# Folding and structural stability of OmpC from *Salmonella typhi*: Role of LPS and environment

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**Outer membrane porins of Gram-negative bacteria are very stable and in general resistant to complete denaturation by heat and other denaturants. Folding and stability of the homotrimeric beta barrel outer membrane protein OmpC, a major cell surface antigen from human pathogen *Salmonella typhi* is examined here using circular dichroism, gel electrophoresis and monoclonal antibodies. The results obtained with the vaccine strain Ty21a (native OmpC), recombinant OmpC (rOmpC) expressed in *E. coli* and the refolded OmpC (rfOmpC) from cytoplasmic inclusion bodies that differ in the amount of bound LPS (Lipopolysaccharide), shed light on the role of LPS and detergents on the refolding and stability of OmpC. From these studies it is suggested that the stability of *S. typhi* OmpC is modulated by the amount and type of LPS association.**

PORINS are outer membrane proteins of Gram-negative bacteria that allow the passive diffusion of small solute molecules and waste materials through their water-filled channel[1]. The trimeric nature of porin with extensive contact surface area renders enormous stability at quaternary structure level, against high temperatures and denaturing reagents like SDS and urea. The inter subunit latching by loop-2 and its role in the porin stability has been reported[2]. The strong network of interstrand hydrogen bonds stabilizes the barrel in the membrane[3]. Porins are resistant to proteolytic activity. These stable porins have potential applications in the development of oral vaccines, biosensors, and nanoreactors[4]. Lipids play an important role in the stability of membrane proteins and *in vivo* folding into membrane[5]. Specific negative charges in the inner core region of LPS and non-lamellar structure of lipid were shown to be contributing for high efficiency of folding of PhoE[6]. Lipid environment helps in maintaining the structural conformation and stability of membrane proteins[7]. The type and the amount of LPS in the purified protein are presumably critical for crystallization of membrane proteins[8]. Though the ability to take up the β-structure is the intrinsic property associated with primary structure itself, detergents help in obtaining native conformation[9]. The nature of detergent plays a major role in solubility, *in vitro* folding and stability of membrane proteins[10]. Non-ionic detergents mimic the hydrophobic environment better than ionic detergents like SDS[11]. Thermal stability of refolded membrane proteins is higher in non-ionic detergents than in ionic detergents like SDS[12]. Refolding studies on various porins such as *H. influenzae* porin[13], *R. capsulatus* porin[14], PorA of *Neisseria meningitides*[15] suggest that refolding can be achieved in the presence of detergent alone and LPS or other membrane components are not required. On the contrary, correct assembly of *in vitro* synthesized OmpF of *E. coli* was achieved only in the presence of LPS and membrane fractions[5]. LPS and outer membrane fractions helped in native-like trimerization even in the absence of detergents. Functional refolding of *Campylobacter jejuni*[16] MOMP porin was shown to refold 95% in the presence of homologous GroEL suggesting the usefulness of chaperones in the efficient refolding of porins *in vitro*.

OmpC is the major surface antigen, expressed throughout the infection period[17] and is a good candidate to display heterologous epitopes on the cell surface[18]. Understanding the role of factors that mediate the refolding and stability of OmpC becomes important, as epitope presentation and recognition require correct folding and high stability. *S. typhi* OmpC, an integral outer membrane protein, is a homotrimer with 357 amino acids and MW 39 kDa/monomer. Crystals of both native and recombinant form, diffracting up to 7 Å were obtained earlier[19]. The poor diffraction was inferred to be due to the presence of bound LPS and the large loops of OmpC on the outer surface. Removal of bound LPS for obtaining better diffracting crystals gave insights into the role of LPS on the stability of OmpC. Our studies on the folding and stability of *S. typhi* OmpC with native, refined and refolded protein using circular dichroism, gel electrophoresis and monoclonal antibodies (mAbs) together suggest the possible role of LPS in modulating porin stability.

## Experimental procedures

### *Bacterial strains, culture conditions*

The bacterial strains that were used in this study[20] are *S. typhi* Ty21a (*galE* mutant) (produces aberrant LPS) and

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HB101/pVF27 (HB101 harbouring the plasmid pVF27). pVF27 (ref. 21) is the vector containing *ompC* gene along with its regulatory elements.

### *Major OMP extraction and porin purification*

OmpC extractions and purification from *S. typhi* Ty21a and HB101/pVF27 were done according to the procedure reported by us[22]. 'Pure' preparations here refer to the OmpC extracted and purified from the crude membrane, containing free and bound LPS. 'Refined' OmpC was obtained by a series of buffer exchanges and ion exchange chromatography to remove free and bound form of LPS.

### *Refolded OmpC (rfOmpC)*

*S. typhi* OmpC was amplified from genomic DNA, cloned under T7 promoter of pET20 b (+) vector (Novagen) and expressed as cytoplasmic inclusion bodies in *E. coli* BL21 (DE3). OmpC was unfolded and refolded into detergent (Polyoxy ethylene N-lauryl ether, $C_{12}E_9$) micelles. Characterization of refolded OmpC with SDS PAGE, tryptic digestion, Western blot and CD analysis suggested that it is equivalent to native OmpC (data not shown).

### *LPS removal and estimation*

LPS estimation of both pure and refined preparation of Ty21a as well as rOmpC was carried out through Manukirthi Biogems (P) Ltd, Bangalore, India. Out of the three methods tried; chromogenic[23], gel clot and KDO[24] (2-keto-3 deoxyoctonate), the chromogenic method resulted in quantitative estimation of LPS. Overexpressed refolded porin (rfOmpC) does not have LPS[13–15] and this was confirmed for the rfOmpC using silver staining in SDS-PAGE.

### *Porin samples used for CD recording*

All purified proteins were in 50 mM sodium phosphate (pH 7.6) and 0.1% $C_{12}E_9$. Pure porin extracts were in 50 mM Tris-HCl (pH 7.7), 0.25% SDS, 5 mM EDTA, 0.4 M NaCl and 3 mM $NaN_3$ and were diluted in buffer containing 50 mM sodium phosphate (pH 7.6) and 0.1% $C_{12}E_9$.

### *Protein concentration used for CD*

Pure Ty21a OmpC: 30 μg/ml (for both λ and T-scan), 160 μg/ml (boiled and cooled for λ scan). Refined Ty21a OmpC: 40 μg/ml (for λ scan), 30 μg/ml (for T-scan), 140 μg/ml (boiled and cooled for λ scan). rOmpC (pure and crude): 30 μg/ml (for both λ and T-scan), 150 μg/ml (boiled and cooled for λ scan). rfOmpC: 50 μg/ml (for both λ and T-scan).

### *Circular dichroism spectra recording*

The far UV spectra were recorded using JASCO spectropolarimeter J-715 version attached with peltier type temperature control system (PTC 348 WI), at Molecular Biophysics Unit, Indian Institute of Science, Bangalore, India. Cuvette with 1 cm path length was used for wavelength and temperature scans. Spectra for boiled and cooled samples were recorded using a 0.2 cm cuvette.

### *Wavelength scans*

Recordings were done from 250 to 205 nm, by stepwise increase in the temperature (unfolding) and stepwise decrease in the temperature (refolding) without taking the samples out of the sample cuvette. The parameters used for wavelength scan recording were: Resolution 0.1 nm, Bandwidth 1.0 nm, Sensitivity 20 mdeg, Response 4 s, Speed 10 nm/min and Accumulation 4.

### *Temperature scan*

For all porin samples spectra were recorded from 25°C up to 90°C at 218 nm. The upper limit for the temperature scan was chosen based upon the melting point of sample used. The parameters used were: Resolution 0.2 nm, Wavelength 218 nm, Tem slope 60°C/h, Bandwidth 2.0 nm, Sensitivity 10 mdeg, Response 4 s. The CD values are expressed in deg $cm^2$ $dmol^{-1}$.

### *Western and dot blots*

Protein from SDS–PAGE (10%) and urea-SDS–PAGE were transferred to PVDF membrane (Immobilon-P 0.45 μm pore size, Millipore) using a semi dry blotting apparatus (Bio-Rad). The transfer was carried out at 100 mA for 1 h in case of transfer from SDS-PAGE and for 1 h 30 min in the case of transfer from urea-SDS-PAGE. Other procedures were as per standard protocols.

## Results

### *Porin extraction and purification*

On SDS–PAGE, boiled samples of Ty21a OmpC show three isoforms with molecular weights ~36, ~37, ~47 kDa due to the presence of LPS (Figure 1). In order to express *S. typhi* OmpC in low/uniform LPS background, *S. typhi*

OmpC was expressed in *E. coli* HB101/pVF27 (rOmpC) and purified using the same method that was used for Ty21a OmpC. Boiled sample of rOmpC showed single band corresponding to ~37 kDa of *S. typhi* Ty21a extract on SDS–PAGE suggesting the lack of heterogeneity in the bound LPS. In order to remove the free and bound form of LPS from crude preparations, porin samples were buffer exchanged and passed through Q Sepharose ion exchange columns. LPS removal was more effective in the case of rOmpC than native OmpC. (Table 1).

### *rfOmpC*

In order to examine the stability of OmpC in the total absence of bound LPS, *S. typhi* OmpC was over expressed as cytoplasmic inclusion bodies. After several trials with different methods, a simple and economic refolding procedure was achieved by diluting 4M urea unfolded OmpC into a buffer containing 0.2% $C_{12}E_9$ and 10% glycerol. Around 10–20% of the refolded OmpC binds to Q Sepharose ion exchange column forming a stable trimer. Like the native OmpC, rfOmpC is SDS and protease resistant. rfOmpC shows CD spectrum similar to that of native Ty21a OmpC. Reaction with mAbs suggests correct refolding (data not shown).

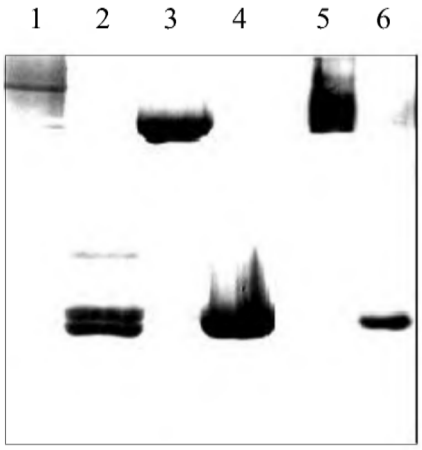


**Figure 1.** 10% SDS–PAGE Lanes 1, 3 and 5 are unboiled samples. Lanes 2, 4 and 6 are boiled samples. Lanes 1 and 2 are Native Ty21a OmpC. Lanes 3 and 4 are refolded OmpC (rfOmpC). Lanes 5 and 6 are pure recombinant OmpC (rOmpC). Reduced anomalous mobility is seen with decreased LPS.

**Table 1.** LPS estimation of both pure and refined preparation of Ty21a as well as rOmpC was carried out by the chromogenic method and resulted in quantitative estimation of LPS given by EU/mg of protein

| Sample | Protein μg/ml | LPS EU/ml | LPS EU/mg of protein |
|---|---|---|---|
| *S. typhi* Ty21a OmpC (pure) | 1 | 93.5 | 93.5 |
| *S. typhi* Ty21a OmpC (refined) | 1 | 77.5 | 77.5 |
| rOmpC (pure) | 0.8 | 48.5 | 61 |
| rOmpC (refined) | 1 | 17.5 | 17.5 |

### *Thermal stability and unfolding of porin*

Wavelength scan at 25°C is indicative of porin enriched with β-strand structure (indicated by $[\theta]_{218}$). In the case of pure and refined Ty21a OmpC, there was no change in the spectra up to 60°C. A significant decrease in β-structure was observed at 80°C and complete collapse of β-structure was noted at 95°C. In the case of pure rOmpC, collapse of β-structure was noticed at 80°C whereas β-structure of refined rOmpC completely collapses at 65°C itself, which is much lower than corresponding Ty21a preparation.

### *Stepwise refolding of unfolded porin*

Stepwise refolding of the porin samples was monitored at different temperatures, after the sample was completely unfolded at 95°C (pure and refined Ty21a OmpC), 85°C (pure rOmpC) and 70°C (refined rOmpC). Spectra were recorded immediately after complete unfolding. The refolding spectra recorded show that the complete refolding of thermal denatured porin is not possible. However, irrespective of the nature of the preparation and presence of LPS and other membrane components in the samples, there is some amount of β-structure regained after 40°C. It is clear that temperature-induced unfolding is not a stepwise reversible process in the presence of non-ionic detergents like $C_{12}E_9$.

### *Thermal transition of β-structure of porin*

The variation of $[\theta]^{T}_{218}/[\theta]^{25}_{218}$ analysed is indicative of the relative change from β-structure at 25°C (Figure 2). Pure Ty21a OmpC shows a very sharp and sudden transition at 79°C whereas refined Ty21a OmpC shows a sharp and sudden transition at 77°C. In both the cases, irrespective of the concentration, the presence of SDS shifted the transition between 60 and 71°C and made it broader. Similar observation was made with the wavelength scan for the samples boiled and cooled in the presence of similar amounts of SDS (0.5 and 5%). Pure rOmpC shows gradual structural transition from 50 to 80°C and relatively sudden transition thereon. The structural transition of refined rOmpC on the other hand started at 62°C itself and was not as sharp as that of refined and pure Ty21a OmpC. Presence of SDS resulted in similar transition regions for the pure Ty21a OmpC and rOmpC, possibly

suggesting the presence of a stable intermediate state in the presence of SDS. When compared to pure and refined preparations of OmpC from native Ty21a and rOmpC, rfOmpC shows much lower thermal stability. A gradual structural transition starts at 60°C itself and structure collapses thereon. It is evident from the temperature scans that thermal stability is dependent on the amount of bound LPS.

### *Use of MAbs to understand porin folding and stability*

The conformation-specific mAbs, P7D8 (Enterobacterial specific) and MPN5 (*Salmonella* specific), were used to understand structural organization, unfolding and refolding of porin. Porin samples were heat denatured and treated in different ways and then blotted directly on to NC membrane in order to understand the refolding of the trimer and the stability of the epitope and the trimer. The dot blot with P7D8 and MPN5 shows that the pure samples of Ty21a OmpC and rOmpC, that were boiled and cooled immediately, did not show any reaction since these samples contain 0.25% SDS. A very light reaction could be noted in the samples that were boiled and cooled at room temperature (slow cooling). Refined samples that were boiled for 5 min and cooled immediately at 4°C were recognized better than the samples that were boiled for 10 min. There was no reaction at all with the samples that were boiled twice before blotting. Moreover when boiled samples of refined Ty21a was recognized by P7D8, refined rOmpC was not recognized, suggesting the lower thermal stability of rOmpC. These results together suggest that stability of trimer is LPS-dependent and the ability to regain the epitopes on refolding depends upon the nature of detergent used.

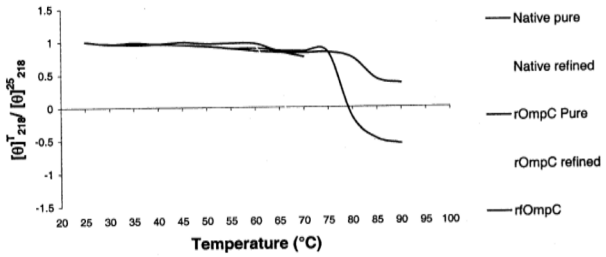


**Figure 2.** Temperature-induced transitions: The ratio of $[\theta]^{25}_{218}/[\theta]^{25}_{218}$ plotted here indicates the change (loss) in β-structure as temperature is increased. $[\theta]^{T}_{218}$ refers to the value of $[\theta]_{218}$ at increasing temperatures. $[\theta]^{25}_{218}$ refers to the value of $[\theta]_{218}$ at 25°C. Comparison of temperature-induced transition for pure and refined preparations of *S. typhi* Ty21a, pure and refined recombinant OmpC, and refolded OmpC in the presence of non-ionic detergent $C_{12}E_9$. Pure native (Dark green), Refined native (Light green), Pure recombinant (Dark brown), Refined recombinant (Light brown) and Refolded OmpC (Blue).

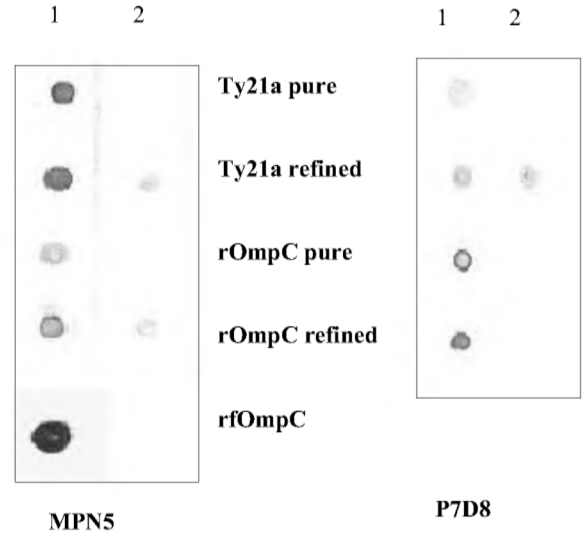


**Figure 3.** Dot blots with MAbs MPN5 and P7D8. Lane 1 is unboiled samples. Lane 2 is samples boiled for 5 min and cooled immediately (sudden cooling) and then kept at 4°C.

## Discussion

OmpC, being a membrane protein is possibly strongly associated with LPS. Though the nature of association is non-covalent, LPS cannot be removed completely by the regular methods of purification. Bound LPS interacts with porin and reinforces the stability of the trimeric structure. The course of purification of the membrane-extracted porin was shown to reduce the thermal stability of the major porin MspA from *M. smegmatis*[25] and the reason was attributed to the removal of lipids. Here we show that refinement of purified porin results in the removal of porin bound LPS and reduces its thermal stability.

*S. typhi* OmpC preparations from different genetic backgrounds that differ in the type and the amount of bound LPS were analysed with CD, conformation-specific monoclonal antibodies and SDS–PAGE, to get insights into the role of LPS and environment on the folding and stability of OmpC.

### *Role of LPS in anomalous mobility*

Unboiled sample of the rfOmpC runs as a single sharp band as against the native OmpC, which runs as a ladder on SDS–PAGE. Even the boiled sample of refolded OmpC shows a single band as against three isoforms observed with the native OmpC under the same condition. The boiled sample of rOmpC, which is expressed in *E. coli* also runs as a single band on SDS–PAGE showing no anomalous mobility (Figure 1). This suggests that LPS is responsible for anomalous mobility of OmpC on SDS–PAGE.

### *Role of LPS on stability*

The protein preparations used contain only porin with bound or unbound form of LPS. There was reduction in the amount of LPS from pure to refined preparations due to the several steps of buffer exchange and also ion-exchange chromatography that gave refined OmpC (Table 1). rfOmpC obtained from cytoplasmic inclusion bodies is unlikely to have any LPS bound to it[14]. From the stepwise unfolding and temperature scan, it can be seen that rfOmpC having no bound LPS showed structure collapse at 60°C itself. Refined rOmpC having very low amount of LPS exhibited complete loss of β-structure at 65°C. In contrast to these, pure rOmpC and the Ty21a OmpC show no significant structural perturbation up to ~77°C. From this analysis, it is seen that stability of porin decreases with reduction in the amount of LPS. Addition of exogenous LPS to the rfOmpC should increase its thermal stability, but this is yet to be demonstrated. This would give an additional support to prove the role of LPS on stability of OmpC. In the case of *E. coli* OmpF it was shown that addition of exogenous LPS and membrane fractions induced *in vitro* trimerization and native conformation[5]. The binding strength of LPS to OmpC is influenced by the kind of LPS and in turn its genetic background. This is evident from the fact that removal of LPS from rOmpC was more efficient than from native Ty21a OmpC (Table 1). The loss in β-structure in the pure preparation of both Ty21a and rOmpC is comparatively less when compared to their corresponding refined preparations. This is indicative of broader transition zone during unfolding of pure preparations as compared to refined samples. Nevertheless the complete transition (end) point seems to be similar in all the preparations.

### *Role of LPS and detergents on refolding*

Stepwise unfolding and refolding studies and temperature jump refolding studies in conjunction with CD analysis reveal that proper refolding of porin cannot be achieved by reducing the temperature either suddenly or in a stepwise manner. Additionally the refolding pathway is influenced by the amount of LPS and the nature of detergent used. Presence of SDS in the boiled and cooled samples possibly did not allow the porin to fold back into native structure but could have induced a coil structure. Even in the presence of $C_{12}E_9$, refolded OmpC gained α-helical structure although it is predominantly β structure. It is possible that irrespective of the type of detergent used, the unfolded porin recovers to the folded β-structure through an α-helical intermediate. This observation is in agreement with the result obtained with folding studies on *E. coli* OmpF with β-OG[12].

OmpC preparations differing in the amount of LPS were subjected to thermal and urea denaturation and probed with mAbs. The ability of these mAbs to react with different oligomeric forms is taken advantage of to follow the conformational changes in the refolding of denatured OmpC[26–30]. Dot blot results show that samples boiled for short time (5 min) and immediately cooled to 4°C were recognized by MPN5 in the case of refined Ty21a OmpC. Under the same conditions, refined rOmpC having lower amount of LPS relatively was not recognized by P7D8 but with MPN5. This suggests that the loss of oligomeric structure, evidenced by the lack of binding of the mAb P7D8, is due to the reduction in the amount of LPS. Samples boiled for 10 min and beyond could not refold and were not recognized by both the mAbs. Pure preparations of Ty21a OmpC and rOmpC on thermal unfolding and cooling did not react with both the mAbs possibly as they were in a buffer containing 0.25% SDS, suggesting the effect of ionic detergent on refolding of thermally denatured OmpC.

Refolding in the presence of ionic detergent, SDS, was not successful as it led to aggregation. Refolding of urea unfolded OmpC from inclusion bodies was achieved in the presence of non-ionic detergent ($C_{12}E_9$) alone. In this method of refolding, majority of the refolded OmpC forms a metastable trimer as it is susceptible to proteolysis and is also broken in the presence of 2% SDS as is seen on SDS PAGE (data not shown). However the unbound fraction is recognized by Salmonella porin specific mAb MPN5. Refolding in the absence of LPS and any other membrane components thus leads to a minor fraction of correctly refolded and stable rfOmpC whereas the major fraction is the formation of an altered conformation which is sensitive to SDS and proteolysis suggesting an unstable trimer. This could be a trapped folding intermediate forming a metastable trimer. This kind of folding intermediate is detected in *in vivo* pulse-chase experiments on *E. coli* PhoE[31] and trimerization of *in vitro* synthesized *E. coli* OmpF[5]. This hypothesis is also supported by other studies[12] that boiled and immediately cooled samples with LPS association show almost recovered β-structure. Moreover, the molecular weight of renatured porin, showing recovered β-structure, was similar to the folded intact trimer. This is referred to as 'reconstituted trimer with altered subunit contacts' associated/held in the detergent micelle. This trimer was susceptible to SDS and proteolytic cleavage.

### *Effect of environment on folding and stability*

Our results suggest that LPS modulates only the porin stability. The folding of *in vitro* synthesized porin[5] into a stable, SDS and protease-resistant trimer conformation suggests that porin folding does not require presence or addition of membrane components but simple non-ionic detergents. This again shows that the detergent induces the folding and the ability to take up the β-structure is an

intrinsic property associated with the porin primary structure itself[9]. The report on refolding and proper assembly of *P. aeruginosa* porin[11] also suggests that non-ionic detergents play a key role in folding and unfolding of porins. Wimley[3] confirms that it is the hydrophobic 'environment' that induces the secondary structure, β-sheet, and not necessarily the individual membrane components. It was also shown that porin inclusion bodies could be perfectly refolded in the presence of non-ionic detergents to give a 3D structure that is exactly the same as that of native porin[14].

In the case of *in vitro* refolding, it could be that the detergent environment forces the unfolded porins to fold back into trimers[12]. Since the presence of chemotropic anionic detergent SDS reduced the trimer stability, it is possible that the non-ionic detergent may mimic the natural membrane environment better and is a more suitable medium to carry out functional studies. It is also possible that the non-ionic detergent, upon boiling, loses its native micelle character and exchange with fresh detergent, on cooling, might help the unfolded protein to gain the native structure to an extent. However, this needs further investigation. Porin folding/refolding is influenced by the hydrophobic environment, as also shown in this study with the presence of ionic (SDS) and non-ionic detergent ($C_{12}E_9$). It is likely that the message to take up the β-structure possibly resides in the sequence[32] but proper folding is assisted or induced by the hydrophobic environment. The hydrophobic environment might force the unfolded protein not to take up any other conformation but favour the retention of the β-structure even after treatment at extreme conditions.

The crystal structure determination of OmpC, OmpC–Fab complexes which are being pursued in our laboratory, along with studies on site directed mutagenesis and deletion analysis based on the available homology model of *S. typhi* OmpC[33], should enable the stability and folding aspects to be examined in a more detailed atomic level.


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