGCACAAGTCATTAATACAAACA-3' and the reverse primer 5'-TTAACGCAGTAAAGAGAGGAC-3'. The PCR reaction was prepared in a total volume of 50 µL and ddH2O, 10xPCR Buffer, dNTP mix (10 mM), MgCl2 (25 mM), *FliC* F-R primers (10 pmol/µL), Taq DNA polymerase (5 U/µL), DNA (100 ng) were added to the reaction mixture and denaturation was carried out first at 95 °C for 2 minutes, then at 94 °C for 1 minute, then annealing at 52 °C for 1 minute and extension at 72 °C for 1.30 minutes for 36 cycles. The resulting PCR product was ligated to the pet SUMO vector.

2.3. Recombinant protein expression

The ligation mixture containing the vector carrying the *FliC* gene was grown overnight at 37 °C on a 15 mL LB agar plate containing 50 g/mL kanamycin. The resulting *E. coli* One Shot Mach1TM-T1R was added and kept on ice for 25-30 minutes. It was then subjected to heat shock for 30 seconds in a water bath adjusted to 42°C. Following transformation, colonies carrying the desired recombinant vector were identified by selecting positive colonies using colony-PCR and cross-PCR. The positive colony was selected and sent for Sanger sequencing analysis. From the colony that tested positive according to the Sanger sequencing result plasmid was isolated using the ProbondTM purification kit. The isolated plasmid was transformed to *E. coli* BL21 (DE3) using a heat shock device for 30 seconds at 42°C after being kept on ice for 30 minutes, and then inoculated onto LB agar plates. Positive colonies were then transferred to 15 mL of LB broth and incubated overnight at 37 °C in a shaking incubator at 200 rpm. At the end of the incubation period, the colonies were transferred to growing LB agar plates. Colonies that yielded positive results were inoculated into 15 mL of LB medium and incubated overnight at 37 °C at 200 rpm in a shaking incubator. Subsequently, 2500 mL of culture broth was transferred to the production culture. The pre-culture was then mixed with 100 mL of LB medium containing 50 µg/mL kanamycin and 1% glucose to produce the 6xHisFliC fusion protein.

When the OD:600 reached approximately 0.4–0.6, one of the production cultures was used as a control without induction with IPTG, while the others were induced with 0.5–1 mM IPTG in the final volume. The fusion protein was produced by incubating the mixture at 200 rpm and 37 °C.

2.4. Recombinant protein purification

To analyze the production of the recombinant fusion protein, cells were harvested by centrifugation at 13,000 rpm for 1 minute after induction. As previous studies have shown, proteins expressed from the pET-SUMO vector are intracellular, so the collected cells were lysed. To lyse the cells, 10 mL of lysis buffer was added to the pellets stored at -20°C. The cells were then rapidly frozen with liquid nitrogen for five seconds. The resulting mixture was allowed to dissolve in a water bath set to 42°C, and this process was repeated 2 or 3 times. The mixture was then centrifuged at 13,000 rpm at +4°C to collect intracellular components. The fusion protein was purified using a ProBond Nickel-Chelation resin column. To initiate purification, the concentrated fusion protein mixture was incubated with 2 mL of Ni-nitrilotriacetic acid resin balanced in denaturing binding buffer and allowed to bind to the probond affinity column at room temperature for 30 minutes. The column was washed with pH 7.8 Guanidinium Lysis buffer (20 mM NaH_2PO_4, 6 M Guanidine Hydrochloride, and 500 mM NaCl) and pH 6 wash buffer (20 mM NaH_2PO_4 and 500 mM NaCl) to allow unlabeled proteins to pass through. The interconnected proteins were extracted from the column using elution buffer at pH 4 (500 mM NaCl and 20 mM NaH_2PO_4). The final sample was added to the dialysis bag and dialyzed twice against the dialysis buffer (20 mM NaH_2PO_4, pH:8.0) was stored at low temperature and used for SDS-PAGE analysis.

2.5. Protein analysis by sds-page

SDS-PAGE was used to determine the purity and molecular mass of the protein. The expression level of the recombinant protein over time and the optimum amount of IPTG were separated on a 10% (w/v) SDS-PAGE gel. Protein lanes were visualized with Coomassie Brilliant Blue G-250.

3.Results

3.1. Development and verification of the recombinant pET-SUMO-FliC vector

The recombinant vector was created according to the standard protocol. First, genomic DNA was isolated from *Salmonella typhimurium* ATCC 14028 strain (Figure 1a). After isolation, the FliC gene was amplified by PCR using gene-specific primers. PCR products were loaded onto agarose gel and electrophoresed. The results were visualized in a UV imager (Figure 1b). According to the results obtained, the lengths of the products were as expected. The gene obtained was then cloned directly in the pET-SUMO vector.

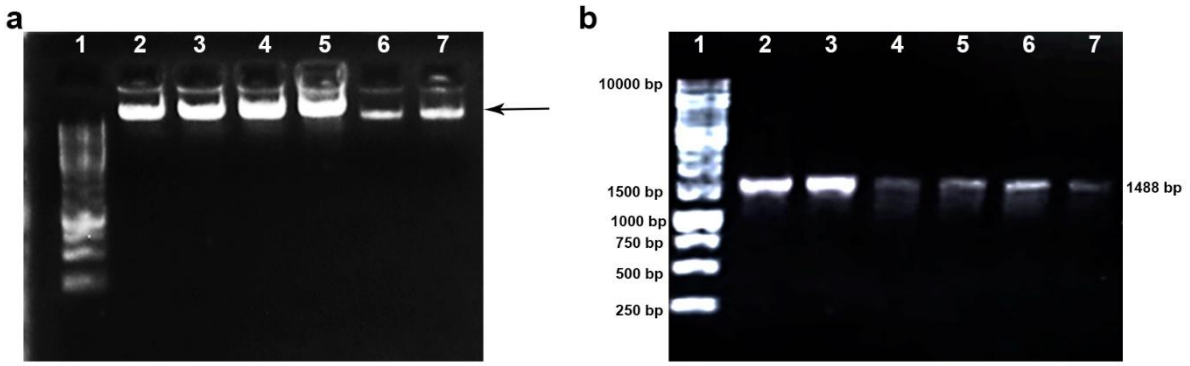
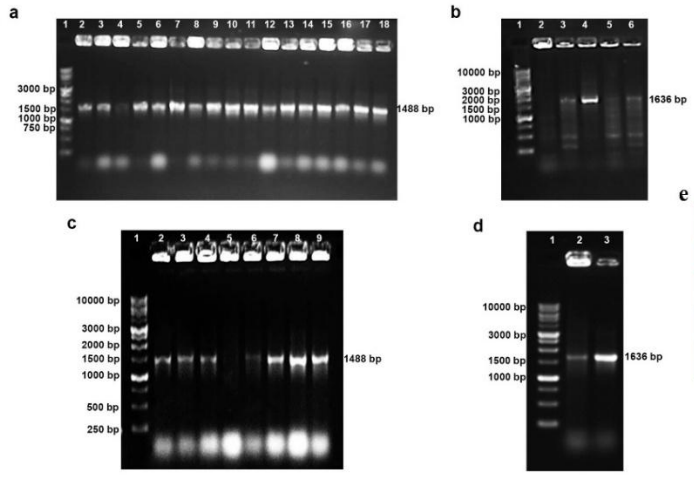


Figure 1. a) Gel image of isolated genomic DNA (1: Marker, Wells 2-7: isolated genomic DNAs) b) Agarose gel electrophoresis image of products obtained after PCR (1: Marker, Wells 2-7: PCR products of the FliC gene)

3.2. Expression of recombinant FliC protein

To express the recombinant FliC protein, the ligation product was converted into competent *E. coli One Shot* Mach1™-T1R cells, followed by isolation of the plasmid from the colony (using gene specific primers) (Figure 2a) and cross-PCR (gene F primer, vector R primer) results (Figure 2b), and the isolated plasmid was transformed into *E.coli* BL21(DE3) cells. Thus, *E.coli* BL21 *(DE3)* cells were introduced to express the coding domains of *Salmonella typhimurium* strain ATCC 14028. After transformation, colony-PCR (using gene spesific primers) (Figure 2c) and cross-PCR (gene F primer, vector R primer) (Figure 2d) results were obtained as expected. Plasmid isolation was performed on the colony thought to be positive after cross PCR, and Sanger sequencing results confirmed that the gene was correctly cloned into the vector (Figure 2). Figure 2e shows the specific sequences and thermodynamic properties of the gene-specific and vector-specific primers used in both PCR with gene primers and cross-PCR analysis to verify the correct orientation and genetic integrity of the cloned insert. Thus, the recombinant expression vector pET SUMO-FliC was obtained (Figure 3). The molecular design of the pET-SUMO-fliC construct was comprehensively validated through a multi-step sequence analysis. The junction sequence and the precise in-frame fusion at the SUMO cleavage site were initially mapped (Figure 3a), while the global plasmid structure and expression cassette were illustrated through circular and linear maps (Figure 3b-c). To ensure genetic fidelity, the 632 bp target fragment (Figure 3d) was subjected to bidirectional Sanger sequencing performed in triplicate using gene-specific forward and reverse primers (Figure 3e). The resulting consensus sequence demonstrated 100% identity with the codon-optimized reference sequence in the BLAST alignment (Figure 3f), confirming the absence of mutations or frame-shifts during the cloning process. IPTG was utilized to induce expression at final concentrations of 0.5 mM and 1 mM. Cells were lysed after IPTG induction using only three cycles of flash freezing in liquid nitrogen and thawing in a 42°C water bath. 10% (w/v) SDS-PAGE was used to evaluate the cell lysates induced by IPTG at different concentrations.



e

Primer	Sequence (5´→3´)	Tm (°C)	Length (bp)
FliC-Forward	ATGGCACAAGTCATTAATACAAACA	56	25
FliC-Reverse	TTAACGCAGTAAAGAGAGGAC	56	21
SUMO Forward	AGATTCTTGTACGACGGTATTAG	60	23
T7 Reverse	TAGTTATTGCTCAGCGGTGG	60	20

Figure 2. a) Gel electrophoresis image of colony PCR products after transformation into *E. coli One Shot* Mach1™-T1R cells (1: Marker Wells 2-18: PCR products of colonies 1-18, respectively) b) Gel electrophoresis image of Cross PCR products after conversion into *E. coli One Shot* Mach1™-T1R *cells* (1: Marker 2: 6th colony, 3: 8th colony, 4: 9th colony, 5: 10th colony, 6: 12th colony) c) Colony PCR gel electrophoresis image after transformation into *E. coli BL21* (DE3) cells (1: Marker Wells 2. 9: PCR products of selected colonies) d) Cross PCR gel electrophoresis image after transformation into *E. coli BL21* (DE3) cells (1: Marker PCR products of 1: 2nd, 2: 3rd selected colonies) e) The sequences, melting temperatures and lengths of the gene-specific primers (*FliC*-Forward, *FliC*-Reverse) and vector-specific primers (SUMO Forward, T7 Reverse) utilized in the gene PCR and cross-PCR analysis to

confirm the correct orientation

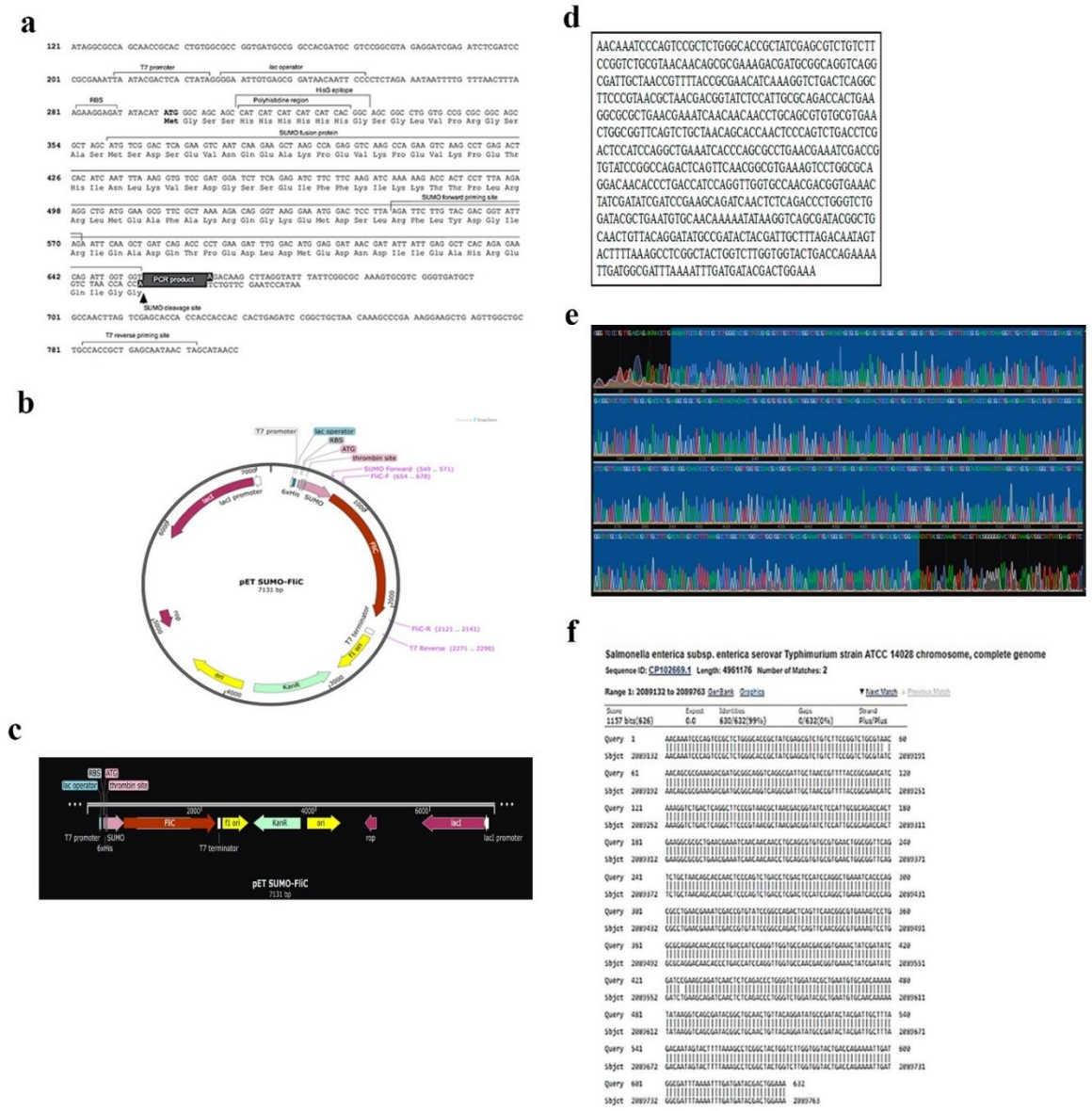


Figure 3. Molecular characterization and sequence validation of the recombinant pET-SUMO-fliC construct. (a Sequence-Level Junction Analysis: Detailed mapping of the N-terminal fusion region. The sequence confirms the precise in-frame alignment between the SUMO tag and the fliC gene, specifically at the C-terminal Gly-Gly (GGT-GGT) cleavage site. Regulatory elements (T7 promoter, RBS) and purification tags (6xHis) are explicitly annotated.b) Circular Plasmid Map: A global view of the pET-SUMO-fliC vector (7131 bp), indicating the relative positions of the antibiotic resistance marker (KanR), origin of replication (ori), and the expression cassette. Sequencing primer binding sites (SUMO Forward and T7 Reverse) are marked to show the strategy used for insert verification c) Linear Expression Diagram: A simplified representation of the recombinant cassette showing the sequential arrangement of purification tags, fusion domains, and the target gene (fliC) d) Represented Fragment: The specific DNA sequence of the fliC gene segment (632 bp) used for the alignment analysis, focusing on the region critical for structural integrity and identity e) Sanger Sequencing Chromatogram: An electropherogram showing high-resolution peaks along fusion boundaries, confirming the absence of

mutations or frameshifts at critical junction points f) BLAST Sequence Alignment: Sequence analysis performed using forward and reverse primers specific to the FliC gene separately revealed the *FliC* gene-specific nucleotide sequence (Query: Queried nucleotide sequence, Subject: Codon-optimised nucleotide sequence)

3.3. Purification and identification of the target protein

Cells were harvested via centrifugation and then lysed with a lysis solution in denaturing conditions. Since the pET-SUMO vector allows intracellular protein production, 10 mL of lysis solution was added to pellets kept at -20°C to lyse the cells. Sonication, one of the most popular techniques to physically lyse bacterial cells, was performed. After this, the samples were frozen in liquid nitrogen for 5 seconds before being allowed to defrost in a water bath adjust to 42°C. Purification of recombinant FliC proteins containing the Poly-His tag was then performed using the ProBondTM Purification System. Purification was performed under native conditions using purification columns. FliC proteins purified by native purification from the column were placed in a dialysis bag and dialyzed twice against dialysis buffer (20 mM NaH2PO4, pH:8.0) for 2 hours. The results obtained from 10% (w/v) SDS-PAGE analysis showed that the molecular weight of the expressed protein was ~70 kDa (Figure 4a). According to this evidence, induction at 37 °C for 6 h with 1 mM IPTG was selected as the optimal condition for recombinant protein production. The purified and dialyzed protein showed to have a high level of expression at the final concentration induced by 1 mM IPTG. The molecular weight of generated protein was determined to be ~70 kDa according to the result of the gel colored with Coomassie Brilliant Blue (Figure 4b).

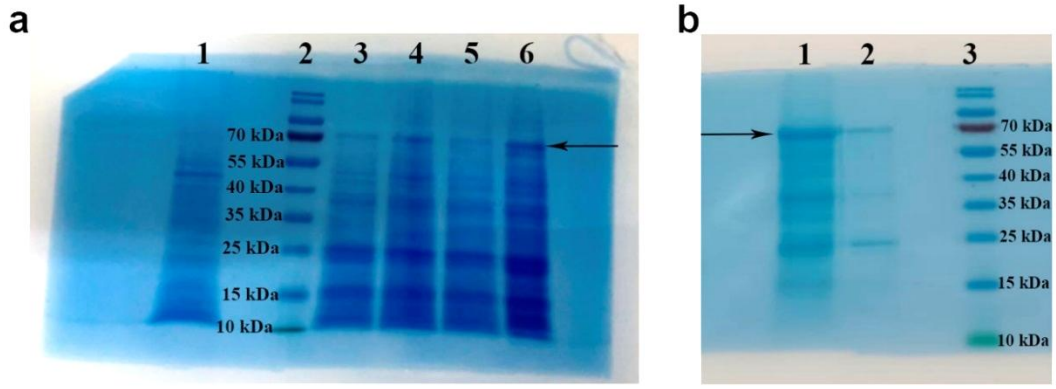


Figure 4. a) SDS-PAGE analysis of recombinant FliC protein (1: Control (IPTG-), 2: Marker 3: Colony B (0.5 mM IPTG), 4: Colony B (1 mM IPTG), 5: Colony C (0.5 mM IPTG), 6: Colony C (1 mM IPTG)) b) SDS-PAGE image of recombinant FliC protein purified by Ni-NTA affinity column (1: culture medium before IPTG-induced purification, 2: purified FliC protein induced by 1 mM IPTG, 3: Marker)

4. Discussion and Conclusion

Proteins are essential biomolecules used in medicine, in scientific investigations and many industrial fields. However, these biomolecules are complex molecules in terms of structure and function. Synthesizing these biomolecules in their native environment is a highly challenging and costly process [18]. For this reason, proteins are generally produced more safely and cost-effectively within host cell in biological processes. Alternatively, these proteins produced in the host cell are called "recombinant proteins" [19]. The first decision to be made when producing these proteins is the choice of the host cell. Bacteria, yeast, fungi and many microorganisms are used for the production of recombinant proteins. Within these hosts, bacteria generally provide an excellent expression system that allows for a fast and inexpensive production process [20]. The most commonly used bacteria for

recombinant protein production is *E. coli*, an enteric bacterium with a long history of safe use, low cost, and rapid growth kinetics in the laboratory and industry [21]. Many recombinant proteins have been produced to date and *E. coli*, still dominates bacterial expression systems [22]. From this point of view, the use of *E. coli* as a host for recombinant FliC production seems highly suitable.

Salmonella is one of the most significant pathogenic bacteria causing foodborne human illnesses and has become a major public health concern. The *Salmonella* serovar causing for intestinal infection, *S. typhimurium*, is responsible for self-limiting gastroenteritis and sometimes causes morbidity or even mortality in newborn infants and immunocompromised individuals [23]. *S. typhimurium* has many virulence factors that help it survive inside the cell. The flagellum is a key structures that helps the bacterial motility. [24]. Flagellin (FliC) is the primary structural protein component of flagella that aids in bacterial movement and feeding. During salmonellosis, flagellin acts as a natural antigen recognized by the innate and acquired immune system. The FliC gene encodes the FliC protein, which is an important component of the flagellin structure required for helical filament formation. In addition to its antigenic properties, this gene also has serve as a vaccine adjuvant [25].

In this study, we cloned the previously isolated and identified antigenic FliC gene from *S. typhimurium* ATCC 14028 strain into pET SUMO vector and realized recombinant protein production.

The pET SUMO expression system is a method for producing high-level recombinant proteins in E. coli. This system employs a small ubiquitin-like modifier (SUMO) to enable the expression of native proteins in *E. coli*. The SUMO tag utilizes its chaperoning properties to ensure proper folding and high-level expression of the target protein. The SUMO protease recognizes the SUMO fusion partner and facilitates precise proteolytic cleavage, leading to the production of native proteins [26]. The pET SUMO system is widely used to produce many therapeutically important proteins. This expression system provides a fast and efficient method for producing flagellin protein. It is therefore thought to be a useful method for the expression of proteins that are otherwise difficult to express in bacterial systems.

In summary, in this study, recombinant FliC protein was successfully produced in an *E. coli* expression system. The highest protein yield was achieved when the culture was induced with 1 mM IPTG. Following affinity column chromatography and analysis on a 10% (w/v) SDS-PAGE gel, the recombinant FliC protein appeared as a major band at approximately 70 kDa. The recombinant fusion protein exhibited an apparent molecular weight of approximately 70 kDa on SDS-PAGE. This is consistent with the calculated theoretical mass of ~65.6 kDa, which includes the FliC protein ~52 kDa [27] and His-SUMO ~13 kDa [28]. In addition, an additional band of ~25 kDa was observed. The appearance of the second band of about 25 kDa is thought to be due to the following reasons ;

1. Isoform formation: In the literature review on this subject, it was seen that there were several studies with similar results. In a study [29] in the purification of the CDK4 gene using the LC-MS strategy, they obtained different protein bands with a molecular weight of 26 and 33 kDa as a result of SDS PAGE analysis. They report that these proteins are CDK4 protein isoforms. In a similar study [30], it was observed that a single p53 gene produced two different proteins, and the second one, which encoded p53, served to suppress p53 expression even though it was expressed from the same gene region. Based on these data, it is thought that the presence of ~25 kDa protein in addition to the target FliC protein band (~70 kDa) produced may be the isoform of the FliC protein.

2. Degradation: The fragility of flagella produced in culture medium often leads to their release into the medium, where they remain even after 0.2 µm filtration. Such structural proteins often exhibit complexity; for example, Komoriya et. al. (1999) identified 12 regions for the 29 kDa flagellin, likely due to post-translational modifications or extracellular protease activity [31]. Similarly, in another study, Mani et. al. [32] found that the protein fraction induced by Xad1 contained a 29 kDa protein that

separated into three distinct isoforms under 2D SDS-PAGE.

The SDS-PAGE analysis of the purified FliC protein revealed a secondary band at approximately 25 kDa, in addition to the expected ~70 kDa fusion protein. Although post-translational modifications can introduce complexity in structural proteins, the IPTG-induced nature of this 25 kDa band strongly suggests it is a proteolytic degradation product of the pET-SUMO-fliC construct rather than an isoform protein. In prokaryotic overexpression systems, high-level production of recombinant proteins often leads to susceptibility to endogenous proteases. Therefore, the ~25 kDa band observed in our study most likely represents a C-terminal or N-terminal degradation product of the full-length SUMO-tagged FliC. While the exact cleavage site remains to be mapped, this fragment reflects the inherent fragility of flagellar proteins during heterologous expression and purification.

Furthermore, the sequence variations identified through our BLAST analysis are considered to be reflective of the natural genetic diversity inherent to the specific bacterial strain utilized in this study. It is well-documented that while pathogens such as Salmonella may appear genomically monomorphic, they actually harbor significant levels of Single Nucleotide Polymorphisms (SNPs) across different strains. A comprehensive study by Roumagnac et al. [33] utilized extensive sequence analysis of global isolates of Salmonella demonstrated that numerous independent mutational events occur and persist within extant haplotypes. This finding confirms how different strains of the same species diverge over time and exhibit regional genetic characteristics. Similarly, Holt et al. [34] demonstrated through high-throughput sequencing technologies that thousands of SNPs exist among different strains of the same species in Salmonella, and these variations can lead to functional diversity in protein-coding sequences. In light of our findings, the variations observed in the BLAST results are interpreted as strain-specific adaptations or neutral genetic drift rather than experimental artifacts. Such variations represent the organism's natural genetic background and are consistent with the structural integrity of the protein successfully expressed in our study.

Peer-review: External, Independent.

Acknowledgements:
We would like to express our sincere gratitude to Prof. Dr. Ahmet Adigüzel, Head of the Microbiology Department at Atatürk University, for providing the Salmonella typhimurium ATCC 14028 strain utilised in this study.

Declarations:

1. Statement of Originality:
This work is original.

2. Author Contributions:
Concept: SK,OE; ***Conceptualization:*** SK,OE; ***Literature Search:*** SK,BA,OE; ***Data Collection:*** SK,BA,OE; ***Data Processing:*** SK,BA,OE; ***Analysis:*** SK,BA,OE; ***Writing – original draft:*** SK,BA,OE; ***Writing – review & editing:*** SK,BA,OE.

3. Ethics approval:
Not applicable.

4. Funding/Support:
This work has not received any funding or support.

5. Competing Interests:
The authors declare no competing interests.

6. GenAI Usage Statement:
No GenAI tools were used at any stage of the study.

7. Sustainable Development Goals:

REFERENCES

[1] Mattick, J. S. (2003). Challenging the dogma: The hidden layer of non-protein-coding RNAs in complex organisms. BioEssays, 25(10), 930-939. https://doi.org/10.1002/bies.10332

[2] Khan, S., Ullah, M. W., Siddique, R., Nabi, G., Manan, S., Yousaf, M., & Hou, H. (2016). Role of Recombinant DNA Technology to Improve Life. International Journal of Genomics, 2016, 1-14. https://doi.org/10.1155/2016/2405954

[3] Unver, Y., Ari, B., Acar, M. et al. A self-inducible heterologous protein expression system in Komagataella phaffii (Pichia pastoris). 3 Biotech 14, 193 (2024). https://doi.org/10.1007/s13205-024-04039-x

[4] Eastwood, T. A., Baker, K., Streather, B. R., Allen, N., Wang, L., Botchway, S. W., Brown, I. R., Hiscock, J. R., Lennon, C., & Mulvihill, D. P. (2023). High-yield vesicle-packaged recombinant protein production from E. coli. Cell Reports Methods, 3(2), 100396. https://doi.org/10.1016/j.crmeth.2023.100396

[5] McElwain, L., Phair, K., Kealey, C., & Brady, D. (2022). Current trends in biopharmaceuticals production in Escherichia coli. Biotechnology Letters, 44(8), 917-931. https://doi.org/10.1007/s10529-022-03276-5

[6] Waugh, D. S. (2005). Making the most of affinity tags. Trends in Biotechnology, 23(6), 316-320. https://doi.org/10.1016/j.tibtech.2005.03.012

[7] Li, M., Su, Z.-G., & Janson, J.-C. (2004). In vitro protein refolding by chromatographic procedures. Protein Expression and Purification, 33(1), 1-10. https://doi.org/10.1016/j.pep.2003.08.023

[8] Chiu, T.-W., Peng, C.-J., Chen, M.-C., Hsu, M.-H., Liang, Y.-H., Chiu, C.-H., Fang, J.-M., & Lee, Y. C. (2020). Constructing conjugate vaccine against Salmonella Typhimurium using lipid-A free lipopolysaccharide. Journal of Biomedical Science, 27(1), 89. https://doi.org/10.1186/s12929-020-00681-8

[9] Reeves, M. W., Evins, G. M., Heiba, A. A., Plikaytis, B. D., & Farmer, J. J. (1989). Clonal nature of Salmonella typhi and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of Salmonella bongori comb. Nov. Journal of Clinical Microbiology, 27(2), 313-320. https://doi.org/10.1128/jcm.27.2.313-320.1989

[10] Takaya, A., Yamamoto, T., & Tokoyoda, K. (2020). Humoral Immunity vs. Salmonella. Frontiers in Immunology, 10, 3155. https://doi.org/10.3389/fimmu.2019.03155

[11] Simpson, K. M. J., Hill-Cawthorne, G. A., Ward, M. P., & Mor, S. M. (2018). Diversity of Salmonella serotypes from humans, food, domestic animals and wildlife in New South Wales, Australia. BMC Infectious Diseases, 18(1), 623. https://doi.org/10.1186/s12879-018-3563-1

[12] Hohmann, E. L. (2001). Nontyphoidal Salmonellosis. Clinical Infectious Diseases, 32, 263-269

[13] Jajere, S. M. (2019). A review of Salmonella enterica with particular focus on the pathogenicity and virulence factors, host specificity and antimicrobial resistance including multidrug resistance. Veterinary World, 12(4), 504-521. https://doi.org/10.14202/vetworld.2019.504-521

[14] Ikeda, J. S., Schmitt, C. K., Darnell, S. C., Watson, P. R., Bispham, J., Wallis, T. S., Weinstein, D. L., Metcalf, E. S., Adams, P., O'Connor, C. D., & O'Brien, A. D. (2001). Flagellar Phase Variation of Salmonella enterica Serovar Typhimurium Contributes to Virulence in the Murine Typhoid Infection Model but Does Not Influence Salmonella -Induced Enteropathogenesis. Infection and Immunity, 69(5), 3021-3030. https://doi.org/10.1128/IAI.69.5.3021-3030.2001

[15] Zarkani, A. A., López-Pagán, N., Grimm, M., Sánchez-Romero, M. A., Ruiz-Albert, J., Beuzón, C. R., & Schikora, A. (2020). Salmonella Heterogeneously Expresses Flagellin during Colonization of Plants. Microorganisms, 8(6), 815. https://doi.org/10.3390/microorganisms8060815

[16] Honko, A. N., Sriranganathan, N., Lees, C. J., & Mizel, S. B. (2006). Flagellin Is an Effective Adjuvant for Immunization against Lethal Respiratory Challenge with Yersinia pestis. Infection and Immunity, 74(2), 1113-1120. https://doi.org/10.1128/IAI.74.2.1113-1120.2006

[17] Butt, T. R., Edavettal, S. C., Hall, J. P., & Mattern, M. R. (2005). SUMO fusion technology for difficult-to-express proteins. Protein Expression and Purification, 43(1), 1-9. https://doi.org/10.1016/j.pep.2005.03.016

[18] Ceylan, H. (2016). İnsan beyin asetilkolinesteraz enziminin klonlanması, e. coli'de ekspresyonu ve enzimin karakterizasyonu.

[19] Lai, J. Y., Klatt, S., & Lim, T. S. (2019). Potential application of Leishmania tarentolae as an alternative platform for antibody expression. Critical Reviews in Biotechnology, 39(3), 380-394. https://doi.org/10.1080/07388551.2019.1566206

[20] García-Ortega, X., Cámara, E., Ferrer, P., Albiol, J., Montesinos-Seguí, J. L., & Valero, F. (2019). Rational development of bioprocess engineering strategies for recombinant protein production in Pichia pastoris (Komagataella phaffii) using the methanol-free GAP promoter. Where do we stand? New Biotechnology, 53, 24-34. https://doi.org/10.1016/j.nbt.2019.06.002

[21] Huang, C.-J., Lin, H., & Yang, X. (2012). Industrial production of recombinant therapeutics in Escherichia coli and its recent advancements. Journal of Industrial Microbiology and Biotechnology, 39(3), 383-399. https://doi.org/10.1007/s10295-011-1082-9

[22] Chen, R. (2012). Bacterial expression systems for recombinant protein production: E. coli and beyond. Biotechnology Advances, 30(5), 1102-1107. https://doi.org/10.1016/j.biotechadv.2011.09.013

[23] Achinas, S., Charalampogiannis, N., & Euverink, G. J. W. (2019). A Brief Recap of Microbial Adhesion and Biofilms. Applied Sciences, 9(14), 2801. https://doi.org/10.3390/app9142801

[24] Wang, F., Deng, L., Huang, F., Wang, Z., Lu, Q., & Xu, C. (2020). Flagellar Motility Is Critical for Salmonella enterica Serovar Typhimurium Biofilm Development. Frontiers in Microbiology, 11, 1695. https://doi.org/10.3389/fmicb.2020.01695

[25] Chilcott, G. S., & Hughes, K. T. (2000). Coupling of Flagellar Gene Expression to Flagellar Assembly in Salmonella enterica Serovar Typhimurium and Escherichia coli. Microbiology and Molecular Biology Reviews, 64(4), 694-708. https://doi.org/10.1128/MMBR.64.4.694-708.2000

[26] Munir, A., Ahmed, N., Akram, M., Fujimura, N. A., Tahir, S., & Malik, K. (2023). Enhanced soluble

expression of active recombinant human interleukin-29 using champion pET SUMO system. Biotechnology Letters, 45(8), 1001-1011. https://doi.org/10.1007/s10529-023-03402-x

[27] Taherkhani, R., Farshadpour, F., Makvandi, M., & Samarbafzadeh, A. R. (2014). Cloning of fliC Gene From Salmonella typhimurium in the Expression Vector pVAX1 and Evaluation of its Expression in Eukaryotic Cells. Jundishapur journal of microbiology, 7(11), e12351. https://doi.org/10.5812/jjm.12351

[28] Zhao, T., Huang, H., Tan, P., Li, Y., Xuan, X., Li, F., … Yang, D. (2021). Enhancement of Solubility, Purification, and Inclusion Body Refolding of Active Human Mitochondrial Aldehyde Dehydrogenase 2. ACS Omega, 6(18), 12004–12013. https://doi.org/10.1021/acsomega.1c00577

[29] Qu, J., Zhang, J., Zellmer, L., He, Y., Liu, S., Wang, C., Yuan, C., xu, N., Huang, H., & Liao, D. J. (2020). About three-fourths of mouse proteins unexpectedly appear at a low position of SDS-PAGE, often as additional isoforms, questioning whether all protein isoforms have been eliminated in gene-knockout cells or organisms. Protein Science, 29(4), 964-976. https://doi.org/10.1002/pro.3823

[30] Chen, J., Ng, S. M., Chang, C., Zhang, Z., Bourdon, J.-C., Lane, D. P., & Peng, J. (2009). P53 isoform Δ113p53 is a p53 target gene that antagonizes p53 apoptotic activity via BclxL activation in zebrafish. Genes & Development, 23(3), 278-290. https://doi.org/10.1101/gad.1761609

[31] Komoriya, K., Shibano, N., Higano, T., Azuma, N., Yamaguchi, S., & Aizawa, S. (1999). Flagellar proteins and type III-exported virulence factors are the predominant proteins secreted into the culture media of Salmonella typhimurium. Molecular Microbiology, 34(4), 767–779. https://doi.org/10.1046/j.1365-2958.1999.01639.x

[32] Mani, T., Joshi, J. B., Priyadharshini, R., Sharmila, J. S., & Uthandi, S. (2023). Flagellin, a plant-defense-activating protein identified from Xanthomonas axonopodis pv. Dieffenbachiae invokes defense response in tobacco. BMC Microbiology, 23(1), 284. https://doi.org/10.1186/s12866-023-03028-z

[33] Roumagnac, P., Weill, F.-X., Dolecek, C., Baker, S., Brisse, S., Chinh, N. T., … Achtman, M. (2006). Evolutionary History of Salmonella Typhi. Science, 314(5803), 1301–1304. https://doi.org/10.1126/science.1134933

[34] Holt, K. E., Parkhill, J., Mazzoni, C. J., Roumagnac, P., Weill, F.-X., Goodhead, I., … Dougan, G. (2008). High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi. Nature Genetics, 40(8), 987–993. https://doi.org/10.1038/ng.195