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Full Length Research Paper

Protein engineering of yellow fluorescent protein insertion in outer membrane protein OmpF from *Salmonella typhi*

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Each monomer of the trimeric outer membrane porin OmpF of *Salmonella typhi* folds as a 16-stranded β-barrel with eight short turns at the periplasmic side and eight loops on the cell surface side. Yellow fluorescent protein (YFP) has been engineered into Loop 7 of OmpF. *HindIII* and *EcoRV* restriction sites were introduced in the region corresponding to Loop 7 of *ompF* gene. Flanking the sites *yfp* gene amplified from pEYFP, was inserted with 15 bases as linker on either side of the insert. The chimeric protein product was successfully expressed in *E. coli* BL21 (DE3). The strategy has provided a hybrid protein in which an outer membrane protein presents a yellow fluorescent protein. This fluorescent chimera can be used as a marker for biological analysis. Moreover, the hydrophilic component makes the engineered membrane protein more amenable for crystallographic analysis, as it improves the crystallisation potential due to the globular component.

Key words: Membrane protein, OmpF, *Salmonella typhi*, yellow fluorescent protein (YFP).

INTRODUCTION

Salmonella typhi is a Gram-negative bacterium belonging to the family Enterobacteriaceae and is the causative agent of typhoid (Germanier, 1984). Outer membrane protein F (OmpF) is a major porin in *S. typhi* responsible for the translocation of antibiotics. The functional unit of OmpF is a homotrimer. Each monomer of molecular weight 37 kDa forms a β-barrel structure having 16 membrane spanning β-strands. The OmpF porin forms three large water-filled channels per trimer, allowing the diffusion of small hydrophilic molecules such as nutrients, antibiotics and waste products across the outer bacterial membrane (Nikaido, 1994). OmpF allows the passage of drugs such as quinolones, tetracyclines, and β-lactams (Cohen, 1989; Tavio, 1999). The understanding of the structure function relationship of *S. typhi* OmpF is important for the development of new drugs against typhoid.

According to the statistics of the databank of transmembrane proteins (PDBTM), there are only 1437 transmembrane protein structures available out of 74,889 total protein structures. The number of β-barrel membrane protein structures are even less when compared with the total transmembrane protein structures available so far. Membrane proteins are difficult to purify and crystallize. Detergents used in solubilisation significantly reduce the amount of polar surface area exposed for crystal contacts. This makes membrane protein crystals fragile and disordered leading to low resolution diffraction data (Prive, 2007). Insertion of a soluble protein into one of the loops of the membrane protein OmpF could increase the effective polar surface area and help in better packing of molecules in the crystals. The C-terminal fusion of Green Fluorescent Protein with outer membrane protein has been reported in *Campylobacter jujeni* MOMP (Dedieu, 2002), Lpp-OmpA of *Escherichia coli* (Shi, 2001) and *Vibrio anguillarum* (Yang, 2008).

However in OmpF, the N- and C-terminii are hydrogen bonded as part of the beta barrel structure. In this situation, sandwich fusion is the only choice. *E. coli* PhoE (Agterberg, 1987), OmpC (Xu, 1999), OmpA

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(Verhoeven, 2009), *S. typhi* OmpC (Puente, 1995) and *Vibrio cholerae* OmpS (Lang, 2000) have been shown to be good sandwich carriers for peptides up to 186 amino acid residues. Here, we report the engineering of yellow fluorescent protein (YFP) containing 239 amino acid residues into the loop 7 of *S. typhi* OmpF for further studies.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains used in this study were *E. coli* BL21 (DE3) and DH5α. The constructs pompF (the *ompF* gene without signal peptide from *S. typhi* Ty21a strain has been cloned in pET20b (+) vector) (D. Balasubramaniam, Madurai Kamaraj University, Madurai, personal communication) and pE*YFP* (gift from Dr.Daniel Oatzen, Aarhus University, Denmark) were used in this study.

Chemicals and media composition

E. coli cells were grown in Luria-Bertani broth with 1% tryptone, 1% NaCl and 0.5% yeast extract, antibiotics kanamycin (50 mg/ml) and ampicillin (100 mg/ml) (Himedia Laboratories Pvt. Limited) were added to media for strain maintenance and 1 mM IPTG (Medox Biotech India Pvt. Limited) as a stock solution was used as an inducer.

OmpF loop engineering

Introducing restriction sites in loop 7 of ompF

The *ompF* gene was digested from pompF construct using *XbaI* and *EcoRI* restriction sites and sub cloned into pOK12 vector and the construct was named as pOKompF. In order to introduce *HindIII* and *EcoRV* restriction sites in the region corresponding to loop7 of *ompF*, the following primers were used(Ocimum Biosolutions (India) Ltd):

Forward primer: 5' TAATGATATCGGCTCGGCCGATCTGG 3',
Reverse primer: 5' ATAAAGCTTGTCGGCGCCGTTCAATGCTTAC 3'

These primers introduce *HindIII and EcoRV sites in loop7*. Using pOKompF as template, inverse PCR (MyCycler™, BIO-RAD) with Phusion DNA polymerase (Finnzymes, Espoo, Finland) was done to amplify *ompF* gene along with the backbone. This 3.2 kb amplicon was purified from agarose gel and subjected to *DpnI* digestion that selectively digests the template DNA leaving behind the PCR amplicon. The amplicon was then treated with *T4 DNA kinase* to add phosphate moiety at the 5' end of both strands of the template. The amplicon was self-ligated with *T4 DNA ligase*. High efficiency competent cells of *E. coli* DH5α were transformed with this ligation mixture. The resulting construct was named as pOKHEF. Presence of restriction sites in the construct was confirmed with restriction digestion with *EcoRV*.

Amplification and cloning of *yfp*

yfp gene was PCR amplified using the following specific primers from pEYFP construct (Ocimum Biosolutions (India) Ltd):

Forward primer: 5'TAATAAGCTTGATGACGACGACAAGATGGTGAGCAAGGGCGAG 3'.
Reverse primer: 5'ACTGATATCCTTGTCGTCGTCATCCTTGTACAGCTCGTCCATGCC 3'.

HindIII and *EcoRV* sites were introduced through primers at 5' and 3' of *yfp* gene respectively. Linker sequence of 15 bases (GATGACGACGACAAG) was also included at 5' and 3' end of the *yfp* gene through these primers. Amplification was done using Taq DNA polymerase that generates 'A' overhang. *yfp* amplicon was purified from agarose gel and ligated with 'T' tailed vector (RBC Biosciences). High efficiency competent cells of *E. coli* DH5α were transformed with this ligation mixture. The resulting construct was named as TAYFP. TAYFP was digested with *HindIII* and *EcoRV* to release *yfp* gene and it was purified from agarose gel.

Fusion of *ompF* with *yfp*

The *HindIII* and *EcoRV* digested *yfp* gene was ligated with pOKHEF. High efficiency competent cells of *E. coli* DH5α were transformed with this ligation mixture. The final construct with pOK12 backbone containing *ompF* with *yfp* incorporated into the region corresponding to Loop 7 was named as pOKFYF.

Construction of FYF expression vector

In order to release *ompF-yfp-ompF* fragment, pOKFYF was subjected to *XbaI* and *EcoRI* double digestion. The fragment corresponding to 1.8 kb was purified from agarose gel. This insert was ligated with pET-20b (+) vector that has been digested with *XbaI* and *EcoRI*. High efficiency competent cells of *E. coli* DH5α were transformed with this ligation mixture. The clones were confirmed by *HindIII* and *EcoRV* digestion for 750 bp *yfp* release and *XbaI* and *EcoRI* for 1.8 kb *ompF-yfp-ompF* and the resulting construct was named as pETFYF. pETFYF plasmid was extracted from DH5α/pETFYF cells and competent cells of *E. coli* BL21 (DE3) were transformed with pETFYF for IPTG induction and expression. All DNA manipulations were carried out by using standard procedures (Sambrook, 1989). Restriction enzymes and modifying enzymes were obtained from New England Biolabs, and were used as recommended by the manufacturer. DNA sequencing was done (Applied Biosystems 3130, Genetic Analyser, USA) for confirmation of all constructs.

Optimization of expression

In order to maximize the expression of protein, a grid screen was done with different concentration of IPTG (0.1, 0.2, 0.3, 0.4 mM). BL21 (DE3)/pETFYF cells were grown till the cell density reached 0.6 OD at A600 nm and induced with the varying amount of IPTG for 5 h. 100 µl of cell culture was centrifuged at 12,000 rpm for 1 min and the supernatant was decanted. In order to perform gel electrophoresis (12% SDS-PAGE), the loading dye (50 µl of 5X) was added to the samples before boiling for 5 min. Quantitative estimation of expressed protein was based on the thickness of the band visualized after staining with coomassie brilliant blue. The highest yield was obtained with 0.1 mM IPTG. The expressed fusion protein was named as FYF.

RESULTS AND DISCUSSION

In the present study, inverse PCR was carried out to

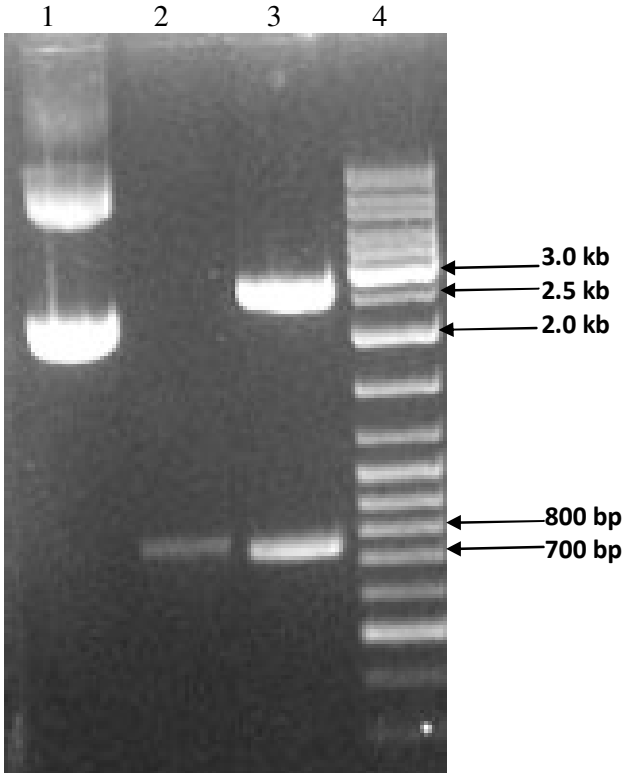

Figure 1. Restriction digestion pattern of TA*yfp*. Lane 1- Undigested TA*yfp* plasmid, Lane 2- +ve control (*yfp* PCR product), Lane 3- TA*yfp* digested with *HindIII+EcoRV* showing 2.7 kb TA vector backbone and 760bp *yfp* release, Lane 4- DNA ladder.

introduce *HindIII* and *EcoRV* restriction sites in the *ompF* gene after 912 and 930 bp respectively. The 3.2 kb PCR amplicon of *ompF* gene along with pOK12 backbone was further treated and re-ligated. In the construct the nucleotide sequence corresponding to six amino acid residues (GADGSA) has been deleted in the middle of the region corresponding to Loop 7 in order to keep the size of the flanking sequence moderate for *yfp* insertion. Loop 7 was chosen as the site of insertion because this loop lies farther away from the trimeric interface and also away from the interface of adjacent monomers of trimer. Moreover, Loop 7 of *E. coli* OmpC, which is a structural homologue to *S. typhi* OmpF, has been reported to be engineered with the insertion of poly His-tag (Xu, 1999). By using pEYFP vector as a template, *yfp* gene was amplified along with linker sequence of 15 bases flanking the gene and *HindIII* and *EcoRV* restriction sites at the 5' and 3' ends respectively. The primers were designed to introduce the linker sequence of 15 bases GATGACGACGACAAG which will give rise to the amino acid sequence of Enterokinase protease cleavage site Asp Asp Asp Asp Lys on both sides of the *yfp* gene. This linker can provide flexibility to the YFP to fold properly when refolding and the protease cleavage site can also be used to remove the YFP from the fusion protein, if needed.

The *yfp* gene along with linker was digested from TA vector using *HindIII* and *EcoRV* restriction sites (Figure 1) and cloned into pOKHEF which was also digested with the same restriction enzymes. Using *XbaI* and *EcoRI* restriction enzymes, the fused gene was cloned into pET20b(+) vector (Figure 2). The BL21(DE3) cells were transformed with pETFYF for overexpression. The full length 66 kDa OmpF-YFP fusion protein (FYF) was successfully expressed with 0.1 mM IPTG concentration at 25°C (Figure 3). YFP protein has been chosen as the protein of choice for insertion mutagenesis because of the fluorescent and robust nature of the molecule. Being a soluble protein, YFP provides large polar surface area and could possibly help in improving crystal contacts. The diffraction quality of the engineered protein is likely to be improved due to better packing of molecules in the crystal. YFP

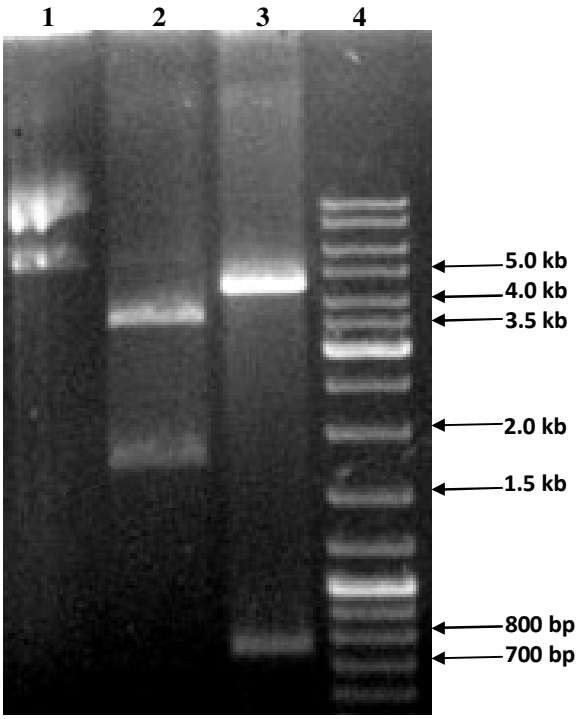

Figure 2. Restriction digestion pattern of pETFYF. Lane 1- Undigested pETFYF plasmid; Lane 2-pETFYF digested with *XbaI+EcoRI* showing 3.7 Kb pET 20b(+) backbone and 1.8 kb *ompF-yfp* release; Lane 3-pETFYF digested with *HindIII+EcoRV* showing 4.8 kb pET 20b(+) with *ompF* gene and 760 bp *yfp* release; Lane 4- DNA ladder.

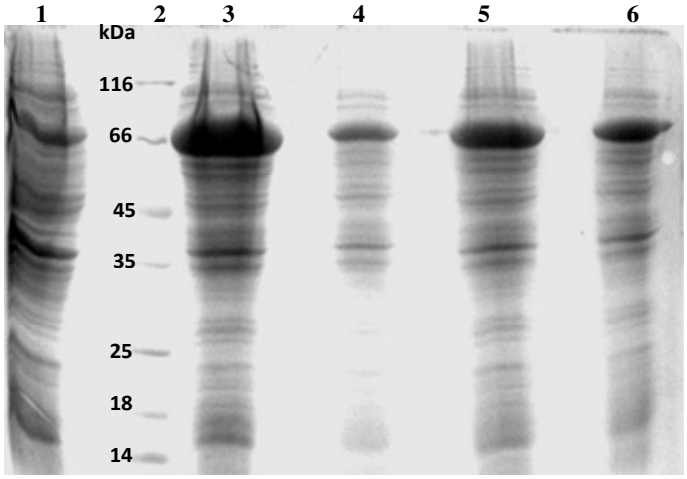

Figure 3. Expression profile of FYF protein. 12% SDS PAGE gel stained with Coomassie stain, showing the expression profile of FYF protein (66 kDa) with different IPTG concentration. All samples were boiled and loaded. Lane 1- Uninduced cells; Lane 2- Protein Marker in kDa; Lane 3- FYF induced with 0.1 mM IPTG; Lane 4- FYF induced with 0.2 mM IPTG; Lane 5- FYF induced with 0.3 mM IPTG; Lane 6- FYF induced with 0.4 mM IPTG.

tagging not only helps as a reporter system but also helps in increasing protein stability. The chimeric protein FYF may be helpful in understanding the folding pathway of OmpF (Enoki, 2004; Craggs, 2009) and the insertion dynamics in macrophages.

The loops of porins are likely to be tolerable in accommodating large alterations, without perturbing the architecture of the barrel. Modification cannot be made in the transmembrane segments which are essential for the structural and functional integrity (Agterberg, 1989; Bosch, 1986). The loops offer stretches of flexible flanking sequence for proper folding of the inserted peptides, without disturbing the tertiary structure of OmpF. *S. typhi* OmpC was shown to be a good candidate for the surface display of epitopes such as VP4 of rotavirus (Sujatha, 2001) and a peptide sequence of CD154 for the activation of lymphoma B cells. Long stretches of His residues, up to 84 amino acids, have been inserted into loop 7 of *E. coli*, to use it as a biosorbent, without affecting the folding and insertion into the outer membrane (Xu, 1999). It was shown in the case of *E. coli* OmpA that insertion of peptides into the loops did not interfere with export and membrane assembly (Puente, 1995). Though, insertion mutagenesis has been done for various porins, insertion of a complete protein into the loop of a porin has not been shown earlier. For the first time, we have successfully cloned a complete YFP into a loop of OmpF. The full length fusion protein FYF has been over expressed. The structure determination of loop insertion mutant of OmpF will be helpful in the development of new drugs against *Salmonella* infection. In terms of protein engineering, the fusion construct will help understand the structural integrity of a membrane protein.

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REFERENCES

Agterberg M, Adriaanse H, Tommassen J (1987). Use of outer membrane protein PhoE as a carrier for the transport of foreign antigenic determinant to the cell surface of *Eschericia coli* K-12. Gene, 59: 145-150

Agterberg M, Adriaanse H, Tijhaar E, Resink A, Tommassen J (1989). Role of the cell surface-exposed regions of outer membrane protein PhoE of *Escherichia coli* K12 in the biogenesis of the protein. Eur J Biochem., 185: 365-370

Bosch D, Leunissen J, Verbakel J, de Jong M, van Erp H, Tommassen J (1986). Periplasmic accumulation of truncated forms of outer-membrane PhoE protein of *Escherichia coli* K-12. J. Mol. Biol., 189: 449-455.

Cohen SP, Mcmurry LM, Hooper DC, Wolfson JS, Levy SB (1989). Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: Decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother., 33: 1318-1325.

Craggs TD (2009). Green fluorescent protein: structure, folding and chromophore maturation. Chem. Soc. Rev., 38: 2865-2875.

Dedieu L, Pages JM, Bolla JM (2002). Environmental regulation of *Campylobacter jejuni* major outer membrane protein porin expression in *Escherichia coli* monitored by using green fluorescent protein. Appl. Environ. Microbiol., 68: 4209-4215.

Enoki S, Saeki K, Maki K, Kuwajima K (2004). Acid Denaturation and refolding of Green Fluorescent Protein. Biochemistry, 43: 14238-14248.

Germanier R (1984). Bacterial vaccines. Academic Press, London

Lang H, Maki M, Rantakari A, Korhonen TK (2000). Characterization of adhesive epitopes with the OmpS display system. Eur J Biochem., 267: 163-170.

Nikaido H (1994). Porins and Specific diffusion channels in bacterial outer membrane. J. Biol. Chem., 269: 3905-3908.

Prive GG (2007). Detergents for the stabilization and crystallization of membrane proteins. Methods, 41: 388-397

Puente JL, Juarez D, Bobadilla M, Arias C F, Calva E (1995). The *Salmonella* OmpC gene: structure and use as a carrier for heterologous sequences. Gene, 156: 1-9

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular cloning: a laboratory manual. Cold Spring Harbor laboratory press, Cold Spring Harbor, New York.

Shi H, Su WW (2001). Display of green fluorescent protein on *Escherichia coli* cell surface. Enzym. Microb. Technol., 28: 25-34

Sujatha S, Arokiasamy A, Krishnaswamy S, Usha R (2001). Molecular modelling of epitope presentation using membrane protein OmpC. Indian J. Biochem. Biophys., 38: 294-297.

Tavio MD, Vila J, Ruiz J, Ruiz J, Sanchez AM, Anta MTJ (1999). Mechanisms involved in the development of resistance to fluoroquinolones in *Escherichia coli* isolates. J. Antimicrob. Chemother., 44: 735-742.

Verhoeven GS, Alexeeva S, Dogterom M, Blaauwen T (2009). Differential bacterial surface display of peptides by the transmembrane domain of OmpA. PLoS One, 8: e6739

Xu Z, Lee SY (1999). Display of polyhistidine peptides on the *Escherichia coli* cell surface by using outer membrane protein C as an anchoring motif. Appl. Environ. Microbiol., 65:5142-5147

Yang Z, Liu Q, Wang Q, Zhang Y (2008). Novel bacterial surface display systems based on outer membrane anchoring elements from the marine bacterium *Vibrio anguillarum*. Appl. Environ. Microbiol., 74: 4359-4365