CHAPTER IV

YSPC: A UNIQUE TRANSLOCATOR EXHIBITS STRUCTURAL ALTERATION IN THE COMPLEX FORM WITH CHAPERONE SYCB
4. YSPC: A UNIQUE TRANSLOCATOR EXHIBITS STRUCTURAL ALTERATION IN THE COMPLEX FORM WITH CHAPERONE SYCB

4.1. Background

In the syc-ysp operon of Ysa-Ysp TTSS of Yersinia enterocolitica biovar 1B, it is observed that SycB is a class II chaperone interacting with its cognate major translocator YspB (and also stabilizing it) and minor translocator YspC. Shigella flexneri secreted YspC but complementation of IpaC could not be done by YspC. Similarly, Ysc injectisome of Y. enterocolitica is capable of secreting both YspB and YspC, but again they cannot function as a substitute for YopB and YopD, because, they cannot form pores in the host cell membrane [Foulquier B et al., 2003]. In our previous study, we have shown that chaperone SycB localizes in the inclusion bodies when expressed recombinantly, but it gets solubilised in the presence of YspC. Individual YspC, when purified recombinantly, shows high solubility, yield and stability and forms a 1:1 complex with SycB. At pH-5.0, SycB releases YspC [Basu A et al., 2012]. With this background, we have proceeded with a study dealing with the translocator YspC and the complex formed by chaperone SycB with YspC. This is the first work concerned with characterization of YspC and YspC-SycB complex by using various biophysical techniques like far UV and near UV CD spectroscopy, intrinsic and extrinsic fluorescence, fluorescence quenching and FRET. Binding of different truncated forms of SycB with YspC has been analyzed and subsequently, the interaction domain of SycB for YspC was also described.

4.2. Materials and Methods

4.2.1. ConSurf Server, Multiple Sequence Alignment and Secondary Structure Prediction

ConSurf server used for the determination of conserved regions in proteins is available at http://consurf.tau.ac.il/ [Ashkenazy H, et al., 2010]. The protein sequence was submitted directly
in the FASTA format instead of uploading a MSA file. The minimal % ID for homologues was reduced to 30% from 35% and rest of the parameters were chosen as default. The phylogenetic tree was generated by ConSurf, and the YspC query sequence was coloured according to the conservation scores. MSA for YspC was done with other group of minor translocators belonging to various Gram negative bacteria, using MultAlin interface [Corpet F, 1988]. Secondary structure prediction servers viz, PSIPRED [Jones DT et al., 1999], Jpred [Cole C et al., 2008], DSC, MLRC, PHD and consensus server [Gupta A et al., 2009] were used to determine secondary structure of YspC.

4.2.2. **Expression and Purification of YspC, YspC-SycB, YspC-ΔSycB\(_{1-114}\) and YspC-ΔSycB\(_{36-114}\)**

Cloning of yspC, sycB, ΔsycB\(_{1-114}\), ΔsycB\(_{36-114}\) were done using pET28a(+), pAcYcDuet as vectors and *Escherichia coli* Top10 as cloning strain. The expression of the individual proteins was checked using expression strain *E. coli* BL21(DE3). The expression vectors (with the desired constructs) used in this study were exactly similar to that used in Chapter III [Basu A et al., 2012]. Detailed procedure and list of primers used in the study were provided in chapter III. pET28a(+)-sycB, pET28a(+)-ΔsycB\(_{1-114}\), pET28a(+)-ΔsycB\(_{36-114}\) were co-transformed with pAcYcDuet-yspC in *E. coli* BL21(DE3). So, that the protein complexes formed between different forms of SycB and YspC, will have histidine tag with SycB; and YspC will be produced without a tag. As mentioned previously, YspC and YspC-SycB complex were soluble at 37°C when induced with 1 mM IPTG in LB supplemented with kanamycin and kanamycin + chloramphenicol [Basu A et al., 2012]. However, YspC-ΔSycB\(_{1-114}\) and YspC-ΔSycB\(_{36-114}\) localised in the inclusion bodies when induced with 1 mM IPTG at 37°C in LB. Further optimization was done by varying the temperature and the IPTG concentration to solubilise the
protein. Finally, YspC-ΔSycB\textsubscript{(1–114)} and YspC-ΔSycB\textsubscript{(36–114)} could be solubilised at 25°C, when induced with 1 mM and the culture was done in TB (see Table 4.1).

All the proteins were purified using Ni–NTA metal affinity chromatography using standard set of buffers (list of buffers used is given in Table 4.2). Induced E. coli BL21(DE3) cells containing YspC, YspC-SycB, YspC-ΔSycB\textsubscript{(1–114)} and YspC-ΔSycB\textsubscript{(36–114)} were dissolved in Buffer A in presence of 2 mM Phenylmethanesulfonyl fluoride (PMSF) and sonicated. The whole cell sonicated lysate was centrifuged at 18,000g for 30 min to isolate the supernatant from the pellet fraction. Ni–NTA column was equilibrated with Buffer B and the supernatant obtained was loaded into the column. Non-specific proteins attached to the column were washed by Buffer C and finally, the bound proteins were eluted by Buffer D. Proteins obtained during elution were analyzed by SDS PAGE and dialyzed against Buffer E for further downstream work.

4.2.3 Size Exclusion Chromatography

Proteins were initially purified by Ni-NTA affinity chromatography and further dialyzed in SEC buffer [25 mM Tris–HCl pH-7.2, 150 mM NaCl, 1 mM EDTA]. HiLoad 16/60 Superdex 200 pg column (G.E. Healthcare) was equilibrated by SEC buffer. YspC was also dialyzed in 25 mM sodium acetate pH-5.0, 150 mM NaCl, 1 mM EDTA and the SEC column was subsequently, and equilibrated in the same buffer. The proteins were loaded into the SEC column at 2 mg/ml concentration and throughout a flow rate of 1 ml/min were maintained. For calibration of the SEC column, following gel filtration markers was used (molecular weight and corresponding elution volume is indicated): Ferritin (440 kDa ~51 ml), Aldolase (158 kDa
~62 ml), Ovalbumin (43 kDa ~76 ml), Carbonic anhydrase (29 kDa ~83 ml), RibonucleaseA (13.7 kDa ~92 ml). The SEC profile was analyzed by AKTAprime software.

### 4.2.4. Far UV and Near UV CD

For the CD experiments YspC and YspC-SycB were dialyzed against 20 mM potassium phosphate and 50 mM NaCl buffer of pH-7.2, 6.0, 5.0, 4.0. Jasco J-815 spectrophotometer was used for collection of far UV, near UV CD spectra and thermal denaturation curve. Far UV CD spectra were recorded from 250 to 190 nm, using 0.1 cm pathlength cuvette with a protein concentration of 5 µM. Similarly, near UV CD spectra were acquired in the wavelength range 300–250 nm with a protein concentration of 25–30 µM using a cuvette of 1 cm pathlength. All the spectra were taken at 20°C with a scan speed of 10 nm/min. Each spectrum was an average of 5 scans with corresponding buffer spectrum used for baseline correction. For each CD spectrum mean residue ellipticity [θ] (degcm²dmol⁻¹) was plotted against wavelength (nm). Thermal denaturation curve was obtained from 10 to 90°C with a temperature increase of 2°C/interval, by plotting [θ] at 222 nm against the temperature. Similar settings like far UV CD were used for the thermal denaturation curve. Percentage of different secondary structural element within the protein was calculated by Dichroweb server [Whitmore L & Wallace BA, 2004].

### 4.2.5. Proteolytic Digestion

Digestion of YspC, SycB and YspC-SycB was carried using trypsin from Proti-Ace kit of Hampton research. Trypsin stock was diluted to 0.1 mg/ml with the buffer provided in the Kit. For 10 mg/ml of protein, 0.01 mg/ml trypsin was added in equal volume for digestion. After
specific time points, the reaction was stopped by addition of SDS PAGE sample lysis buffer and proteolytic digestion products were analysed by SDS PAGE.

4.2.6. ANS Fluorescence

YspC and YspC-SycB were dialyzed in different buffers like 25 mM Tris HCl pH-7.2 + 50 mM NaCl, and 20 mM Sodium citrate (pH-6.0, 5.0, 4.0) + 50 mM NaCl. ANS was also solubilised in different buffers and its concentration was measured from the optical density obtained at 350 nm (molar extinction coefficient of 4,950 cm$^{-1}$M$^{-1}$). Jasco FP-6500 fluorimeter was used to measure the ANS emission spectrum from 400 to 600 nm with a scan speed of 50 nm/min. The excitation wavelength was set at 350 nm and 1 cm path length cuvette was used for measurement. Further, 4 µM protein and up to 40 µM ANS was incubated and 3 accumulations for each spectrum were monitored from 400 to 600 nm. The corresponding buffer spectrum was subtracted in each case.

4.2.7. Tyrosine Fluorescence

Intrinsic tyrosine fluorescence was determined for YspC and YspC-SycB. The proteins were dialyzed in the aforesaid buffers (mentioned in case of ANS fluorescence). Phosphate buffer quenches tyrosine fluorescence, so, it could not be used [Chen RF & Cohen PF, 1966]. When excited at 275 nm, the emission spectrum was monitored from 280 to 400 nm with a scan speed of 50 nm/min. 4 µM protein and 1 cm pathlength cuvette was used for experimental purpose. To determine the ANS quenching of tyrosine fluorescence, ANS was added up to a concentration of 40 µM. Again excitation wavelength was set at 275 nm and emission spectrum was scanned from 280 to 540 nm. From each protein and ANS spectrum the corresponding buffer spectrum was subtracted.
4.3. Results

YspC, a 444 amino acid residues long protein of molecular weight 48255.3 Da, has a theoretical pI of 6.12. It is a highly soluble translocator protein. It could be purified by metal affinity chromatography followed by SEC. It possesses a single transmembrane region from residue 241–265 as predicted by TMHMM [Krogh A et al., 2001]. YspC was purified by Ni–NTA affinity chromatography using histidine tag at the N-terminus, so, recombinant histidine tag YspC has a molecular weight of ~50.5 kDa.

4.3.1. YspC Shows Significant Variation from Other Minor Translocators

ConSurf is a bioinformatic tool or package for analyzing the evolutionary relationship among different protein or nucleic acid sequences [Ashkenazy H et al., 2010]. When the primary sequence of YspC was analysed by ConSurf, five different protein (other than input sequence) sequences were retrieved from BLAST analysis (BLAST E value -0.0001 -default parameter). UNIREF90 database was used for the process and to avoid repetition of same sequence, the maximal identity percentage was kept at 95%; while the minimal identity percentage was reduced from default 35% to 30%, to obtain a significant number of homologues. Proteins retrieved from BLAST analysis belong to Yersinia sp and Providentia sp (with identities ranging from 25% [UniRef90_D4C1Y7] to 94% [UniRef90_A1JQ85]), and they are members of the IpaC/SipC family (pfam-PF09599). These sequences were then subjected to MSA with YspC (input sequence) using MAFT, integrated within the ConSurf package. Phylogenetic tree was generated revealing the phylogenetic distances between these proteins (Figure 4.1). In Consurf, for generation of phylogenetic tree, neighbour joining method was used and their exact distances were calculated in the Newick format. Figure 4.2 shows the sequence conservation
analysis of YspC by ConSurf, primarily highlighting the significant conserved residues. The colour grade given in the Figure 4.2 in ConSurf, depicts the extent of conservation of amino acids at specific positions. From Figure 4.2, it was clear that YspC has conservation in and around the transmembrane region (201–288), and in part of the C-terminus region (366–413). Phylogenetic analysis revealed that YspC and A1JQ85 (YspC variant) are the only two sequences with a recent common ancestor in comparison to others (YspC distance 0.02398, A1JQ85 distance 0.03020). Other members in the phylogenetic tree are distantly related to YspC, hence, they have significant variation in their protein sequence in comparison to YspC, as inferred from their corresponding distances (details are given in Figure 4.1). Minor translocator proteins like IpaC, SipC belonging to the IpaC/SipC family show sufficient sequence conservation amongst them. Although YspC belongs to this family of translocators, it shows little homology in sequence to the proteins of IpaC/SipC family as determined from MSA. Similarly, when a different group of minor translocators like AopD, PopD and YopD were aligned using Multalin server, they show significant homology amongst them; again they exhibit little homology to YspC (Figure 4.3). Therefore, YspC stands alone not possessing homology to the other five common minor translocators belonging to other gram negative bacterium, confirming its unique nature. Thus, YspC seems to evolve differently from its homologues and probably this accounts for its uniqueness among the minor translocators.

YspC and YspC-SycB were purified by a two step purification process. Metal affinity chromatography purification was followed by a SEC. When YspC was isolated from YspC-SycB complex, it eluted at a volume corresponding to 72.5 kDa in superdex column (YspC monomer is ~48.3 kDa) and no higher order oligomeric form of YspC was seen [Basu A et al., 2012]. However, when expressed and purified in isolation, at pH-7.2, two peaks were observed
in SEC profile of YspC. The 1\textsuperscript{st} peak eluting with a maximum corresponding to a molecular weight of \(~432\) kDa, followed by a 2\textsuperscript{nd} peak eluting at a volume corresponding to \(~73\) kDa, whereas histidine tag YspC is 50.5 kDa (Figure 4.4a). In Figure 4.4a, the 1\textsuperscript{st} peak of YspC\textsubscript{(oligomer)} has lesser YspC concentration, when compared to 2\textsuperscript{nd} peak of YspC\textsubscript{(monomer)} in spite of comparable peak heights. This is in compliance with the fact that proteins are quantified during SEC by their absorbance at 280 nm, where, oligomers or soluble aggregates of protein often show much higher absorbance compared to the monomeric form due to the light scattering at 280 nm [Nomine Y \textit{et al.}, 2001]. At pH-5.0, however, we could not see any oligomeric peak, instead, we observed YspC eluting at a volume corresponding to \(~74\) kDa (Figure 4.4b), quite similar to the profile of YspC, released from SycB at pH-5.0 [Basu A \textit{et al.}, 2012].

4.3.2. The Secondary Structural Elements in YspC are Moulded Differently While Forming Complex with SycB

Bioinformatic predictions regarding the secondary structural content of YspC were done by Jpred, PSIPRED and consensus server for secondary structure prediction. Jpred and PSIPRED predicted that YspC possesses 66.66\% and 70.94 \% of \(\alpha\)-helix, 6.3\% and 0.004 \% of \(\beta\)-sheet, respectively, rest of the structures are random coil. Consensus server for secondary structure prediction used its various algorithms to predict that YspC possesses 67.57\% helix, 3.38\% strand and rest, random coil structure. To compare the bioinformatic predictions with the secondary structural elements present in the solution state, we have used far-UV CD spectroscopy to determine the secondary structural elements of YspC and YspC-SycB complex. Far UV CD spectra were deconvoluted by Dichroweb server [Whitmore L & Wallace BA, 2004]. Table 4.3 shows the estimated values of various structural forms of YspC and YspC-SycB complex. At pH 7.2, YspC possesses 51.2\% \(\alpha\)-helix, 16.5\% \(\beta\)-sheet structure. However, in
the complex form with SycB, YspC-SycB shows 67.8% α-helicity, 3.9% β-sheet (Figure 4.5a).
The summation of individual YspC and SycB CD spectra at pH-7.2, shows an α-helicity of
56.8%, 10.8% β-sheet structure and 32.4% other structure (including random coils and turns)
[Basu A et al., 2012]. Also, we could observe high amount of β-sheet experimentally when
compared to the estimates of secondary structure prediction server.

Figure 4.5a shows, at pH-7.2 and 6.0, the secondary structure of YspC and YspC-SycB complex
exhibits less variation. However, in case of SycB, a more significant variation in secondary
structure was seen between pH-7.2 and 6.0 [Basu A et al., 2012]. YspC shows significantly less
and irregular variations in secondary structural content with changes in pH as shown in the
Table 4.3, but at all pHs there is some amount of β-sheet structure present in YspC. In contrast,
YspC-SycB shows a gradual decrease in α-helicity, with simultaneous increase in random coil
structure with the reduction in pH from 6.0 to 4.0.

In case of YspC-SycB complex, the CD spectra at pH-5.0 and 4.0 are almost equivalent to the
summed CD spectra of individual YspC and SycB as seen from Table 4.3 and reference [Basu
A et al., 2012]. At pH-4.0, YspC-SycB still shows an α-helicity of 34.1%, this is mainly due to
the helicity of YspC; since SycB is almost unfolded at pH-4.0. It is to be noted that YspC gets
released from SycB at pH-5.0 [Basu A et al., 2012].

4.3.3. YspC Shows Tertiary Structure Signal Unique for a Translocator Protein

Near UV CD (300-250 nm) is known to show signals in the 270-290 nm region typically
originating from tyrosines and 4 those in the 280-300 nm region from tryptophan, and signals in
the 250-270 nm regions are attributed to phenylalanine. These general facts were used to study
the tertiary folding of the aforesaid proteins/complexes. YspC possesses 4 tyrosines and 10
phenylalanines, whereas SycB contains 14 tyrosines and 10 phenylalanines. Both the proteins
lack tryptophan. So, YspC-SycB complex would possess 18 tyrosines and 20 phenylalanines. At pH-7.2, both YspC and YspC-SycB displayed defined tertiary structure, as signal seen from near UV CD spectroscopy. The maximum of the CD spectrum for YspC was recorded at 270 nm, whereas YspC-SycB shows a maximum at 272 nm (Figure 4.5b). At pH-5.0, YspC still possesses some tertiary structure with decreased signal intensity.

4.3.4. **YspC Exhibits High Tm and Cooperative Melting Behaviour Which Changes in the Complex Form**

Due to the α-helical nature of the proteins, ellipticity values at 222 nm were plotted against temperature to obtain the thermal denaturation curve. As we have seen in case of YspC from the far UV CD spectroscopy that the secondary structural change is insignificant between pH-7.2 and 5.0. So, YspC exhibits a Tm of ~51°C at pH-7.2 and that slightly reduces to ~50°C at pH-5.0. The sigmoidal thermal curve depicts a cooperative melting behaviour. YspC-SycB also shows a comparable melting temperature (Tm ~53°C) at pH-7.2 (Figure 4.5c). Similar, range of melting temperatures were observed for PopD-PcrH (~55°C) [Schoehn G et al., 2003], IpaC-IpgC (~56.3°C) [Birket SE et al., 2007], AopD-AcrH (~57.7°C) [Tan YW et al., 2009]. Interestingly, YspC and SycB individually have cooperative melting behaviours at pH-7.2. But the thermal denaturation curve of YspC-SycB complex at pH-7.2 did not show a proper sigmoidal nature.

4.3.5. **Proteolytic Digestion Bears the Signature of Modified Structural Folds of YspC When in the Complex with SycB**

Proteolytic digestion of YspC, SycB and YspC-SycB was carried out for different time duration, using trypsin. It was seen that YspC, when digested for 30 minutes, produced three bands around 26 kDa MW protein marker. With increase in digestion time, two bands
disappeared and at about 150 minutes a stable band was found to remain just below 26 kDa. Prominent stable bands around 17, 14 and 12 kDa, were also seen throughout the digestion period from 30 to 150 minutes (Figure 4.6a). The exact masses of 14 and 12 kDa bands could be characterized by mass spectrometry. The masses of these two bands are 14550.4297 Da and 12638.7012 Da, respectively (Figure 4.7). On the other hand, similar digestion of YspC-SycB shows a stable band of YspC below 43 kDa in the time course starting from 30 to 150 minutes. SycB in the complex form shows two stable bands above 17 kDa and just below the undigested SycB, from 60 to 150 minutes (Figure 4.6b). Individual SycB also shows two stable digestion bands just below the undigested SycB from 30 to 150 minutes. A 3rd band appears at 17 kDa and becomes prominent at 150 minutes (Figure 4.6c). This difference in the digestion pattern of YspC and YspC-SycB support the differential moulding of YspC in individual and complex form. Similar observation was made in case of IpaC, forming a complex with IpgC in their corresponding trypsinolysis data [Birket SE et al., 2007]. Black arrows in Figure 4.6 represent the fragments obtained during digestion of YspC, YspC-SycB and SycB.

4.3.6. YspC in Individual and in Complex Form with SycB Exhibits Solvent Exposed Hydrophobic Domains

ANS is a hydrophobic dye, which can bind to the solvent exposed ordered hydrophobic surfaces in a protein molecule. When ANS was excited at 350 nm, it gave an emission maximum (λ\text{max}) at 525 nm. When YspC was incubated with ANS, the emission maximum shows a blue shift to 482 nm. At pH-7.2 and pH-6.0, YspC-ANS spectra are almost similar; only at pH-6.0, there is a slight increase in the emission intensity implying increased solvent exposure of hydrophobic regions (Figure 4.8a). In case of YspC-SycB complex, at pH-7.2 and 6.0, the ANS binding profiles are identical with λ\text{max} at 480 nm and similar emission intensity (Figure 4.8b).
So, YspC shows solvent exposed hydrophobic patches in individual and complex form. Below pH-6.0, we see a significant increase in the emission intensity of YspC, showing that more hydrophobic patches were getting solvent exposed (Figure 4.8a).

4.3.7. Binding of YspC Masks the Tyrosine Residues in the Two Predicted TPR Regions of SycB

Individually SycB possesses 14 tyrosine residues and YspC possesses 4 tyrosines. So, YspC-SycB complex altogether contains 18 tyrosine residues. Tyrosine fluorescence of YspC and YspC-SycB were measured in a range of pH-7.2–4.0. When excitation was done at 275 nm, an emission maximum (λ_{max}) at 304 nm was observed for YspC and YspC-SycB shows a λ_{max} at 305 nm. In both cases, the tyrosine fluorescence did not show any significant change with reduction in pH. Unlike tryptophan fluorescence, tyrosine fluorescence is less affected by the change in pH [Guzow K et al., 2002]. When ANS is incubated with YspC and YspC-SycB, and excitation was done at 275 nm, the emission maximum intensity (304 nm for YspC and 305 nm YspC-SycB) reduced and a red shift was observed in the emission spectra with a new emission maximum arising at ~480 nm at pH-7.2 (Figure 4.8c & 4.8d). The new emission maximum corresponds to the ANS-protein complex. So, ANS is binding within the FRET distance of tyrosine and quenching its fluorescence [Murali J & Jayakumar R, 2005]. We could determine the FRET efficiency, which is a measure of ANS quenching of tyrosine fluorescence. The FRET efficiency is calculated as the ratio of F₀ and F, where F₀ corresponds to the fluorescence of tyrosine residues in absence of a quencher and F is the tyrosine fluorescence in presence of ANS-quencher. Also, the number of tyrosine multiplied by the reciprocal of FRET efficiency would give us an indirect estimate of the buried tyrosines.
At pH-7.2, a FRET efficiency of 1.39 was seen for YspC and 1.14 for YspC-SycB, which indirectly indicated that 1 tyrosine in case of YspC and 2 tyrosines in YspC-SycB are exposed; hence, ANS could bind in its vicinity and quench the fluorescence. However, at pH-7.2, SycB shows a FRET efficiency of 1.65 and also the corresponding homology model proposed that 5 or more tyrosines are exposed to the solvent [Basu A et al., 2012]. So, within the complex the exposed tyrosine residues of SycB were masked by YspC. Since, the tyrosine residues are mainly localised in the two predicted TPR regions of SycB, the two TPRs of SycB may be involved in its interaction with YspC. At pH-6.0, YspC shows a FRET efficiency of 1.43 and YspC-SycB shows 1.16, both of which are comparable to the FRET efficiencies observed at the physiological pH-7.2. However, at pH-5.0 and 4.0, a high increase in FRET efficiency (1.97 and 2.18, respectively) and corresponding increase in ANS (λ\text{max}) was observed in YspC. This shows unfolding of tertiary structure, so, that tyrosine residues become easily accessible to ANS. However, SycB shows much higher FRET efficiencies compared to YspC at pH-5.0 and 4.0 [Basu A et al., 2012].

4.3.8. The Two TPR Regions of SycB Interacts with YspC Forming a Physiologically Altered Complex

As predicted from ANS fluorescence spectroscopy, tyrosine fluorescence and FRET, the accessible tyrosine residues in the two TPR regions of SycB are masked while it is forming a complex with YspC. To determine whether the two predicted TPR regions of SycB are sufficient for its interaction with YspC, we have designed two constructs; one containing the N-terminal helix and the two TPRs (ΔSycB\((1–114)\)) and the other containing only the two TPRs (ΔSycB\((36–114)\)). YspC-SycB complex was soluble at 37°C, when induced with 1 mM IPTG in LB [Basu A et al., 2012]. However, at similar condition YspC & ΔSycB\((1–114)\) and YspC & ΔSycB\((36–114)\)
both localized in the inclusion bodies. To solubilise the proteins, we reduced the temperature of induction and the IPTG concentration. We also used an enriched medium for cell growth. With further optimization, we could find that at 25°C, when induced with 1 mM IPTG in TB medium both YspC & ΔSycB_{(1–114)} and YspC & ΔSycB_{(36–114)} localized in the cell free supernatant. Co-expressed YspC & ΔSycB_{(1–114)} and YspC & ΔSycB_{(36–114)} formed complexes, which were purified by metal affinity chromatography and SEC (Figure 4.9a & 4.9b). It is interesting to note that lack of the TPR-like region and the C-terminal helix and coil alters the physiological localization of YspC-ΔSycB_{(1–114)} and YspC-ΔSycB_{(36–114)} within the host cell.

YspC formed a complex with ΔSycB_{(1–114)} which proves that either the N-terminal, or the two TPRs, or both, of SycB interacted with YspC. However, formation of a complex between YspC and ΔSycB_{(36–114)} confirmed that the two TPR region of SycB are sufficient for its interaction with YspC. Although the two TPRs of SycB showed very poor solubility when expressed in isolation, they formed complex with YspC [Basu et al., 2012]. Interestingly, both YspC-ΔSycB_{(1–114)} and YspC-ΔSycB_{(36–114)} eluted as higher order oligomeric peaks with the maxima in the molecular weight corresponding to ~453 and ~495 kDa, respectively (Figure 4.9a & 4.9b). YspC-SycB complex shows an oligomeric form and a heterodimeric form; this heterodimeric form was non-existent in YspC-ΔSycB_{(1–114)} and YspC-ΔSycB_{(36–114)} complex.

4.4. Discussion

Ysa-Ysp TTSS is an important system for virulence of \textit{Y. enterocolitica} biovar 1B and responsible for the gastro-intestinal phase of infection within the host. Ysa-Ysp TTSS is comparatively less explored than Ysc-Yop TTSS, which primarily overcomes the host immune response and results in the systemic phase of the infection [Cornelis GR \textit{et al.}, 1998; Cornelis
GR & Wolf-Watz H, 1997; Haller JC et al., 2000; Young GM, 2007]. syc-ysp Operon is a part of Ysa-Ysp system encoding proteins like SycB, YspB and YspC. It is shown that YspC is a secretory protein, possessing one transmembrane region and showing some homology to IpaC of S. flexneri. Therefore, it is predicted to have a translocon forming activity. Moreover, it interacts with its cognate class II chaperone SycB. So, YspC is annotated as a minor translocator protein [Foulter B et al., 2003; Matsumoto H & Young GM, 2006]. While we tried to determine the evolutionary relationship between YspC and other translocator proteins using ConSurf, from the phylogenetic tree, we could determine that YspC shows divergence from a unique node, when compared to its homologues. Some proteins belonging to the IpaC/SipC family from Yersinia sp and Providentia sp, are distantly related to YspC. Similarly, from the MSA, it is determined that YspC shows little homology to PopD, YopD, AopD group of minor translocator. IpaC and SipC, another group of minor translocators from Shigella and Salmonella, respectively, show high homology amongst them; but again they show little homology to YspC. From ConSurf analysis, it is determined that YspC possesses conservation in the transmembrane region and its surrounding sequences, and in the C-terminal region. However, strikingly less conservation is noticed in the N-terminal of YspC. Interestingly, it is seen that the N-terminal of IpaC is required for secretion purpose, PopD and YopD interacts with their chaperone through the N-terminal and also the N-terminal region of AopD gets protected by chaperone AcrH. For other group of translocators, conservation is observed throughout the length of the protein without any preference for a particular region. However, the C-terminus of YopD possesses a putative coiled coil domain; and an α-helical amphipathic domain, which is responsible for interaction of YopD with LcrV and formation of YopD
oligomers [Ashkenazy H et al., 2010; Costa TRD et al., 2010; Faudry E et al., 2007; Schoehn G et al., 2003; Tan YW et al., 2009; Tobias T et al., 2002].

During the course of purification of recombinant YspC, we noticed that solubility, stability and yield of YspC are very high. Contrary to this, other minor translocators like PopD localizes in the inclusion bodies, AopD can be solubilised, only in the presence of 2% TritonX-100 and shows non-specific heterogeneous aggregation during SEC [Faudry E et al., 2007; Schoehn G et al., 2003; Tan YW et al., 2009]. IpaC also localizes in the inclusion bodies at 37°C, when expressed recombinantly in *E. coli* [Picking WL et al., 1996]. During SEC, YspC shows a higher Stokes’ radius compared to a globular protein. However, YspC-SycB shows almost a globular nature, when subjected to SEC [Basu A et al., 2012]. So, YspC is undergoing some structural alteration, while forming a complex with SycB. Similarly, PopB is a molten globule, but forms an almost globular complex with PcrH, as seen from SEC profile. At pH-5.0, SEC profile of individual YspC and YspC isolated from SycB are similar, with the existence of a single monomeric form of YspC [Basu A et al., 2012].

From the far UV CD spectroscopy, we noticed formidable changes in secondary structural content of YspC, when it forms a complex with SycB (Table 4.3). In other words, strand, turns and random structures in YspC are transformed into organized helical form in the YspC-SycB complex. Similar, observation is made in case of IpaC too. Its secondary structural elements changes significantly, in complex form with IpgC [Birket SE et al., 2007]. At pH-7.2, YspC possesses a well defined tertiary structure, which is unlikely for a translocator protein, since, it needs to traverse through a 6-7 nm wide needle complex with a 2.5 nm wide hollow conduit [Hoiczyk E & Blobel G, 2001]. However, unfolding of translocator proteins occur, before their translocation through the injectisome.
At pH-5.0, YspC still possesses some tertiary structure with reduced signal intensity. The translocator proteins like PopB, PopD are shown to have molten globule conformation at all pH [Faudry E et al., 2007; Schoehn G et al., 2003]. Also, a fragment of YopD(150-287) from Y. pestis is partially unfolded in the native state [Raab R & Swietnicki W, 2008]. Therefore, in absence of its chaperone SycB, YspC can be highly soluble and maintain a stable structure. So, SycB binds to YspC and may restrict its translocation, unless some activation signal of TTSS is encountered. Interestingly, in a homologous operon in S. flexneri ipgc mutants show lower levels of IpaB and IpaC. Also, it is established that IpgC is required for the stability of IpaB and IpaC [Menard R et al., 1994]. YspC shows stable digestion fragments following trypsinolysis. However, the variation in the digestion pattern of YspC and YspC-SycB support the differential moulding of YspC in individual and complex form. Similar observation is made in case of IpaC from the corresponding trypsinolysis data. While IpaB forms a complex with IpgC, it is digested more slowly and forms two stable bands, when compared to the digestion of individual IpaB [Birket SE et al., 2007]. These stable fragments obtained after digestion of proteins have significant value for crystallization. Proteolysis of IpaB-IpgC complex has allowed identification of IpaB fragments, which proved useful for further crystallization purpose [Adam PR et al., 2012; Lunelli M et al., 2009].

ANS binding to YspC and YspC-SycB, shows a blue-shift in the maximum emission intensity ($\lambda_{\text{max}}$) to 482 and 480 nm, respectively. SycB also shows a blue shift in the $\lambda_{\text{max}}$ to 482 nm upon ANS binding. So, YspC and SycB, both in individual and complex form show solvent exposed hydrophobic patches. However, the ANS binding at pH-7.2 and 6.0, are identical in case of YspC as well as YspC-SycB. This is contradictory to the behaviour shown by SycB, which shows a consistent increase in emission intensity with reduction in pH from 7.2 to 4.0.
At pH-6.0, the emission intensity of SycB increases significantly, showing that more ordered hydrophobic patches got solvent exposed [Basu A et al., 2012]. However, when SycB forms a complex with YspC, it attains a structural rigidity. So, YspC-SycB do not show any increase in fluorescence emission intensity at pH-6.0, when compared to pH-7.2. Till now very less information is available regarding the structural details of translocator-chaperone complexes. 3-D experimental structures are available for peptides of translocators bound to class II chaperones [Job V et al., 2010; Lunelli M et al., 2009]. Also, fragments of translocators have been structurally elucidated by X-ray crystallography and NMR spectroscopy [Adam PR et al., 2012; Raab R & Swietnicki W, 2008; Tobias T et al., 2002], but structures of full length translocators and translocator-chaperone complexes are still not available. From the FRET efficiency, a prediction can be made that YspC shows a much compact structure compared to SycB. So, the five or more exposed tyrosines in SycB (predicted from the FRET data and the model of SycB) are masked, while YspC forms a complex with SycB. Since, 12 tyrosines out of 14 are located in the two TPR regions, so, we verified the interaction of YspC with the two TPRs of SycB. These TPR repeats are special regions involved in interaction with other proteins. YspC shows interaction with both the constructs of SycB: one harbouring the N-terminal and the two TPR regions, and another is having only the two TPR regions. Both constructs lacks the TPR-like region and the C-terminal helix and coil present in the homology model of SycB [Basu A et al., 2012]. However, both YspC-ΔSycB\textsubscript{(1–114)} and YspC-ΔSycB\textsubscript{(36–114)} complex shows altered physiological localization compared to YspC-SycB, when recombinantly expressed in *E. coli*. They are solubilised at 25°C in enriched TB medium. IpgC, the homologue of SycB, interacts with IpaB (peptide) by pockets P1, P2, P3. These pockets are mainly formed by the residues in the first two TPR regions of the IpgC molecule, as depicted.
by the PDB structure [Lunelli M et al., 2009]. In *P. aerginosa*, TPRs of PcrH forms the concave cleft involved in interaction of PcrH with PopD peptides [Job V et al., 2010]. YspC-SycB complex shows an oligomeric form and a heterodimeric form during SEC. This heterodimeric form is non-existent in YspC-ΔSycB(1–114) and YspC-ΔSycB(36–114) complex. So, lack of the TPR-like region, and the C-terminal helix and coil alters the physiological states of the complexes. However, further experiments need to be done to establish the role of TPR-like region and C-terminal helix and coil of SycB in maintenance of the heterodimeric form of the complex.
Figure 4.1. Generation of Phylogenetic tree of YspC using ConSurf. Phylogenetic tree shows the evolutionary relation of YspC with its homologues belonging to the IpaC/SipC family. The input protein sequence refers to YspC. The phylogenetic distances amongst various proteins are mentioned on the branches.
Figure 4.2. Prediction of conserved residues within YspC using ConSurf. All the amino acids of YspC are assessed for conservation by ConSurf. The adjoining conservation scale marks residues as variable, average (in between) and conserved, based on the colour scheme.
Figure 4.3. Multiple sequence alignment of YspC and other five minor translocator proteins like IpaC, SipC, AopD, PopD, and YopD, edited in ESPript. SipC and IpaC were aligned individually and with YspC. Similarly, AopD, PopD and YopD were aligned individually and with YspC.
Figure 4.4. Size exclusion chromatography profile of YspC at pH-7.2 and pH-5.0. a) The SEC profile of YspC at pH-7.2 and the corresponding SDS PAGE. L1 corresponds to the protein molecular weight marker, whereas L2 and L3 represent the protein present in Peak1 and Peak2 of the SEC profile, respectively. b) The SEC profile of YspC at pH-5.0 and L1 in the SDS PAGE corresponds to the protein molecular weight marker, whereas L2 is the protein present in Peak of the corresponding SEC.
Figure 4.5. Far UV and near UV CD spectra and thermal denaturation curve of YspC and YspC-SycB. a) Far UV CD spectra of YspC and YspC-SycB from pH-7.2 to 4.0. b) Near UV CD spectra of YspC and YspC-SycB at pH-7.2 and 5.0. c) Thermal denaturation curves of YspC at pH-7.2 and 5.0, and YspC-SycB at pH-7.2, were obtained by plotting $[\theta]$ values corresponding to 222 nm against the raise in temperature from 10 to 90°C.
Figure 4.6. Trypsinolysis of YspC, SycB and YspC-SycB depicts a variable proteolytic digestion pattern of individual YspC and YspC-SycB complex. a–c) SDS PAGE showing trypsinolysis of YspC, YspC-SycB and SycB, respectively. M denotes the protein molecular weight marker, ctrl denotes the undigested protein. The digestion was carried out with time points of 30, 60, 90, 120, 150 minutes. Black arrows mark the stable fragments obtained during proteolytic digestion of all the proteins.
Figure 4.7. Mass spectrum of YspC digested by trypsin, using MALDI-TOF. YspC was digested by trypsin for 2 hours and subjected to native mass spectrometry. Within a range two peaks were detected corresponding to two digested fragments with masses 14550.4297 Da and 12638.7012 Da, respectively.
Figure 4.8. ANS binding profile of YspC and YspC-SycB. Tyrosine fluorescence and ANS quenching suggest that the two TPR regions of SycB are sufficient for its interaction with YspC. a), b) The fluorescence emission spectra of YspC and YspC-SycB, respectively, when treated with molar excess of ANS. c) Black bar shows tyrosine fluorescence emission at λmax 304 nm, white bar represents the tyrosine fluorescence emission at λmax 304 nm in presence of ANS (quencher) and grey bar shows ANS emission at λmax 482 nm due to FRET, for YspC. d) Black bar shows tyrosine fluorescence emission at λmax 305 nm, white bar represents the tyrosine fluorescence emission at λmax 305 nm in presence of ANS (quencher) and grey bar shows ANS emission at λmax 480 nm due to FRET, for YspC-SycB.
Figure 4.9. Fragments of SycB containing the Two TPR regions shows *in vivo* interaction with YspC. a) SEC profile of Ni–NTA purified YspC-ΔSycB(1–114) and corresponding SDS PAGE showing the complex obtained after SEC in L2. b) SEC profile of Ni–NTA purified YspC-ΔSycB(36–114) and corresponding SDS PAGE showing the complex obtained after SEC in L2. L1 is the protein molecular weight marker in (a) and (b).

While it could be established that the two TPR regions of SycB were sufficient for its interaction with YspC, but SEC profile shows the lack of the heterodimeric form of the complexes in both the cases, which was observed in case of YspC-SycB complex.
Table 4.1. List of clones and their induction condition

<table>
<thead>
<tr>
<th>Clone</th>
<th>Host</th>
<th>Status</th>
<th>Induction Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Growth medium</td>
</tr>
<tr>
<td>pet28a(+)-yspC</td>
<td>BL21(DE3)</td>
<td>Transformed</td>
<td>LB</td>
</tr>
<tr>
<td>pAcYcDuet-yspC and pet28a(+)-sycB</td>
<td>BL21(DE3)</td>
<td>Co-transformed</td>
<td>LB</td>
</tr>
<tr>
<td>pAcYcDuet-yspC and pet28a(+)-ΔsycB(1-114)</td>
<td>BL21(DE3)</td>
<td>Co-transformed</td>
<td>TB</td>
</tr>
<tr>
<td>pAcYcDuet-yspC and pet28a(+)-ΔsycB(36-114)</td>
<td>BL21(DE3)</td>
<td>Co-transformed</td>
<td>TB</td>
</tr>
</tbody>
</table>


Table 4.2. Buffers used for protein purification

<table>
<thead>
<tr>
<th>BUFFERS</th>
<th>COMPOSITION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Buffer A</strong></td>
<td>25mM Tris-HCl pH-8.0, 150mM NaCl, 5mM imidazole, 2% glycerol</td>
</tr>
<tr>
<td><strong>Buffer B</strong></td>
<td>25mM Tris-HCl pH-8.0, 150mM NaCl, 10mM imidazole, 2% glycerol</td>
</tr>
<tr>
<td><strong>Buffer C</strong></td>
<td>25mM Tris-HCl pH-8.0, 150mM NaCl, 40mM imidazole, 2% glycerol</td>
</tr>
<tr>
<td><strong>Buffer D</strong></td>
<td>25mM Tris-HCl pH-8.0, 150mM NaCl, 250mM imidazole, 2% glycerol</td>
</tr>
<tr>
<td><strong>Buffer E</strong></td>
<td>25mM Tris-HCl, pH-7.2, 150mM NaCl, 1mM EDTA</td>
</tr>
</tbody>
</table>

*This buffer is used during SEC*
Table 4.3. Percentage of α-helix, β-sheet and other structures in YspC, YspC-SycB at different pH, calculated by Dichroweb server

<table>
<thead>
<tr>
<th>Protein</th>
<th>pH</th>
<th>α-helix%</th>
<th>β-sheet%</th>
<th>others% (random coil, turn)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YspC</td>
<td>7.2</td>
<td>51.2</td>
<td>16.5</td>
<td>32.3</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>53.8</td>
<td>14.7</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>57.6</td>
<td>12.2</td>
<td>30.2</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>50.3</td>
<td>13</td>
<td>36.6</td>
</tr>
<tr>
<td>YspC-SycB</td>
<td>7.2</td>
<td>67.8</td>
<td>3.9</td>
<td>28.3</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>65.7</td>
<td>5.1</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>53.7</td>
<td>9.8</td>
<td>36.4</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>34.1</td>
<td>12.2</td>
<td>53.7</td>
</tr>
</tbody>
</table>