Investigating the Efficiency of Recombinant FliC-Loaded *Bacillus subtilis* Spores in Mice Immunization against *Salmonella enterica* Serovar Typhi

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Abstract

Background: Bacterial spores are among the most efficient vaccine delivery vehicles. Because of their safety and efficacy, *Bacillus subtilis* spores are increasingly used in this regard. The negatively charged surfaces of the spores allow antigens to be adsorbed onto these structures. In this study, a candidate vaccine against *Salmonella enterica* serovar Typhi was adsorbed onto *B. subtilis* spores and the immunogenicity of the formulation was investigated in BALB/c mice.

Methods: This work was performed during 2018-2019 in Islamic Azad University of Lahijan. FliC protein was recombinantly expressed in *E. coli* BL21 (DE3) cells and purified by affinity chromatography. On the other hand, *B. subtilis* strain PY79 (ATCC1609) was cultured in DSM medium and after the sporulation, FliC protein was adsorbed onto the spores in three different pH values (4, 7 and 10) and the adsorption was verified using dot-blot assay. FliC-adsorbed spores were then administered to BALB/c mice through the subcutaneous route. Mice immunization was evaluated by serum IgG assessment and challenge study.

Results: FliC protein was successfully expressed and purified. Sporulation was controlled by phase-contrast microscopy. Serum IgG assay showed significant stimulation of the mice's humoral immune system. Immunized mice were able to resist bacterial infection.

Conclusion: The results showed the efficiency of spores as natural adjuvants for the stimulation of mice immune system. The formulation can be exploited for the delivery of recombinant vaccines against bacterial pathogens.

Keywords: *Salmonella* typhi; Vaccine candidate; *B. subtilis* spores

Introduction

Vaccine efficiency is largely dependent on the exploited adjuvant, especially in subunit recombinant vaccines, where, in comparison to whole-cell vaccines, there are a few antigens to be presented to the immune system (1). There are two main types of adjuvants: delivery vehicles (2), such as emulsions, mineral salts, virosomes and liposomes; and immunostimulants, such as sapo-

nins, Toll-like receptor (TLR) agonists and cytokines (3).

Because of the low efficiency of the licensed human adjuvants, particularly in immunocompromised people, there is a great need to develop new potent and cost-effective adjuvants, which can induce a strong and long-lasting immune response (1).

Spores of *Bacillus subtilis*, a Gram-positive bacterium, are extensively used as a probiotic in the food industry (4-6). Because of the safety of this bacterium and its potential to stimulate immune responses, it has been largely investigated for its adjuvanticity (7-9). Indeed, because of the negative surface charge of the spores, they are appropriate vehicles for the delivery of recombinant proteins (10). Proteins can be adsorbed onto the spores' surfaces or the bacterium can be engineered to express the desired protein following the bacterial sporulation (9, 11). Therefore, *Bacillus subtilis* spores have the two mentioned roles of adjuvants: they are potent immunostimulants and they can be as the carriers for the delivery of recombinant vaccines.

Salmonella infections are among the main causes of morbidity and mortality all over the world. *Salmonella enterica* serotype Typhi (*S* Typhi), which causes typhoid fever, is one of the invasive serovars of *Salmonella*. Annually, there are 21.7 million illnesses due to typhoid fever in the world (12). Currently, there are two commercially available, WHO-approved vaccines against *Salmonella*: Ty21a, a live-attenuated vaccine and a gluco-conjugated vaccine. However, the efficiency of these vaccines is about 50%. Many types of research are conducting to develop a more efficient vaccine (13). FliC is a structural protein, which is present in some pathogenic bacteria, such as pathogenic *Salmonella* species (14), *E. coli* (15) and *Clostridium difficile* (16) and its immunogenicity has been proven.

In this research, FliC protein, a potent immunogen from *Salmonella*, was recombinantly expressed in *E. coli*. The protein was purified and adsorbed onto *B. subtilis* spores and the immunogenicity of the formulation was investigated in BALB/c mice.

Materials and Methods

This work was performed during 2018-2019 in Islamic Azad University of Lahijan, Guilan, Iran.

Chemicals, enzymes, media and bacterial strains

LB (Luria-Bertani) broth and LB agar were prepared from Difco Laboratories (USA). Mouse anti-His tag and HRP-conjugated anti-mouse IgG antibodies were purchased from Sigma (Germany). PCR master mix, as well as 1 kb DNA Ladder, was prepared from GoldBio (China). Ethanol and glacial acetic acid were purchased from Mojallali co. (Iran). Antibiotics (kanamycin and ampicillin) were purchased from Sigma (Germany). Prestained protein ladder and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from SinaClon (Iran). *Bacillus subtilis* strain PY79 (ATCC1609) was provided by Iranian Research Organization for Science and Technology, Tehran, Iran. *E. coli* BL21 (DE3) star was purchased from Royan Institute, Tehran, Iran.

FliC protein expression and purification

The sequence of *fliC* gene was taken from Gene-Bank (Accession Number: AY353376) (https://www.ncbi.nlm.nih.gov/genbank/). The gene was codon-optimized using ExpOptimizer Codon Optimization Tool (https://www.novoprolabs.com/tools/codon-optimization). For further analysis, GenScript's OptimumGene™ was used. The codon-optimized sequence was synthesized in pET28a (+) by Bioneer Company (South Korea).

E. coli BL21 (DE3) cells were competent for the transformation by CaCl₂ method (17). The synthesized construct (pET28a-*fliC*) was transferred to competent cells using the heat shock method (18). Colony-PCR was exploited to confirm the transformation of the bacteria. T7 promoter primers were used for amplification of the *fliC* gene. The PCR program was set as follows: initial denaturation, 95 ℃ for 5 min (one cycle); 30 cycles of denaturation at 95 ℃ for 40 sec, annealing at

50 ℃ for 40 sec and extension at 72 ℃ for 1 min; and one cycle of final extension at 72 ℃ for 5 minutes.

FliC protein expression was performed as previously described (19). Expression was induced by addition of 1 mM IPTG. The expression was continued for 2, 4 and 16 h after addition of the inducer and finally, the result of the expression was analyzed on a 12% SDS-PAGE. The expression of FliC protein was confirmed using Western blotting (20). Here, HRP-conjugated mouse anti-His tag antibody (Abcam, USA, 1:5000) was used as the secondary antibody. The expressed protein was purified by a nickel column (Sigma, Germany) under non-denaturing condition (21) and the results were analyzed on a 12% SDS-PAGE.

Spore preparation

B. subtilis strain PY79 (ATCC1609) was exploited for the delivery of FliC antigen. Highly purified endospores were prepared from the bacteria using the method described (22). Shortly, *B. subtilis* was cultivated in DSM medium (for 1 liter: KCl (1 g); 1 M $MgSO_4$ (1 ml); 10 mM $MnCl_2$ (1 ml); 1 M $CaCl_2$ (0.5 ml); 1 mM $FeSO_4$ (1 ml) and H_2O) at 37 ℃ for 2 hours. Then, the bacterial culture was centrifuged at 10000 × gr for 10 minutes. The supernatant was discarded and the pellet (containing the endospores) was suspended in lysis buffer (50 mM Tris-HCl, 50 μg/mL lysozyme). The mixture was incubated at 37 ℃ for 1 hour. After that, the spores were washed with distilled water and used for the delivery of FliC recombinant protein.

Adsorption of FliC protein onto spores

FliC protein was adsorbed onto the bacterial spores according to Huang et al. (10). Shortly, 10^9 spores were suspended in PBS (200 μl, pH 7.4). Then, 0.2 mg of FliC protein was added to this suspension and the pH was adjusted at three different values: 4, 7 and 10 by addition of HCl or NaOH. The mixture was incubated at room temperature for 1 hour. After that, the mixture was centrifuged at 10000 × gr for 10 minutes. The supernatant was used for the quantification of the adsorbed protein and the pellet was resuspended in PBS buffer.

Immunization of Mice

Animal experiments were conducted according to institutional guideline of Animal Care and Use. 15 BALB/c female mice were divided into 2 groups: 10 mice in the test group, which received FliC-loaded *B. subtilis* spores; and 5 mice in the control group, which received the same amount of bacterial spores (without FliC). For the first immunization, FliC-loaded spores (containing 15 μg FliC) were administered subcutaneously to the test group. Second, third and fourth immunizations were performed in the same manner at days 14, 28 and 42, respectively.

Humoral immunity assessment

For humoral immunity assay, serum IgG was assayed by ELISA using anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) (Sigma, Germany) as previously described (19). For each sample, the endpoint titer value was determined (23). Peripheral blood was obtained from facial vein 7 d after each administration.

Challenge of mice and microbial culture of the liver and spleen

To evaluate the immunogenicity of the FliC-loaded spores, 14 and 28 d after the last immunization, mice were challenged by IP injection of 10 LD_{50} of live *S* Typhi (19). After 48 h, the mice were killed by cervical dislocation and sterilized in 70% ethanol. Then, under sterile conditions, the liver and spleen of the mice were harvested. The organs were completely ground and homogenized in 1500 μl Triton X-100. Then, 10 μl of the sample were cultured in a TSA (Trypticase Soy Agar) medium. The culture was incubated at 37 °C for 24 h and the number of colonies per plate was counted and recorded (20).

Statistical analysis

All statistical analyses were done using GraphPad PRISM version 7 software (GraphPad Software, La Jolla, CA). T-test was used to investigate the statistical significance of the results.

Results

Preparation of FliC protein

Codon optimization of *fliC* gene resulted in desired properties for the efficient expression of the protein. Following the codon optimization, Codon Adaptation Index (CAI) changed from 0.66 to 0.98, Codon Frequency Distribution (CFD) changed from 3% to 0 and the sequence's GC content changed from 47.34% to 50.95% (Table 1 and Fig. 1). According to mfold server, the resulted mRNA was stable inside the cell. Analyzing the sequence by the ProtParam program showed that the expressed protein is stable in *E. coli* expression system.

**Table 1:** The main parameters of the sequence before and after the codon-optimization

Parameter	Before Codon-Optimization	After Codon-Optimization
GC %	47.34	50.95%
CAI	0.66	0.98
CFD	3%	0
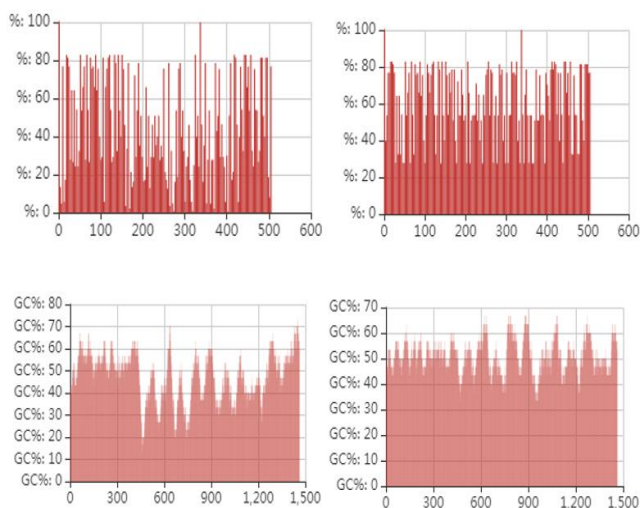
Fig. 1: CAI of the sequence before (A) and after (B) the codon-optimization. CAI changed from 0.73 to 0.98. GC percent of the sequence changed from 47.34% (C) to 50.95% (D)

Following the transformation of the bacteria and the formation of colonies on LB agar plate, colo-ny-PCR was performed to investigate the presence of *fliC* gene in the transformed bacteria. As it has been shown in Fig.2, out of 8 examined colonies, 5 colonies carried *fliC* gene. The PCR reaction was performed by using T7 promoter and terminator universal primers, so a 264 bp fragment was added to the amplified sequence and the sequence has a size of about 1800 bp.
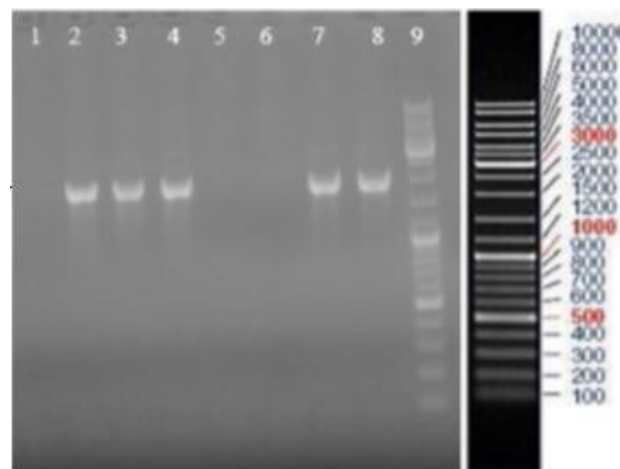

Fig. 2: Colony PCR of the colonies; Lanes 1-8: eight selected colonies from the plate; Lane 9: 1 kb DNA Ladder

Expression, purification and validation of the recombinant protein

The expression of FliC protein was analyzed on a 12% SDS-PAGE (Fig. 3A). Two transformed clones were selected for analysis of the protein expression. Three different induction times, 2, 4 and 16 h were examined to obtain the best induction time. As it is seen in Fig. 3A, the best result was obtained when the induction lasted for 2 hours. To verify the expressed protein, Western blotting was performed and the expressed protein is the protein of interest, i.e. FliC protein (Fig.3B). FliC protein was purified using Ni-NTA under denaturing conditions (24). The protein was purified following the addition of buffer E (100 mM NaH2PO4, 10 mM Tris-HCl, 8 M urea, pH 4.5). The Bradford method was used to determine protein concentration (25) (Fig. 4).
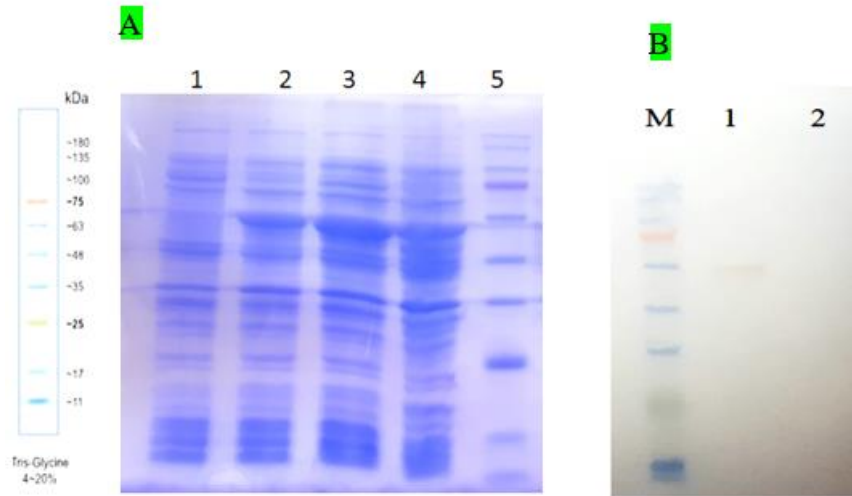
Fig. 3: Analysis of FliC recombinant protein expression. A: SDS PAGE showing the different induction times following the addition of IPTG: Lane 1, Un-induced sample; lane 2, 2 h induction; lane 3, 4 h induction; lane 4, 16 h induction; lane 5, protein size marker. B. Western blotting using anti-His tag antibody. Lanes 1, IPTG-induced sample; lane 2, un-induced sample; M, protein size marker
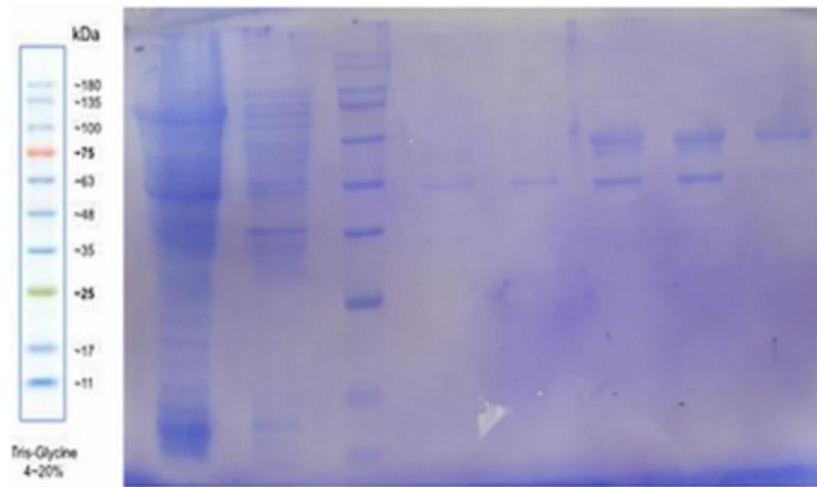

Fig. 4: 12% SDS-PAGE gel showing the different steps of FliC protein purification: Lane 1, cell lysate; lane 2, flow-through from the column; lane 3 and 4, washing the column with buffer B (8 M urea, pH 8); lane 5, washing the column with buffer C (8 M urea, pH 6.3); lane 6, washing the column with buffer D (8 M urea, pH 5.8); lane 7, washing the column with buffer E (8 M urea, pH4.5); PPL, prestained protein ladder

IgG antibody response

The adjuvanticity of antigen-loaded spores in terms of the antigen-specific humoral immunity was investigated and total antigen-specific IgG was assessed (Fig. 5). The figure shows that the delivery of the antigen using the spores has strongly stimulated the production of IgG antibody. The average optical density (OD) in the test group was 2.3, which was significantly higher than the control group ($P<0.001$).
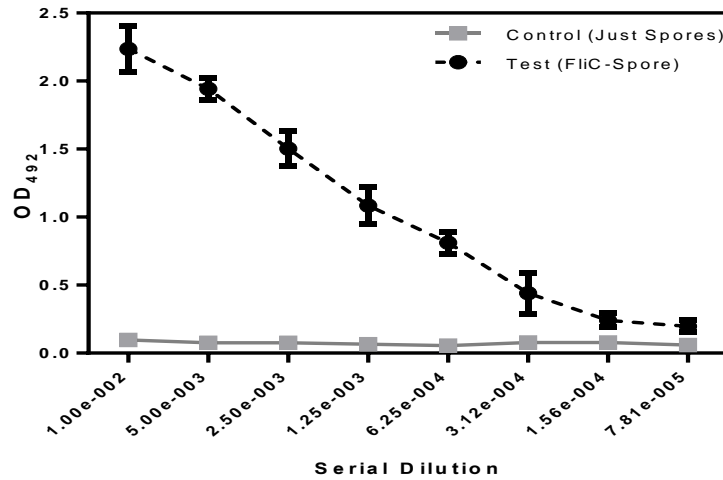
Fig. 5: Serum IgG response following the immunization period. Indirect ELISA was performed to obtain the IgG antibodies titer in which FliC protein was coated in the plate and rabbit anti-mouse IgG conjugated with HRP were used as the secondary antibody for the detection

Animal Challenge

Two days following the treatment of the mice with live *S* Typhi bacteria, the mice were killed; liver and spleen were extracted, homogenized and cultured on TSA media. The average number of bacteria in control and test groups was calculated as 4.46×10^8 and 2.25×10^8, respectively. Statistical analysis using t-test showed a significant difference between the two groups, which indicates the efficiency of the vaccine candidate against *S* Typhi ($P < 0.0001$).

Discussion

Finding appropriate adjuvants that can carry the vaccines and, at the same time, stimulate the innate immune responses is a main issue in vaccine development (26, 27). There have been many delivery vehicles, such as micro and nanoparticles (28, 29), and immunostimulatory compounds, such as natural and synthetic immunomodulators tested for this purpose (30, 31).

In recent years, *Bacillus subtilis* spores have been used as natural potent adjuvant to enhance the efficiency of the vaccines (10, 32). Owing to many desirable properties, such as safety, stability, ability to evoke innate immunity, the proper surface charge, ease of production, cost-

effectiveness, etc., these spores have had promising results in vaccine delivery application.

Causing more than 200000 annual death, typhoid fever is a health problem, especially in areas with low sanitation (33). Vaccination is a good strategy to protect against the disease and at the moment there are commercial vaccines to avoid typhoid fever; however, because of the relatively low efficiency of the available vaccines, there is a great need to work on the development of new ones (34). Many *Salmonella* antigens and immunogens have been introduced, including FliC, a flagellar protein with a potent immunization property (35, 36). In the present study, *B. subtilis* spores are proper vehicles for the delivery of FliC protein, a vaccine candidate against *Salmonella enterica* serovar Typhi. Loading the live spores with this protein elicited IgG production (with an average OD of 2.3) in immunized mice. The challenge of test and control animals showed that the delivery of FliC protein by *B. subtilis* spores can confer protective immunity to the mice ($P < 0.0001$).

This is the first study that exploits *B. subtilis* spores as a delivery vehicle as well as an immunostimulatory agent for a vaccine candidate against *S. enterica* serovar Typhi. Indeed, it is the first time that the immunogenicity of recombinant FliC protein is investigated against *S. enterica*

serovar Typhi. However, the efficiency of *B. subtilis* spores (both killed and live ones) has been proved in different studies. Surface display of immunogens is a mostly used strategy for this regard. For example, Dai et al. expressed OmpC protein of *Salmonella* serovar *Pullorum* on the surface of *B. subtilis* spores and showed that the oral administration of the formulation to mice stimulates the humoral immune response and protective immunity against *S. enterica* serovar Typhimurium is elicited (37). The expression of a surface immunogenic protein (Sip) of *Streptococcus agalactiae* and its administration to tilapia is able to cause an effective humoral immune response and confer good protective response to the bacterial infection (38). However, there are studies loaded specific antigens on the spores and evaluated the immunogenicity of the formulation. Song et al. used HA from killed H5N1 virions to load killed *B. subtilis* spores and use the formulation to immunize mice. The formulation was able to stimulate both innate and acquired immunity. All treated mice were able to tolerate 20 LD_{50} of H5N2 virus (39).

Although recombinant FliC protein can stimulate the antibody production in mice and these antibodies were able to detect the native FliC in clinical samples, however, as stated before, this is the first report that investigates the immunogenicity and protective effect of FliC protein against *S. enterica* serovar Typhi. The efficiency of FliC protein as a vaccine candidate against *S. enterica* serovar *enteritidis* has been investigated and proved. The recombinant FliC protein from *S. enteritidis* can protect the chickens against homologous challenges (40).

Conclusion

The results of our work showed the efficiency of FliC-loaded *B. subtilis* spores as a vaccine candidate against *S* Typhi.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Conflict of interests

There is no potential conflict of interests relevant to this paper.

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