

Cloning, expression, purification, and characterization of recombinant flagellin isolated from *Pseudomonas aeruginosa*

Gholamreza Goudarzi · Morteza Sattari ·
Mehryar Habibi Roudkenar · Mehran Montajabi-Niyat ·
Ahmad Zavarán-Hosseini · Kamran Mosavi-Hosseini

Received: 1 February 2009 / Revised: 1 April 2009 / Accepted: 7 May 2009 / Published online: 23 May 2009
© Springer Science+Business Media B.V. 2009

Abstract *Pseudomonas aeruginosa* as an opportunistic pathogen causes lethal infections in immunocompromised individuals. This bacterium possesses a polar flagellum made up of flagellin subunits. Flagella have important roles in motility, chemotaxis, and establishment of *P. aeruginosa* in acute phase of infections. Isolation, cloning, and expression of flagellin were aimed at in this study. Flagellin gene (*fliC*) of *P. aeruginosa* strain 8821M was isolated by PCR and cloned into a pET expression vector. The recombinant flagellin (46 kDa) was overexpressed as inclusion bodies (IBs). IBs were solubilized in guanidine hydrochloride (GuHCl) followed by affinity-purification and renatured using Ni²⁺-Sepharese resin. Recombinant flagellins reacted with the serum from a rabbit previously immunized with native flagellin. In addition, polyclonal antiserum raised against the recombinant flagellin was shown to significantly inhibit the cell motility of *P. aeruginosa* strain 8821M in vitro.

Keywords *fliC* · Inclusion bodies · *Pseudomonas aeruginosa* · Recombinant flagellin

Introduction

Pseudomonas aeruginosa as a Gram-negative bacterium causes severe nosocomial and community acquired infections at various body sites including the urinary tract, surgical or burn wounds, the cornea, and the lower respiratory tract (Driscoll et al. 2007; Feldman et al. 1998). *P. aeruginosa* takes advantage of various underlying host conditions to establish acute or chronic infections (Döring and Pier 2008). This bacterium possesses a single polar flagellum which confers motility and chemotaxis, facilitates adherence to cells and inanimate surfaces and contributes to colonization and invasion during the early phase of infection in predisposed hosts (Spangenberg et al. 1996). Recent findings highlight a major role of the flagellin monomer in the recognition of bacteria by the host and in the induction of immune responses by toll-like receptor-5 (TLR-5) (Ramos et al. 2004; Tseng et al. 2006; Steiner 2007). *P. aeruginosa* strains express either a-type or b-type flagellin (Spangenberg et al. 1996; Brimer and Montie 1998). This classification is based on the antigenicity and apparent molecular weight of the flagellin subunits encoded by *fliC* (Allison et al. 1985; Spangenberg et al. 1996). b-Type flagellins were found to comprise a homogeneous group of proteins, whereas the heterogeneous

G. Goudarzi · M. Sattari (✉) · M. Montajabi-Niyat
Department of Bacteriology, Medical Sciences School,
Tarbiat Modares University, Tehran, Iran
e-mail: Sattarim@modares.ac.ir

M. H. Roudkenar · K. Mosavi-Hosseini
Research Center, Iranian Blood Transfusion Organization,
Tehran, Iran

A. Zavarán-Hosseini
Department of Immunology, Medical Sciences School,
Tarbiat Modares University, Tehran, Iran

a-types were divided into several subtypes (Spangenberg et al. 1996). All a-type flagellins were reported to have an a_0 common cross-reacting antigenic component and usually also have one or more additional antigenic components which play at most a minor role in inheriting antigenicity (Ansorg 1978; Allison et al. 1985; Brimer and Montie 1998). Although the a-type and b-type genes differ by 35% in primary structure, sequence alignments demonstrate a high homology between all *P. aeruginosa* flagellins, which substantiates the intention to use flagella antigens as antipseudomonal vaccines (Spangenberg et al. 1996). Comparisons of the nucleotide and amino acid sequences of flagellins from different species have shown the N and C-termini to be highly conserved and the central hypervariable region to be less conserved (Wilson and Beveridge 1993; Brimer and Montie 1998). Most of the structural and functional features of flagella are determined by the N- and C-terminal conserved regions, whereas the antigenic or serological variation is found in the central portion of the flagellin (Namba et al. 1989; Wilson and Beveridge 1993). These data support flagellin as an antigen candidate to design an anti-flagellin vaccine. In the present study, we report high yield expression of recombinant flagellin (r-flagellin) fused to a C-terminal His-tag in *E. coli*. We also report the recognition of recombinant flagellin by hyperimmune sera raised in animals immunized with the native flagella protein.

Materials and methods

Bacterial strains, vector and media

Pseudomonas aeruginosa 8821M was kindly provided by Dr. Isabel Sa-Corria (Instituto Superior Tecnico, Lisboa, Portugal). *E. coli* strains Top10F and BL-21(DE3) pLysS were obtained from Invitrogen and Novagen (USA), respectively. Plasmid pET-28a as expression vector was purchased from Novagen. Bacteria were cultured in LB broth or on agar (Merck, Germany) with or without 30 μ g kanamycin/ml (Sigma, USA).

Isolation of *fliC*

The genomic DNA from *P. aeruginosa* 8821M was extracted using a genomic DNA extraction kit

(Fermentas, Lithuania). The specific primers were designed according to *fliC* sequences of *P. aeruginosa* strain JJ692 from NCBI (GenBank Accession No: AY275678). The full coding sequence of *fliC* was amplified by polymerase chain reaction (PCR) using specific primers containing *Nco*I and *Xho*I sites. The sequence of the forward primer was: 5'-ACTCC ATGGCCTTGACCGTCAAC-3' and reverse was: 5'-ATTCTCGAGGCGCAGCAGGCTCAGAAC-3'. Amplifications were carried out in 25 μ l volumes containing 0.5 μ M of each primer, 2.5 μ l 10 \times PCR buffer, 1.5 mM MgSO₄, 0.2 mM nucleotide (dATP, dCTP, dGTP, and dTTP), 2.5 U of *Pfu* DNA polymerase (Fermentas) and 200 ng genomic DNA. PCR was carried out in a master gradient thermocycler (Eppendorf, Germany). The gene amplification conditions were as follows: 94°C (4 min), 35 cycles consisting of 94°C (60 s), 56.2°C (60 s), and 72°C (60 s) and an additional extension time at 72°C (10 min). PCR product was separated by electrophoresis on 1% (w/v) agarose gel (Fermentas) and the desired fragment was recovered from the gel using high pure PCR purification kit (Roche, Germany).

Cloning and construction of expression plasmid

The purified fragment and the vector were digested by respective restriction enzymes. The fragment was ligated to the pET-28a vector. The ligation product was transformed into competent *E. coli* Top10F and transformants were selected on LB agar plates containing 30 μ g kanamycin/ml (Sambrook and Russell 2001). The selected clones were further analyzed by restriction enzymes and PCR and finally sequenced by a commercial facility using universal forward and reverse T7-promoter and T7-terminator primers (TAG Copenhagen A/S Symbion, Denmark).

Expression and isolation of inclusion bodies

For expression, the recombinant plasmid, pET-28a/*fliC*, was transformed into competent *E. coli* BL-21 (DE3) pLysS. *E. coli* cells harboring expression vector pET-28a/*fliC* were grown in LB medium supplemented with kanamycin (30 μ g/ml) and chloramphenicol (34 μ g/ml) at 37°C to an OD₆₀₀ = 0.8. For induction, IPTG (Sigma, USA) was added to a final concentration of 1 mM and the culture was grown at 37°C for 4 h. The cells were subsequently harvested and suspended

in Lysis Buffer (20 mM sodium phosphate, 10 mM EDTA, 1% (v/v) Triton X-100, pH 7.5) followed by freezing, thawing, and sonication on ice in the presence of PMSF (1 mM) as a protease inhibitor. To chelate the EDTA, 10 mM MgSO₄ was added followed by adding DNase (0.01 mg/ml) at RT for 20 min. Afterward, centrifugation was performed. The pellet was thoroughly resuspended in 0.1 culture volume of the same buffer without Triton-X100. The inclusion bodies (IBs) were collected by centrifugation at 10,000×g for 10 min and stored at 4°C.

Solubilization, refolding, and purification of recombinant protein

The washed IBs were denatured and solubilized with Guanidinium Buffer (20 mM sodium phosphate, 500 mM NaCl, 6 M guanidine hydrochloride, pH 7.4) for 1 h. The solubilized proteins were purified using Ni²⁺-Sepharose resin (Invitrogen, USA) according to the manufacturer's instructions with some minor modifications. Purifications were performed under *hybrid* conditions (Denaturing and renaturing conditions). Briefly, the resin was equilibrated with denaturing binding buffer (20 mM sodium phosphate, 500 mM NaCl, 8 M urea, pH 7.8). After applying the samples to the column, a linear urea gradient from 8 M to 0 M of refolding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) was used at flow rate of 0.6 ml/min. The weakly bound contaminant proteins were washed away from the column using wash buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 8.0). Finally, protein of interest was eluted in elution buffer (20 mM sodium phosphate, 500 mM NaCl, 250 mM imidazole, pH 8.0). The purified r-flagellin was dialyzed against 20 mM Tris-HCl, pH 7.4 for removing imidazole and analyzed by 12% (w/v) SDS-PAGE followed by Coomassie brilliant blue G-250 staining. The protein concentration was determined by the Bradford method with bovine serum albumin as standard (Bio-RAD, USA).

Isolation and purification of native flagellin

LB agar plates containing 0.2% (w/v) glucose were inoculated with the *P. aeruginosa* 8821M and incubated for 24 h at 37°C. Flasks containing LB broth medium were inoculated with the bacterial colonies and incubated at 37°C in an orbital shaker incubator at

100 rpm for 24 h. Bacterial cells were harvested by centrifugation for 20 min at 7,000×g and 4°C. The resulting pellet was resuspended in 50 mM sodium phosphate buffer (pH 7.4) at a ratio of 100 ml of buffer per 8 g of wet weight cells. The cell suspension was then blended in a commercial blender for 1.5 min to shear off the flagella. The suspension was centrifuged at 10,000×g at 4°C for 20 min to remove the cells (Montie et al. 1982). The supernatant was saturated in 5% (w/v) increments with ammonium sulfate. After each addition of ammonium sulfate, the solutions were stirred at 4°C for 4–5 h. The precipitated proteins were collected by centrifugation. Each of the protein fractions was redissolved in a small amount of 50 mM sodium phosphate buffer (pH 7.4) prior to being dialyzed against the same buffer. The pH of the dialyzed fractions was then adjusted to pH 3.0 with 1 M HCl and maintained at that pH under constant stirring for 30 min at room temperature. The proteinaceous suspension was ultracentrifuged at 100,000×g for 1 h at 4°C to sediment the acidic-insoluble material. Finally, the pH of the supernatant was adjusted to 7.4 with 1 M NaOH (Brett et al. 1994; Ibrahim et al. 1985). Product was verified using 12% (w/v) SDS-PAGE. The protein concentration was determined by Bradford assay with bovine serum albumin as standard (Bio-RAD, USA).

Antibody production

Female New Zealand White rabbits (Razi vaccine & serum research institute, Karaj, Iran) were immunized either with 100 µg of native or recombinant flagellin in complete Freund's adjuvant (Sigma, USA) administered subcutaneously, boosted two times with 100 µg of purified flagellin in incomplete Freund's adjuvant at 2 and 4 weeks. 10 days after the second immunization, the animals were exsanguinated by cardiac puncture under anesthesia and serum samples were collected and stored at –70°C until required for use. All animal experiments were done in accordance with institutional and national ethical guidelines.

Western blot analysis

The proteins separated by SDS-PAGE were blotted onto polyvinylidene difluoride (PVDF) membrane (Hi-bond Amersham Biosciences, USA) by using a semidry blotter unit (Labconco, Kansas City, Mo.).

The membrane was blocked by 1% (w/v) skim milk according to standard procedures. The native immune serum was diluted to 1:1,000 in phosphate-buffered saline (PBS)-0.1% (v/v) Tween 20 and incubated 3 h at 4°C with shaking. Block membranes were washed with PBS-Tween 20 and then incubated with affinity-purified goat anti-rabbit immunoglobulin G (heavy and light chain) horseradish peroxidase (HRP) conjugate antibody (Bio-Rad), at a 1:2,500 dilution in PBS-Tween 20. Membranes were then washed three times with PBS-Tween 20 and development using DAB solution (Sigma, USA).

Motility inhibition assay

To verify the functionality of recombinant polyclonal antibody, three Petri dishes were filled with 10 ml of motility agar (LB with 0.3% (w/v) agar, Soft agar) which had been rehydrated with either anti-native; anti-recombinant polyclonal or preimmune rabbit serum diluted 1:20. Twenty microliters of a cell suspension of *P. aeruginosa* strain 8821M ($OD_{600} = 0.2$) in PBS was dispensed into the central well (5 mm in diameter) of each plate. For each assay, triplicate plates per serum were examined. The plates were incubated at 37°C. The mean diameters of bacterial spreading with sharp and less distorted rings were measured after incubation for 18 h (Brett et al. 1994).

Results

Construction of the pET-28a/*fliC*

Specific primers were designed to amplify *fliC* gene from the *P. aeruginosa* strain 8821M. The expected size of the PCR product, approximately 1,200 bp, was obtained (Fig. 1). Existence of insert (*fliC*) in recombinant vector, pET-28a/*fliC*, was also detected by digestion using *Nco*I and *Xho*I restriction enzymes (Fig. 2) and finally the identity and orientation of *fliC* in the construct were confirmed by DNA sequencing (Data not shown).

Expression and purification of recombinant flagellin

The *E. coli* BL21 (DE3) pLysS was transformed with the recombinant expression vector, pET-28a/*fliC*, and

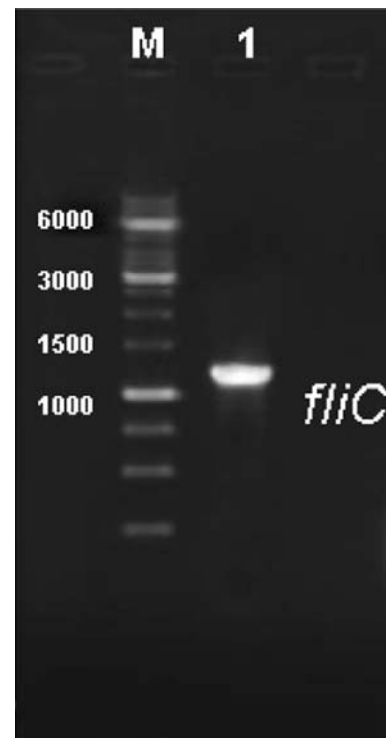


Fig. 1 Electrophoresis of PCR product on agarose gel (1% w/v). Lane 1, Single expected band of *fliC* (approximately 1,200 bp); lane M, 1 kb DNA size marker

induced with IPTG and accumulated large amounts of a protein migrating in SDS-PAGE with an apparent molecular weight of approximately 46 kDa (Fig. 3, lane 3). Both supernatant and the pellet of cell lysates were tested for the presence of recombinant proteins. The majority of the expressed protein was detected in inclusion bodies. The IBs were partially purified after washing with sodium phosphate buffer containing 1% (v/v) Triton X-100 (Fig. 3, lane 2). Recombinant flagellin was completely solubilized in 6 M guanidine hydrochloride lysis buffer and after loading into the Ni^{2+} -Sepharose column was purified and refolded without any protein precipitation (Fig. 3, lane 1). This purification yielded 70 mg of highly purified r-flagellin protein from 1 l of induced culture.

Isolation and purification of native flagellin

The flagellar filaments of *P. aeruginosa* 8821M were isolated by mechanical shearing and differential centrifugation and precipitated by ammonium sulfate. SDS-PAGE analysis of these crude preparations

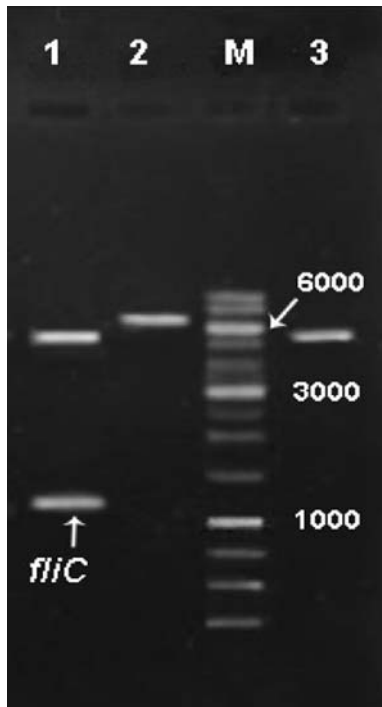


Fig. 2 Detection of recombinant vector by restriction enzyme digestion. The plasmids were extracted and digested with appropriate restriction enzymes. *Lane 1*, recombinant vector, pET28a-*fluC*, digested with *Xho*I and *Nco*I; *lane 2*, pET28a-*fluC* digested with *Xho*I (6,570 bp); *lane 3*, pET-28a digested with *Xho*I (5,369 bp); *lane M*, 1 kb DNA size marker. The products were electrophoresed on 1% w/v agarose gel

showed that flagellar filaments precipitate in 10% (w/v) of the ammonium sulfate with an apparent M_r of approximately 46 kDa (Fig. 4, lane 5). Whereas, in the higher concentrations of ammonium sulfate, there was no track of any protein bond (Fig. 4, lanes 2–4). Although the above procedures removed most contaminants, SDS-PAGE displayed some residual proteins at M_r s of approximately 18, 40, 55, and 60 kDa. These contaminants were easily removed by a modified version of the acid pH dissociation, ultracentrifugation and neutral pH reassociation method outlined by Ibrahim et al (1985). Finally, a homogeneous preparation of native flagellin was accomplished and SDS-PAGE analysis after this step showed a single band with a M_r of approximately 46 kDa (Fig. 4, lane 1). Protein quantitation of the purified native flagellin preparations determined that 200–250 μ g of flagellin protein could be routinely isolated from a 1-l cell culture grown overnight.

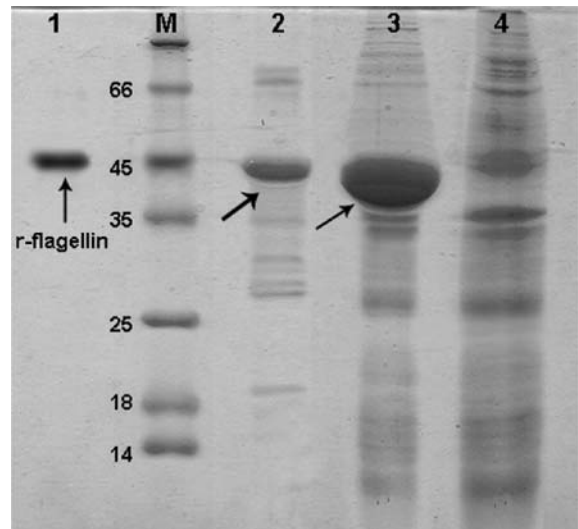


Fig. 3 Detection of expressed and purified recombinant flagellin on SDS-PAGE (12% w/v). The gel was stained with Coomassie blue G-250. Samples were resuspended directly in SDS loading buffer and boiled for 5 min. *Lane 1*, purified r-flagellin from Ni^{2+} -Sepharose column; *lane 2*, inclusion bodies after washing and solubilization in 6 M GuHCl; *lane 3*, pellet of IPTG induced bacteria; *lane 4*, pellet of un-induced bacteria; *lane M*, standard protein size marker (kDa). The r-flagellin proteins have been shown by arrow

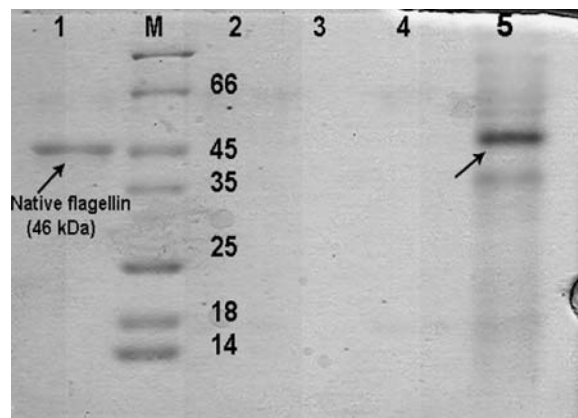


Fig. 4 SDS-PAGE (12% w/v) of native flagellin preparation from *P. aeruginosa* 8821M. The flagellar filaments were detached and precipitated by ammonium sulphate and further purified by pH disassociation and ultracentrifugation. The gel was stained with Coomassie blue G-250. *Lane 1*, purified native flagellin (46 kDa) after pH disassociation and ultracentrifugation; *lanes 2–4*, crude preparations in saturation 15–25% (v/v) of the saturated ammonium sulphate solution, respectively; *lane 5*, crude preparation in saturation 10% (v/v) of the ammonium sulphate solution; *lane M*, protein size marker (kDa)

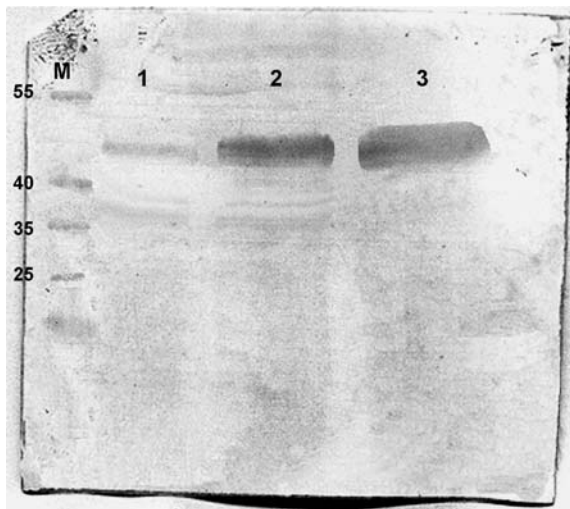


Fig. 5 Western blot of recombinant flagellin protein probed with rabbit serum anti-native flagellin (1:1,000). Lane 1, pellet of un-induced bacteria; lane 2, pellet of IPTG induced bacteria; lane 3, purified r-flagellin; lane M, pre-stained protein size marker (kDa). HRP-conjugated anti-rabbit IgG (1:2,500) and DAB were used

Western blot analysis

To detect antigenicity of expressed protein Western blot analysis was performed. The major band observed in SDS-PAGE (46 kDa) was confirmed as r-flagellin protein by western blot analysis with rabbit serum anti-native flagellin which indicates apparent molecular mass of 46 kDa and its immune-reactivity (Fig. 5).

Motility inhibition

To study the functional activity of anti-recombinant flagellin, a motility test was performed. Rabbit anti-recombinant polyclonal serum inhibited motility of *P. aeruginosa* 8821M as well as anti-native polyclonal serum in motility agar (Fig. 6) were compared with pre-immunized rabbit serum, which indicates that antibody raised against r-flagellin was biologically active.

Discussion

In this study, we report the cloning of flagellin in a pET-28a vector and its expression in *E. coli*. The a-type flagellin antigens have M_r S of 45 to 52 kDa, while all of the b-type antigens characterized have a

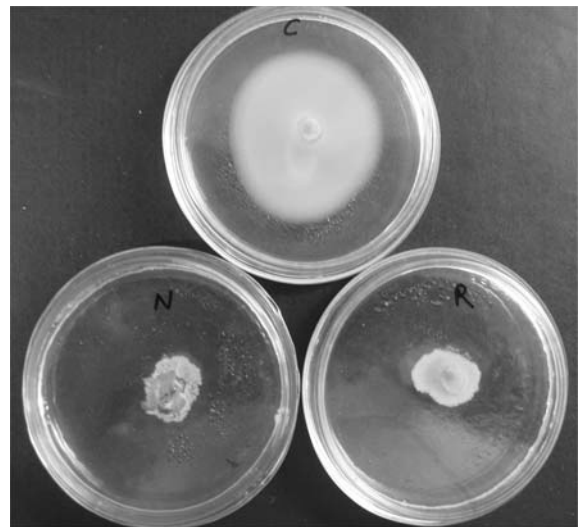


Fig. 6 Motility inhibition assay. Anti-recombinant polyclonal serum (R) inhibited motility of *P. aeruginosa* 8821M as well as anti-native polyclonal serum (N) in motility agar (LB with 0.3% w/v agar) compared with pre-immune rabbit serum (C)

M_r of 53 kDa with approximately 1,460 bp (Arora et al. 2004; Kelly-Wintenberg and Montie 1989). Winstanley et al. (1996) have amplified part of the flagellin gene of various *P. aeruginosa* strains using PCR. They compared the flagellin gene sequences of two *Pseudomonas putida* strains with the flagellin gene sequence of *P. aeruginosa* PAK. From this comparison, primers specific for N-terminal (CW46) and C-terminal (CW45) conserved regions were designed. PCR amplification of thirty-seven presumed a-type isolates of *P. aeruginosa* by using CW46 and CW45 yielded products of 1.02 kb. In another study, these primers were utilized to amplify the major portion of the *fliC* gene to compare of PCR fragment sizes in various a-type strains (Brimer and Montie 1998). Due to the positioning of the oligonucleotide primers on the flagellin gene, the entire *fliC* sequences are not amplified by this procedure. PCR product amplified with our specific designed primers contains approximately 1,200 bp length which is accordance with a-type of flagellin. In addition, the molecular weight of the expressed r-flagellin (approximately 46 kDa) also confirms the presence of a-type flagellin in strain 8821M. Previous studies had demonstrated unusual glycosylation of flagellin in certain strains of *P. aeruginosa* (Brimer and Montie 1998), whereas, our SDS-PAGE analysis (on the both native and r-flagellin preparations)

showed no difference between their molecular weights. It seems no glycosylation or post translational modifications accrue in this strain. However, a glycosylation assay such as the biotin-hydrazide method could be performed to confirm this. Traditionally, preparation of native flagellin for immunological and in vitro studies is time-consuming, needs large volumes of bacterial culture, sophisticated technical equipment and the performance of several purification steps that usually lead to a low yield. Therefore, developing a method to overcome to conventional approaches to obtain high yields is an asset. In the present study, recombinant DNA technology was applied to obtain flagellin. Results similar to those reported in our study have been reported by Kelly-Wintenberg et al. (1989). In that study, plasmids with DNA fragments containing the structural gene of the flagella from a library of *P. aeruginosa* PAO1 were cloned and expressed into *E. coli* by triparental mating method, whereas in our study, the flagellin gene was isolated from *P. aeruginosa* by using specific primers and PCR, then cloned in the pET vector and overexpressed in *E. coli* as IBs aggregates. pET-28a vector carries six histidine residues in C-terminal of fusion protein. The His-tag facilitates purification of recombinant protein by Ni²⁺-Sepharose resin. IBs were solubilized effectively in GuHCl, loaded on the Ni²⁺-Sepharose trapped column under denaturant conditions then purified and refolded under renaturant conditions. Our efforts at refolding the solubilized proteins using dialysis in which urea concentration is gradually decreased were led to precipitation and aggregation of many proteins. In contrast, the on-column purification and refolding approach effectively refolded desired recombinant protein without protein aggregation or precipitation. Our results also revealed that when using a column purification method, denaturant concentration reduction must be stepwise to enhance the yield of refolded recombinant proteins. In Western blotting, the reaction of rabbit anti-native serum with recombinant flagellin demonstrates the presence of common epitopes between native and recombinant flagellin proteins. In addition, our motility inhibition test results show that the anti-serum raised against the recombinant flagellin is capable of inhibiting the bacterial motility in vitro similarly to the native flagella antiserum. Together, these results suggested that recombinant flagellin can

be used in immunization and protection studies. As mentioned earlier, polyclonal antiserum raised against the r-flagellin preparation inhibits cell motility of *P. aeruginosa* 8821M in vitro. The mechanism of this inhibition has not been well characterized whoever, previous studies suggested that antibodies binding to the flagellar filaments cause bacterial agglutination and disruption of the rotational movements of the structure and lead to loss of colony spreading on the plates (Montie et al. 1982; Brett et al. 1994). Since motility has been demonstrated to be an important factor in microbial pathogenesis (Montie and Holder 1982), disruption of such a function by immobilizing antibodies in vivo may prove to be an advantageous prophylactic measure against bacteria. We believe that, this is the first report of overexpression of recombinant flagellin with a His-tag in heterologous system. Highly purified recombinant protein was obtained after purification. The r-flagellin carried antigenic epitopes as well as native flagellin and polyclonal antibody raised against it inhibited the bacterial motility. In many studies, the efficacy of native flagellin as a vaccine has been proved in protection and survival of animals and humans (Arora et al. 2005; Holder and Naglich 1986; Döring et al. 2007). In this work, we also verified that recombinant flagellin showed similar immunological properties as compared to native protein. Since *P. aeruginosa* only contains two major antigenic types of flagella, we suggest producing b-type flagellin as a recombinant protein. The immunoprotection of these two recombinant proteins can be evaluated as a bivalent vaccine or in combination with other antigenic substances in experimental infection models in vivo.

Acknowledgments We thank Professor Ian Alan Holder (Departments of Microbiology and Surgery, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267) for his scientific support and revising of the manuscript.

References

- Allison JS, Dawson M, Montie TC et al (1985) Electrophoretic separation and molecular weight characterization of *Pseudomonas aeruginosa* H-antigen flagellins. Infect Immun 49:770–774
- Ansorg R (1978) Flagella specific H antigenic schema of *Pseudomonas aeruginosa*. Zentbl. Bakteriol. Parasitenkd. Infekkrankh Hyg Abt 1(224):228–238

- Arora SK, Wolfgang MC, Ramphal R et al (2004) Sequence polymorphism in the glycosylation island and flagellins of *Pseudomonas aeruginosa*. *J Bacteriol* 186(7):2115–2122
- Arora SK, Neely AN, Ramphal R et al (2005) Role of motility and flagellin glycosylation in the pathogenesis of *Pseudomonas aeruginosa* burn wound infections. *Infect Immun* 73(7):4395–4398
- Brett PJ, Mah DCW, Woods DE (1994) Isolation and characterization of *Pseudomonas pseudomallei* flagellin proteins. *Infect Immun* 62(5):1914–1919
- Brimer CD, Montie TC (1998) Cloning and comparison of *fltC* genes and identification of glycosylation in the flagellin of *Pseudomonas aeruginosa* a-type strains. *J Bacteriol* 180(12):3209–3217
- Đoring G, Pier GB (2008) Vaccines and immunotherapy against *Pseudomonas aeruginosa*. *Vaccine* 26:1011–1024
- Đoring G, Meisner C, Stern M (2007) A double-blind randomized placebo-controlled phase III study of a *Pseudomonas aeruginosa* flagella vaccine in cystic fibrosis patients. *Proc Natl Acad Sci USA* 26(104):11020–11025
- Driscoll JA, Brody SL, Kollef MH (2007) The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs* 67:351–368
- Feldman M, Bryan R, Prince A et al (1998) Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun* 66(1):43–51
- Holder IA, Naglich JG (1986) Experimental studies of the pathogenesis of infection of *Pseudomonas aeruginosa*: immunization using divalent flagella preparations. *J Trauma* 26(2):118–121
- Ibrahim GF, Fleet GH, Walker RA (1985) Method for the isolation of highly purified *Salmonella* flagellins. *J Clin Microbiol* 22(6):1040–1044
- Kelly-Wintenberg K, Montie TC (1989) Cloning and expression of *Pseudomonas aeruginosa* flagellin in *Escherichia coli*. *J Bacteriol* 171(11):6357–6362
- Montie TC, Holder IA (1982) Loss of virulence associated with absence of flagellum in an isogenic mutant of *Pseudomonas aeruginosa* in the burned—mouse model. *Infect Immun* 38(3):1296–1298
- Montie TC, Craven RC, Holder IA (1982) Flagellar preparations from *Pseudomonas aeruginosa*: isolation and characterization. *Infect Immun* 35(1):281–288
- Namba K, Yamashita I, Vonderviszt F (1989) Structure of the core and central channel of bacterial flagella. *Nature* 342:648–654
- Ramos HC, Rumbo M, Sirard JC (2004) Bacterial flagellins: mediators of pathogenicity and host immune responses in mucosa. *Trends Microbiol* 12(11):509–517
- Sambrook J, Russell DW (2001) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- Spangenberg C, Heuer T, Tummeler B et al (1996) Genetic diversity of flagellins of *Pseudomonas aeruginosa*. *FEBS Lett* 396:213–217
- Steiner TS (2007) How flagellin and toll-Like receptor 5 contribute to enteric infection. *Infect Immun* 75(2):545–552
- Tseng J, Do J, Machen TE et al (2006) Innate immune responses of human tracheal epithelium to *Pseudomonas aeruginosa* flagellin, TNF- α and IL-1 β . *Am J Physiol Cell Physiol* 290:678–690
- Wilson DR, Beveridge TJ (1993) Bacterial flagellar filaments and their component flagellins. *Can J Microbiol* 39:451–472
- Winstanley C, Coulson MA, Wepner B et al (1996) Flagellin gene and protein variation amongst clinical isolates of *Pseudomonas aeruginosa*. *Microbiology* 142:2145–2151