

Overexpression, Purification, and Immunogenicity of Recombinant Porin Proteins of *Salmonella enterica* Serovar Typhi (*S. Typhi*)

Verma, Shailendra Kumar¹, Vandana Gautam¹, Konduru Balakrishna², and Subodh Kumar^{1*}

¹Division of Microbiology; Defence Research and Development Establishment, Jhansi Road, Gwalior-474002, India

²Division of Microbiology, Defence Food Research Laboratory, Siddarthanagar, Mysore-570011, India

Received: December 16, 2008 / Revised: April 8, 2009 / Accepted: April 9, 2009

Porin proteins of Gram-negative bacteria are outer membrane proteins that act as receptors for bacteriophages and are involved in a variety of functions like solute transport, pathogenesis, and immunity. *Salmonella enterica* serovar Typhi (*S. Typhi*), a Gram-negative bacterium, is the causative agent of typhoid fever. Porins of *S. Typhi* have been shown to have a potential role in diagnostics and vaccination. In the present study, the major outer membrane proteins OmpF and OmpC from *S. Typhi* were cloned in pQE30UA vector and expressed in *E. coli*. The immunogenic nature of the recombinant porin proteins were evaluated by ELISA by raising hyperimmune sera in Swiss Albino mice with three different adjuvants (*i.e.*, Freund's adjuvant and two human-compatible adjuvants like montanide and aluminium hydroxide gel) and proved to be immunogenic. The recombinant OmpF and OmpC generated in this work may be used for further studies for vaccination and diagnostics.

Keywords: Immunogenicity, montanide, OmpF, OmpC, porin protein, *Salmonella Typhi*

Salmonella enterica serovar Typhi (*S. Typhi*), the causative agent of typhoid fever, is a Gram-negative bacterium. Like other Gram-negative bacteria, *S. Typhi* is covered by a membranous structure outside the cytoplasmic membrane and lipopolysaccharide (LPS) called outer membrane. Porins are the outer membrane proteins (OMPs) of Gram-negative bacteria and play a role in the physiology of the bacterium by allowing the small hydrophilic molecules to pass through the channel. Their expression is preferential according to the environmental conditions of the bacterial growth [17, 5]. These proteins are resistant against high temperature and

denaturing agents. OmpF and OmpC are two major porin proteins of *S. Typhi*. Expression of *ompC* and *ompF* genes in *S. Typhi* is under the control of *EnvZ* and *ompR*, a two-component signal transduction system encoded by the *ompB* locus [6]. The functional and mature OmpC is a homotrimer. The OmpC monomer has 357 aa without signal peptide, with a molecular mass of 39 kDa. This porin protein is expressed under low and high osmolarity conditions [14, 18]. LPSs have been shown to provide structural stability to OmpC [1]. OmpF has preferentially been shown to be expressed under low osmolarity conditions.

S. Typhi is an obligate human pathogen, and typhoid fever continues to be a major health problem in the world [11]. Because of the lack of availability of good diagnostic tools, particularly that are field-based, correct diagnosis of typhoid fever remains a grey area. Earlier studies have indicated that the increase in titer of antibody to porins in sera of typhoid fever patients could be of diagnostic value [3]. Specific humoral and cellular immune responses are mounted against *Salmonella* OMPs [2, 21]. *S. Typhi* OMPs have shown to provide protection in mice upon challenge with virulent strains [9]. The mutants of OmpC and OmpF of *S. Typhimurium* showed attenuated virulence [4], and OmpC and OmpF of *S. Typhi* were shown to confer lifelong, specific bactericidal antibody response [21]. The porin proteins may also have potential use in the development of oral vaccines, biosensors, and nanoreactors [7]. Antibody responses are important to achieve protection against *Salmonella* infection [9]. Because of the ability of the porins to elicit antibody response that has shown to be protective in nature, several groups have studied native *S. Typhi* porins prepared by conventional method as vaccine candidates against typhoid fever [19, 22].

Since the porin proteins of *S. Typhi* can be invaluable in studies related to typhoid diagnostics and vaccination, and as the purification of native porin proteins by conventional methods can be a tedious and time consuming procedure,

*Corresponding author

Phone: +91-751-234-1848, Ext. (333); Fax: +91-751-234-1148;
E-mail: subodh_kumar@email.com

there is a need to develop an easy and rapid method that can be easily scaled up. Here, we report a method of cloning of *ompF* and *ompC* of *S. Typhi*, their overexpression in *E. coli*, and purification to homogeneity of the recombinant products. We further show the abilities of the recombinant porin proteins to elicit antibody response in a mouse model with different adjuvants [*i.e.*, Freund's adjuvant and two human-compatible adjuvants (montanide and aluminium hydroxide gel)]. The increased antibody response to the recombinant porins is also shown in typhoid patient sera.

MATERIALS AND METHODS

Materials

Montanide ISA 720 was a kind gift from Seppic, France. Qiagen genomic kit, Ni²⁺-NTA Fast Flow, Chelating Sepharose column, anti-His-tag antibody, pQE30UA expression vector, and *E. coli* host M15 cells were purchased from Qiagen GmbH, Hilden, Germany. *Taq* polymerase, dNTPs, PCR buffer, and restriction endonucleases (*PvuI* and *PstI*) were purchased from MBI, Fermentas, Ontario, Canada. Kanamycin, ampicillin, IPTG, aluminium hydroxide gel, Freund's complete/incomplete adjuvants, and GenElute PCR cleanup kit were purchased from Sigma, U.S.A. Nitrocellulose membrane (0.45 µm) was purchased from Millipore. The BCA Protein Assay Kit was from Pierce, Rockford, U.S.A.

Bacterial Strains and Patient Sera

An Indian clinical isolate, strain SKST of *S. Typhi* [11], was used in the present study. The genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. *E. coli* M15 cells were used for expression of the recombinant plasmids. Sera of five typhoid fever patients proven to be culture and Widal positive, and sera of five normal healthy controls were included in the study. The sera were a kind gift from Dr. Pallab Ray of Department of Medical Microbiology, PGIMER, Chandigarh, India.

PCR Amplification and Cloning of *ompF* and *ompC* Genes

The amplification of the *ompF* and *ompC* gene was carried out using the primers 5'tagaagcgcaaatcctggcagcg3' (939–962 bp) and 5'tcagaactggaagtaataccgac3' (2,027–2,004 bp, Accession No. X89757), and primers 5'gttaaagtactgtccctcctg3' (410–430 bp) and 5'tagaactggt aaaccagacc3' (1,540–1,520 bp, Accession No. M31424), respectively. PCR reactions of 25 µl each for *ompF* and *ompC* contained 0.5 µmol/l of each primer, 0.2 mmol/l of each dNTP, 0.5 units of *Taq* polymerase, and 1.5 mmol/l MgCl₂ in 1× PCR buffer with 500 pg of template DNA. The reaction procedure consisted of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 62°C (*ompF*) or 58°C (*ompC*) for 1 min, and extension at 72°C for 2 min. The DNA was denatured for 4 min in the beginning and finally extended for 5 min at 72°C. PCR products were purified using a GenElute PCR cleanup kit and were ligated in the linear pQE30UA vector as per the manufacturer's instructions. The ligated products were transformed into chemically competent *E. coli* M15 cells. The recombinant clones were selected on LB plates containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml). Colonies appearing on the plate were screened for recombinant plasmid *pompF* or *pompC* by minipreparation of plasmid

DNA. The desired insert in recombinant plasmids *pompF* and *pompC* was confirmed in right orientation by restriction digestion with *PvuI* and *PstI* endonuclease, respectively, and by DNA sequencing.

Expression of Recombinant Porins (OmpF and OmpC)

Competent M15 host cells of *E. coli*, transformed with recombinant plasmid construct *pompF* or *pompC*, were selected on LB agar plates containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The selected transformants were inoculated into 5 ml of LB medium containing the above-mentioned antibiotics and allowed to grow overnight at 37°C in a shaker incubator at 200 rpm. The overnight grown cultures were further inoculated in 10 ml of antibiotics containing LB broth to the final concentration of 1%. The cultures were grown at 37°C at 200 rpm until the OD₆₀₀ reached ~0.6–0.8. At this stage, the cultures were induced with 0.75 mM (OmpF) or 0.5 mM (OmpC) isopropylthiogalactoside (IPTG) and further grown for 4 h using the same conditions. Prior to IPTG induction, 1-ml aliquots were prepared from both the cultures and were used as uninduced controls. After 4 h of the induction, 1-ml aliquots of each induced culture were prepared and pelleted at 12,000 ×g for 2 min at 4°C. The induced and uninduced culture pellets of OmpF and OmpC were lysed in Laemmli buffer and analyzed by 12% SDS–PAGE [13].

Western Blot with Anti-His-Tag Antibody

In order to detect recombinant OmpF or OmpC in *E. coli*, induced and uninduced lysates were resolved on 12% SDS–PAGE. The resolved proteins were electroblotted onto nitrocellulose membrane using the wet transfer method [24]. Membranes were blocked overnight at 4°C in 3% BSA and incubated with mouse anti-His-tag monoclonal antibody at a dilution of 1:1,000 in blocking solution (1% BSA) for 1 h at 37°C. After three washings with PBS-T, membranes were incubated with rabbit anti-mouse IgG horseradish peroxidase (HRP) conjugated at a dilution of 1:5,000 for 1 h at 37°C. After washings with PBS-T, membranes were developed with 3,3'-diaminobenzidine in PBS containing 8.8 mM H₂O₂.

Localization of Recombinant Porins

To determine the solubility of the recombinant proteins, test-tube cultures were set up and induced with appropriate IPTG concentrations at log phase for 4 h. Aliquots (1 ml) of the induced culture were harvested by centrifugation at 12,000 ×g for 2 min. The cell pellet was resuspended in 1 ml of cell lysis buffer (pH 8.0) containing 10 mM Tris, 5 mM EDTA, 100 mM NaCl, 5 mM DTT, and 100 µg/ml lysozyme. The cell suspension was sonicated on ice and the resulting cell lysate was centrifuged at 12,000 ×g for 30 min at 4°C. The clear supernatant and remaining pellet were collected and analyzed by 12% SDS–PAGE.

Preparation of Inclusion Bodies from One Liter of Bacterial Culture

Two tubes of LB medium (10 ml, with antibiotics) were inoculated with M15 cells transformed with *pompF* or *pompC* plasmids. The cultures were grown overnight at 37°C with shaking (200 rpm) and used for inoculation of LB broth (1 l containing antibiotics) to a final concentration of 1%. The cultures were then grown at 37°C at 200 rpm until the OD₆₀₀ reached 0.6–0.8 and induced with IPTG [0.75 mM (OmpF) or 0.5 mM (OmpC)]. After induction of 4 h, the cells were harvested at 8,000 ×g (Sorvall SLC-6000 rotor) for 15 min at 4°C. The cell pellets of OmpF and OmpC were resuspended

in 50 ml of cell lysis buffer of pH 8.0 (10 mM Tris, 5 mM EDTA, 100 mM NaCl, 5 mM DTT, 100 µg/ml lysozyme) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed by sonication. The resulting cell lysates were centrifuged at 26,000 ×g (Sorvall SLC-1500 rotor) for 30 min at 4°C. The cell pellets in the form of inclusion bodies (IBs) were washed with IB wash buffer of pH 6.0 (10 mM Tris, 5 mM EDTA, 200 mM NaCl, 1 M urea, 1% TritonX-100) and centrifuged as before. The supernatants were decanted and each IB pellet was resuspended in 30 ml of IB solubilization buffer of pH 8.0 (10 mM Tris-HCl, 100 mM NaH₂PO₄, 100 mM NaCl, 8 M urea), stirred on a magnetic stirrer for overnight at 37°C, and centrifuged at 26,000 ×g for 30 min at 4°C. The supernatant was collected.

Purification of Recombinant Porins by Immobilized Metal Affinity Chromatography (IMAC)

Both of the recombinant proteins were purified by IMAC using a Ni²⁺-NTA Fast Flow, chelating Sepharose column [8]. The column was equilibrated with 20 ml of IB solubilization buffer (pH 8.0). The supernatant was allowed to bind to the column for 30 min. The column was washed by passing 50 ml of column wash buffer of pH 6.0 (10 mM Tris, 100 mM NaCl, 100 mM NaH₂PO₄, 8 M urea). The protein bound to the column was eluted by passing 15 ml of elution buffer of pH 4.0 (10 mM Tris, 100 mM NaCl, 100 mM NaH₂PO₄, 8 M urea). One ml fractions were collected and analyzed by 12% SDS-PAGE. The pooled volume of OmpF was dialyzed against 1 l of 10 mM PBS (pH 7.2) that was replaced four times over a period of 24 h. The dialyzed sample was concentrated to 2 ml using an Amicon ultracell with 10 kDa cutoff membrane. OmpC was found to precipitate in 10 mM PBS (pH 7.2), and therefore it was dialyzed against 10 mM Tris (pH 8.0) containing 50 mM NaCl and 4 M urea. The protein concentration was determined with reference to standard BSA in the BCA assay kit [23]. The endotoxin content of both purified proteins was determined by a Limulus Amebocyte Lysate (LAL) QCL-1000 kit (Cambrex).

Stability of Recombinants OmpF and OmpC

To determine the stability of the purified recombinant porin, 1-ml aliquots of OmpF and OmpC (500 µg/ml) were incubated at 37°C and 4°C for 4 months. Both proteins were incubated in elution buffer with 8 M urea as well as in dialysis buffer without urea (OmpF) or with 4 M urea (OmpC). The degradation of both proteins was checked by SDS-PAGE and their concentration was determined by a BCA kit.

Formulation of Recombinant Porins (OmpF and OmpC) with Adjuvants

OmpF and OmpC were formulated with aluminium hydroxide gel, Freund's complete/incomplete adjuvant (FCA/FIA), and montanide ISA720. In the case of FCA/FIA, one part of adjuvant was mixed with one part of each antigen (v/v). For montanide ISA720, 2.3 parts of adjuvant was mixed with one part of each antigen (70:30, w/w). The taken materials were mixed by leur-lock syringes until an emulsion was formed. For aluminium hydroxide gel (13 mg/ml), the pH was first adjusted to 7.0 with NaOH and then mixed with OmpF or OmpC in the ratio of one part adjuvant and one part of each antigen (v/v). The formulation was incubated for 6 h at 4°C on a rocking platform, and centrifuged at 3,000 rpm for 10 min. The unadsorbed protein in the supernatant was tested by a BCA kit. More than 90% of each recombinant protein (OmpF and OmpC) was

found adsorbed to aluminium hydroxide gel. The pellet was resuspended in normal saline and was used for immunization of mice.

Immunization of Mice and Measurement of Anti-OmpF/OmpC IgG Antibody

Swiss albino mice, male, 4–6-week-old (n=10/group), were used for immunization. The mice were maintained and used in accordance with the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals. The study had the approval of the Institutional Ethics Committee. Animals were divided into three groups; *viz.*, recombinant OmpF with Freund's complete/incomplete adjuvant (FA group), recombinant OmpF with aluminium hydroxide gel (alum group), and recombinant OmpF with montanide adjuvant (montanide group). Similar groups were formed for immunization with recombinant OmpC. Each animal received 20 µg of purified recombinant OmpF or OmpC protein. Immunization was done by subcutaneous administration of antigen on days 0, 14, and 21. The first dose in the FA group was administered with complete adjuvant and the rest of the doses were given with incomplete adjuvant. Sera were collected prior to first dose and 7 days after the last dose. Quantitation of antibodies to the recombinant OmpF or OmpC was performed using ELISA by a previously described method [12].

Seroreactivity of Recombinant Porin Proteins

Evaluation of the seroreactivity of antibody present in the typhoid patients sera (five no) with purified recombinants OmpF and OmpC was performed by indirect ELISA. Each recombinant purified antigen was coated (0.1 µg/well) on the microtiter plate. Sera samples were tested at 1:500 dilution. Sera from five healthy (typhoid negative) persons was used as control in the same format.

Statistical Analysis

A statistical analysis to compare the groups was performed using the *t*-test (Sigma Stat, Jandel Scientific, U.S.A.).

RESULTS

Construction of *pompF* or *pompC* Plasmids for Expression of OmpF/OmpC Proteins

The DNA fragments of 1,089 bp and 1,131 bp encoding OmpF and OmpC proteins, respectively, were amplified by PCR using specific designed primers. The nucleotide sequences of porin proteins of OmpF and OmpC of *S. Typhi* amplified from the clinical isolates described in this report have been submitted to GenBank under Accession Nos. DQ224404 and FJ009676, respectively. These amplified products were cloned into pQE30UA vector individually and the recombinants were screened by restriction digestion of the minipreps. The recombinant vectors (*pompF* and *pompC*) were generated by fusing the *ompF* and *ompC* gene sequences in-frame with the translation initiation codon and the 6× His-tag of pQEUA30 under the control of IPTG-inducible T5 promoter, as shown in Fig. 1A. The prepared constructs were predicted to encode 382 and 396 amino acids with 42.1 kDa (OmpF) and 43.17 kDa (OmpC) proteins including 6× His-tag at the N-terminus.

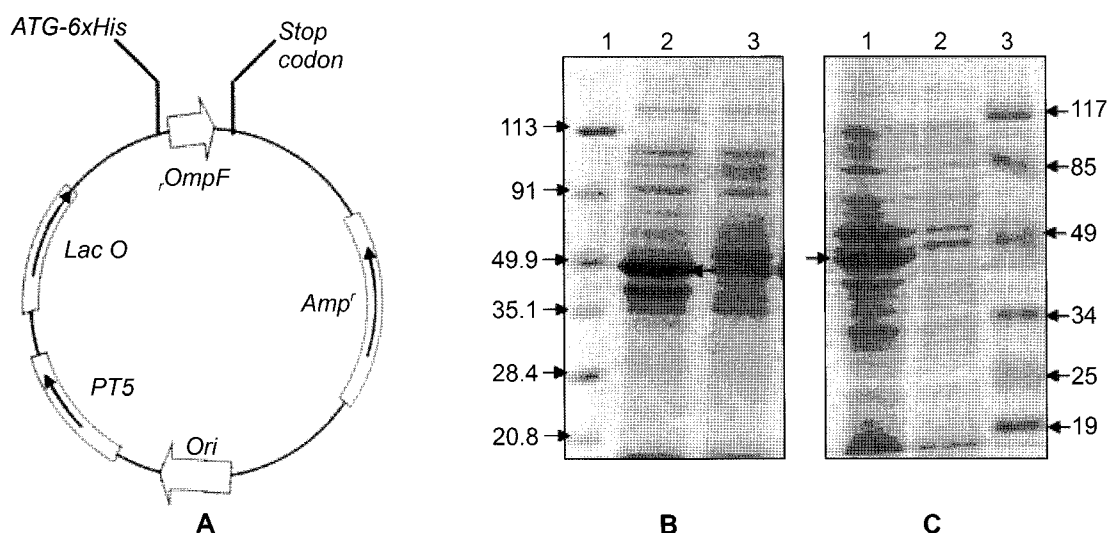


Fig. 1. A. Map of recombinant plasmid *pompF* or *pompC*.

In this plasmid, the gene encoding *S. Typhi* porin proteins, OmpF or OmpC, were cloned in-frame with the initiator codon provided by pQE30UA. Abbreviations are as follows: *Lac O*, Lac operator gene; *Amp^r*, Ampicillin marker; *Ori*, replication origin sequences. The arrows indicate direction of transcription. **B.** 12% SDS-PAGE analysis of OmpF protein expression. The panel depicts protein molecular mass marker (lane 1), and Coomassie-stained polypeptide profiles of *E. coli* lysate induced with IPTG (lane 2) and uninduced lysate (lane 3). The arrow at the right of the panel indicates the position of OmpF. **C.** 12% SDS-PAGE analysis of OmpC protein expression. The panel depicts Coomassie-stained polypeptide profiles of *E. coli* lysate induced with IPTG (lane 1) and uninduced lysate (lane 2); protein molecular mass marker (lane 3). The arrow at the left of the panel indicates the position of OmpC.

Overexpression of Recombinant Porin Proteins OmpF and OmpC

The prepared recombinant constructs for the expression of OmpF and OmpC were transformed into chemically competent M15 *E. coli* host cells, which provide T5 RNA polymerase for expression of heterologous genes. Small-scale cultures of the positive clones were subjected to IPTG induction to identify clones capable of expressing the predicted 42.1 and 43.17 kDa recombinant proteins. A typical induction experiment comparing the OmpF protein profiles of IPTG-induced *E. coli* lysate (lane 2) and uninduced lysate (lane 3) is shown in Fig. 1B. The recombinant OmpC protein profiles of IPTG-induced *E. coli* lysate (lane 1) and uninduced lysate (lane 2) are shown in Fig. 1C. It is evident that IPTG induction results in the expression of the unique 42.1 and 43.17 kDa proteins. One of the representative clones (*pompF* and *pompC*) expressing optimum quantity of recombinant OmpF or OmpC was selected and further verified by DNA sequencing. Expression conditions were optimized using different concentrations of IPTG (0.2–1.0 mM) and duration of induction (1–5 h). The IPTG concentrations of 0.75 mM (OmpF) and 0.5 mM (OmpC) with 4 h induction were found most effective to get the optimum yield of the recombinant proteins.

Western Blot with Anti-His-Tag Antibody

The bacterial lysates of induced and uninduced cultures of both OmpF and OmpC were separated on 12% SDS-PAGE with a prestained protein molecular mass marker and were transferred onto the nitrocellulose membrane. Western blot

analysis with anti-His-tag antibody detected 42.1 and 43.17 kDa proteins in the induced bacterial lysates, representing the recombinant OmpF and OmpC, respectively, but the same bands were not seen in the uninduced bacterial lysates (Figs. 2A and 2B).

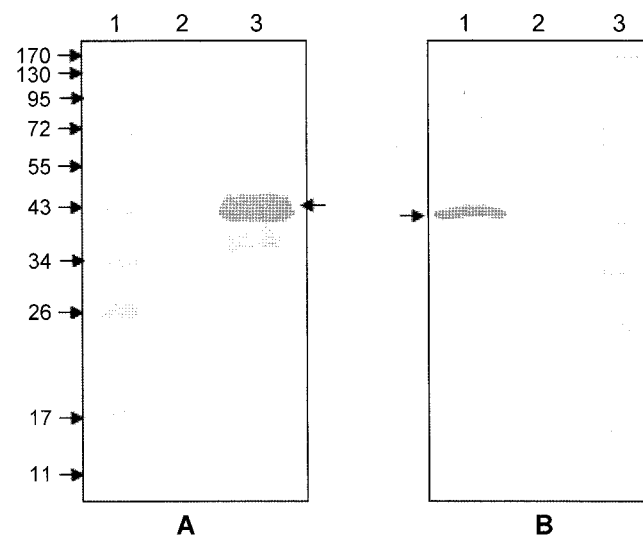


Fig. 2. Western blot analysis of recombinant OmpF [A] and OmpC [B] with anti-His-tag monoclonal antibody.

A. Lane 1, protein molecular mass markers (kDa); lane 2, uninduced *E. coli* lysate; lane 3, *E. coli* lysate induced with IPTG. **B.** Lane 1, *E. coli* lysate induced with IPTG; lane 2, uninduced *E. coli* lysate; lane 3, molecular mass markers (kDa). The *E. coli* lysates were run on 12% SDS-PAGE and transferred onto the nitrocellulose membrane.

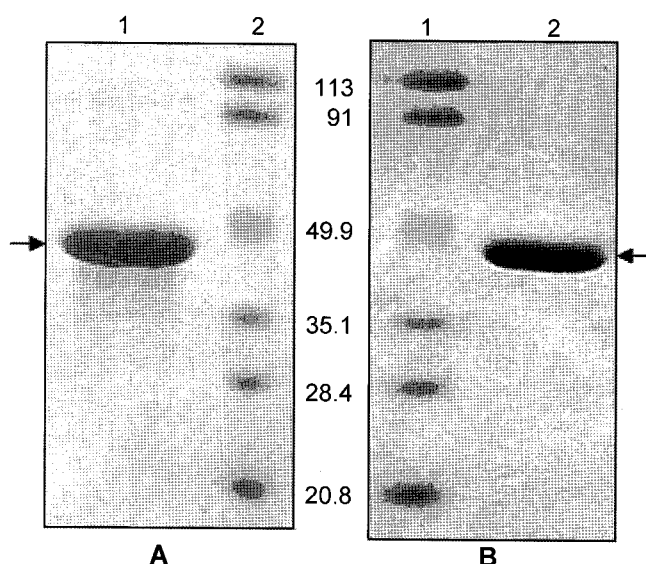


Fig. 3. SDS-PAGE (12%) analysis of recombinant OmpF (A) and OmpC (B) purified by IMAC using Ni-NTA resin.

A. Lane 1, purified recombinant OmpF protein; lane 2, molecular mass marker. B. Lane 1, molecular mass marker; lane 2, purified recombinant OmpC protein.

Purification of Recombinant OmpF and OmpC Porin Proteins

Lysis under native conditions revealed the association of both recombinant proteins with pellet fraction, demonstrating that the recombinant OmpF or OmpC protein is insoluble. No discernible recombinant protein was found in the supernatant (data not shown). Lysis under denaturing conditions revealed the presence of the major proportion of the 42.1 or 43.17 kDa recombinant protein in the supernatant. The purification of recombinant OmpF or OmpC was carried out by solubilizing in 8 M urea and purified by IMAC. Both recombinant proteins OmpF (Fig. 3A) and OmpC (Fig. 3B) could be eluted using the elution buffer of pH 4.0, and SDS-PAGE analysis showed it to be almost pure. The protein yields of 30 and 25 mg/l of bacterial culture of recombinant OmpF and OmpC, respectively, were collected, as shown in Table 1. The endotoxin content in both the proteins was determined by an LAL QCL-1000 kit and was found to be less than 2.5 EU/25 mg of OmpF or OmpC.

Stability of Recombinant OmpF and OmpC

The concentration of incubated proteins in both buffers at different temperatures was observed to be the same as

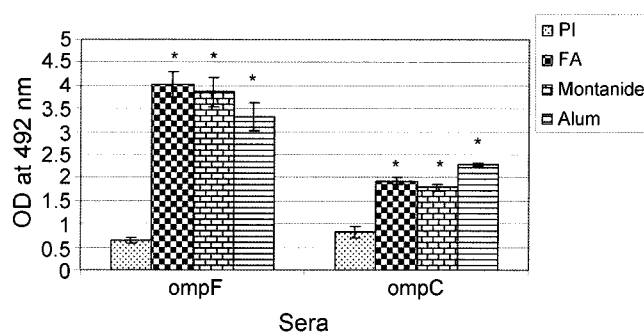


Fig. 4. Comparative evaluation of recombinant OmpF and OmpC specific IgG antibody titers in immunized Swiss albino mice by ELISA.

Three groups of animals were formed and each group contained 10 mice. Mean IgG titers are shown at 1:200 dilutions. PI, Pre-immune sera; FA, Freund's adjuvant group; Montanide, montanide adjuvant group; Alum, aluminium hydroxide gel group. The error bars indicate mean standard error of four independent ELISAs. The groups were compared statistically using the *t*-test. *= $p < 0.05$, when compared with PI.

originally stored. No degradation was observed in SDS-PAGE for both the proteins stored at two different temperatures.

Titer of the Recombinant OmpF- or OmpC-Specific IgG Antibody

To check the titer of OmpF- and OmpC-specific IgG antibody in Swiss albino mice, sera were collected from all animals before immunization (day 0) and 7 days after the last dose. The IgG antibody titer was measured by ELISA in each pooled sera at various dilutions (1:100 to 1:12,800). A significant rise ($p < 0.05$) in IgG antibody against recombinant OmpF was observed in mice of the FA group, Montanide group, and Alum group. The recombinant OmpF with aluminium hydroxide gel, however, produced a lower titer of IgG antibody in comparison with the other two adjuvants (Fig. 4). In comparison with OmpF, lower OD values were obtained with OmpC at 1:200 dilution; however, like OmpF, significantly higher levels of anti-OmpC IgG antibodies ($p < 0.05$) were generated in all the three groups, even at the last tested dilution (data not shown). Interestingly, with OmpC, the highest titers were observed in the Alum group, unlike OmpF (Fig. 4).

Seroreactivity with Typhoid Sera

The reactivity of recombinant porin proteins was determined in five typhoid patients and normal healthy sera by indirect

Table 1. Purification summary of OmpF and OmpC from inclusion bodies.

Steps	Recombinant OmpF	Recombinant OmpC
Bacterial pellet from 1 l culture (wet weight)	3.06 g	3.0 g
Purified IB from 1 l culture (wet weight)	0.93 g	0.9 g
Purified IB from 1 l culture (wet weight)	0.81 g	0.78 g
Purified proteins (concentration) from 1 l of shake flask culture	30.0 mg	25.0 mg

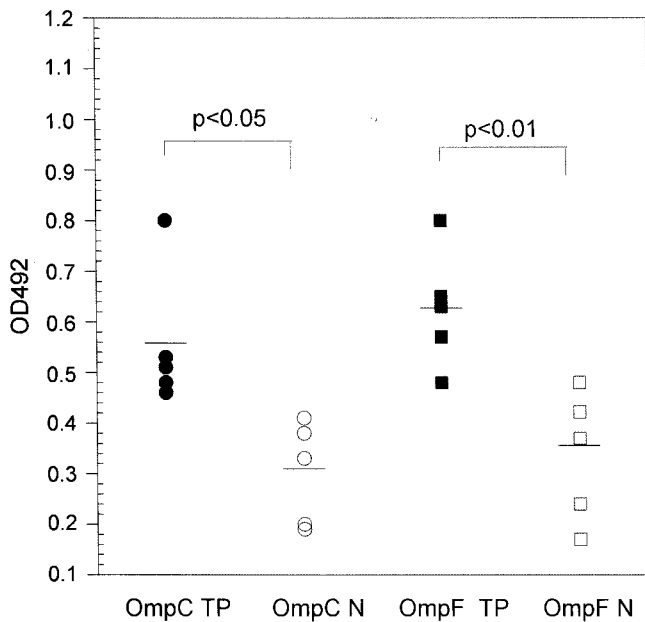


Fig. 5. Evaluation of reactivity of recombinant porins (OmpF and OmpC) with antibodies present in sera of typhoid patients (five individuals) by indirect ELISA.

Reactivity was also evaluated with normal human sera (five individuals). OmpC TP, OmpC with typhoid patients' sera; OmpC N, OmpC with normal human sera; OmpF TP, OmpF with typhoid patients' sera; OmpF N, OmpF with normal human sera. The bar shows the mean value. The groups were compared statistically using the *t*-test.

ELISA. A significant difference between the OD values in sera of typhoid patients and healthy control could be observed both for OmpF ($p < 0.01$) and OmpC ($p < 0.05$), as shown in Fig. 5.

DISCUSSION

Porins are outer membrane proteins of Gram-negative bacteria that play a major role in the physiology of the bacterium, by allowing the passive diffusion of small solute molecules and waste materials through their water-filled channel [16]. Outer membrane porins of *S. Typhi* are immunologically important surface antigens and have been shown to have potential use in diagnostics and as vaccine for typhoid fever [15]. We initiated this work with an aim to develop an easy and rapid method for purification of the recombinant porin proteins, so that they can be used for studies related to diagnostics or vaccine development of typhoid fever.

In an earlier published report, it has been stated that heterologous expression of OmpC in *E. coli* with signal peptide is toxic to cells, whereas the removal of signal peptide leads to the formation of cytoplasmic inclusion bodies [10]. In contrast to this report, we have cloned the OmpF and OmpC with a signal peptide and no cytotoxicity was revealed by these recombinant proteins to the cells,

although the overexpression of recombinants OmpF and OmpC proteins in *E. coli* led to the formation of the cytoplasmic inclusion bodies. For the purification of the recombinant proteins, the IBs were solubilized using denaturant (urea). Urea shows concentration-dependent binding to protein [20]. The concentration of urea (denaturant) plays a major role to solubilize the aggregated proteins, and the modulation of denaturant concentration determines the degree of unfolding by disrupting the intramolecular interactions. In the present study, 8 M urea was found to be suitable to solubilize the IBs to get the optimum yield of the recombinant porins. The recombinant porins were purified by IMAC under denaturing conditions (8 M) to yield 30 and 25 mg/l of shake flask culture of recombinants OmpF and OmpC, respectively, as described in the Materials and Methods section. The obtained recombinant porins were found to be stable at 37°C and 4°C for 4 months.

As both the porin proteins have been purified under denaturing conditions, we decided to evaluate the immunogenic nature of the obtained recombinant porins, so that these can have potential use in vaccine or diagnostics studies. In this context, the immunogenicity of the recombinant porins was evaluated in Swiss albino mice with three different adjuvants (Freund's adjuvant and human-compatible adjuvants like aluminium hydroxide gel and montanide). A rise in titer of anti-OmpF and anti-OmpC IgG antibodies was observed in immunized mice. In the case of recombinant OmpF, IgG titer ($p < 0.05$) was observed with Freund's adjuvant, followed by montanide adjuvant and aluminium hydroxide gel. In contrast to OmpF, the high titer ($p < 0.05$) of recombinant OmpC-specific IgG antibody was observed in mice immunized with aluminium hydroxide gel followed by Freund's adjuvant. The recombinant OmpC with montanide adjuvant produced a lower titer ($p < 0.05$) of IgG antibody. The marked IgG responses in mice immunized with OmpC and OmpF support the immunogenic nature of these recombinant porins and suggest that these may be used as good immunogens for vaccine studies. The role of native porins in typhoid diagnostics has already been defined [3]. To evaluate the usefulness of recombinant porin proteins in diagnostics, anti-OmpF and anti-OmpC antibodies were examined in the sera of five typhoid patients. A significant rise in anti-OmpF ($p < 0.01$) and anti-OmpC ($p < 0.05$) antibodies was observed when compared with normal human sera, suggesting the potential use of the recombinant porin proteins in typhoid diagnostics. Our results are in accordance with the previously reported studies [3] on the possible use of native porins as diagnostic molecules of typhoid fever. Since the number of patients sera included in the study is very small, the results should be interpreted carefully. A detailed study on the usefulness of recombinant porin proteins in typhoid diagnostics is under way.

In conclusion, the recombinant OmpC and OmpF of *S. Typhi* porins were expressed in *E. coli* and purified. The

recombinant proteins proved to be immunogenic and useful in typhoid diagnostics. Since the expression system uses *E. coli* as the heterologous host, the process is easy and amenable to inexpensive scale-up.

Acknowledgments

The authors are thankful to Dr. R. Vijayaraghavan, Director of Defence Research and Development Establishment (DRDE), Ministry of Defence, Government of India for his support and constant inspiration, and providing necessary facilities for this study. We are also thankful to Seppic, France and Dr. Pallab Ray of PGIMER, Chandigarh, India for providing montanide ISA720 adjuvant and typhoid fever patient sera, respectively.

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