Regulation of the multi-functional protein YscU in assembly of the Yersinia type III secretion injectisome

Ho Ngoc Hoang Oanh
To Dad and Mom
To my dear Tuân
and to my little Cam
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List of publications

The thesis is based on the following published papers and manuscript:


IV. Oanh Ho, Frédéric H. Login, Per Rogne, Tomas Edgren, Mattias Hedenström, Tobias Karlberg, Naresh Sunduru, Mikael Elofsson, Herwig Schüler, Hans Wolf-Watz and Magnus Wolf-Watz. (2017). Targeting dissociation of the substrate specificity switch protein YscU in the *Yersinia* type III secretion system with small molecules. *Ongoing Manuscript*

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Abstract

*Yersinia pseudotuberculosis* is a Gram-negative zoonotic pathogenic bacterium causing gastroenteritis in human and animals. It shares a conserved virulence plasmid encoding for a needle-like secretion machinery, or type III secretion system, which can be found in other pathogenic Gram-negative bacteria. The type III secretion system (T3SS) is a macromolecular assembly that enables pathogenic effector proteins (or *Yersinia* outer proteins, Yops) to be transported into eukaryotic host cells. This export machinery is assembled in a highly ordered stepwise mechanism. The activation of T3SS is also dependent on calcium concentration, temperature, and pH of the growth media as mimic factors for host cell's contact. The T3SS-associated inner-membrane protein, YscU, of *Yersinia* is proposed to function as a substrate specificity switch protein and forms a basal structure of T3SS. YscU has four α-helical transmembrane domain and a soluble cytoplasmic domain YscUC which undergoes auto-proteolysis at a conserved NTPTH motif. The auto-proteolysis process, which is required for the assembly of the injectisome and secretion of Yops, results in a 10-kDa C-terminal poly peptide fragment, denoted YscUCN and 6-kDa N-terminal fragment YscUCN. In this thesis, we showed that YscUC dissociation was important for Yops secretion and resulted in unfolded YscUCN and oligomeric YscUCN. By combination in vivo and in vitro methods, growth media conditions as calcium, temperature, and pH were indicated to control secretion by regulation of YscUC dissociation. The calcium-binding isotherm to YscUC was fit best with a one-site binding model resulting in $K_D = 800 \, \mu M$, which is identical to calcium level that blocks secretion in vivo. YscU is also the key protein for the T3SS pH dependence, demonstrated by thermal unfolding profile and secondary structure of protein were altered between pH 7.4 and 6.0. In addition, bacterial inner membrane was proposed to assist the YscUCN folding, monitored by using lipid bilayer as a mimic environment in nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy. This binding is important for Yops secretion and YscUC is anchored on bacterial membrane upon dissociation. The other substrate specificity switch protein YscP has function as a “molecular ruler” controlling length of the secretion needle. Previous genetic experiments have suggested that YscP and YscU interact physically, when mutation at defined residues on yscU (suppressor mutants) rescued Yops secretion in null-yscP mutant. In this research, direct binding of YscU and YscP was proved as weak but important interaction with $K_D = 430 \, \mu M$ by application of NMR and the binding interface of YscP was centred on the last helix of YscUC. Furthermore, we found that the YscP interaction could inhibit YscU auto-proteolysis. Studying the dissociation kinetic of suppressor YscUC variants at temperature 30 and 37°C provides strong support to a model where YscU is a temperature sensor for T3SS and YscUC dissociation is required for Yops secretion. Interestingly, the NPTH motif is conserved through most of YscU family members, meaning that role of dissociation may be conserved also in other bacterial injectisomes. To this end, the dissociation of YscU can be used as a therapeutic target in drug discovery. We attempted to identify the small molecules that can hinder YscU dissociation. The small compound methyl(l-5-ethyl-1-2-phenyl-1,3-thiazolidin-4-yl)acetate was found to be able to inhibit dissociation and to crystallize full YscUC, which has never been successfully done before. Finally, we found that the inner-rod protein YscI is binding to YscU with a 1:1 stoichiometry as shown with pulldown assays and isothermal titration calorimetry. Taken together we have made several discoveries that expand the functional palette of YscU and all these functions were shown to have biological relevance with Yops secretion levels. In light of the strong sequence conservation between T3SS utilizing pathogenic bacteria the findings are likely to be general characters.
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>T₃SS</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>CD</td>
<td>Circular dichroism</td>
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<tr>
<td>DMPC</td>
<td>1,2-dimyristoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DMPG</td>
<td>1,2-dimyristoyl-sn-glycero-3-phospho-(1′-rac-glycerol)</td>
</tr>
<tr>
<td>Far UV-CD</td>
<td>Circular dichroism in far ultraviolet</td>
</tr>
<tr>
<td>FlhB</td>
<td>Flagellar secretion switch protein</td>
</tr>
<tr>
<td>FliK</td>
<td>Flagellar needle length regulator protein</td>
</tr>
<tr>
<td>HSQC</td>
<td>Heteronuclear single quantum coherence spectroscopy</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Tₘ</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>Yop</td>
<td><em>Yersinia</em> outer protein</td>
</tr>
<tr>
<td>YscI</td>
<td><em>Yersinia</em> inner rod protein</td>
</tr>
<tr>
<td>YscP</td>
<td><em>Yersinia</em> needle length regulator protein</td>
</tr>
<tr>
<td>YscU</td>
<td><em>Yersinia</em> secretion switch protein</td>
</tr>
<tr>
<td>ΔHₘ</td>
<td>Change in enthalpy at equilibrium at melting point</td>
</tr>
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1. Introduction

1.1. Yersinia bacteria

*Yersinia bacteria* belongs to the genus of the large and diverse Gram-negative Enterobacteriaceae (1). The Enterobacteriaceae are rod-shaped bacteria and typically have a length of 1-5 µM. This large family includes *Yersinia, Shigella, Salmonella, Escherichia coli* causing similar and other less common species such as *Proteus, Enterobacter, Serratia,* and *Citrobacter*. *Yersinia* genus has 11 species, of which *Yersinia pestis, Yersinia pseudotuberculosis,* and *Yersinia enterocolitica* are the causative agents for infectious diseases in humans (1).

*Yersinia enterocolitica* enters human bodies through the gastrointestinal tract. Patients acquire these infections by uptake of contaminated food or water. Symptoms may be watery or bloody diarrhea and fever that require treatment with antibiotics (2). *Yersinia pestis,* which causes the deadly bubonic plague in the 6th, 14th and 19th century, was discovered independently by Alexandre Yersin and Kitasato Shibasaburo in 1894 (3). Nowadays, pathogenic *Yersinia pestis* can be treated by combination of antibiotics and specific supports as circulatory, ventilator, or renal support (4). *Yersinia pseudotuberculosis* is the oldest member of *Yersinia* genus (5), and causes tuberculosis-like symptoms through the gastrointestinal absorption of contaminated food products (6,7). These bacteria do not require any treatment since the infections are self-limiting; therefore, *Yersinia pseudotuberculosis* is a suitable bacterial model for study in laboratory conditions. Despite of being causative agents for different diseases, members of *Yersinia* genus are genetically similar.

1.2. Type III secretion system

Higher eukaryotic cells such as plants, animals and human are exposed to bacterial infections, which can lead to severe and lethal diseases. Most of the pathogenic bacteria are classified as Gram-negative bacteria that employ the different export apparatus to transport bacterial virulence factors to the extracellular milieu. The type III secretion system was firstly named by Salmond and co-workers in 1993 and is a widespread way to translocate toxin proteins into eukaryotic host cells (8,9). Genetic analysis of bacterial virulence factors has shown that the export apparatus is highly conserved during evolution whereas the effector proteins are species dependent, explaining why a single pathogenicity mechanism can give rise to a diverse set of diseases in plants, animals and human (10,11). The T3SS contains an
organelle denoted the injectisome (or needle complex) which is a multiprotein assembly that connects the bacterial cytoplasm with the eukaryotic host cells by a hollow tube-like structure (Figure 1). During the past few years, an intensive progress has been done to understand this bacterial organelle’s involvement in pathogenicity.

Early genetic studies showed that the injectisome is related to the flagellum, and was firstly visualized by Macnab and co-workers (12). Flagella, which are present in both Gram-positive and Gram-negative bacteria, were suggested as an ancestor of Gram-negative bacterial T3SS (13,14). Both injectisome and flagella have significant similar basal structures that are the multi-rings complex, embed on the cytoplasm and spanned through the bacterial envelope (11,15). A hollow needle, a filament or a pilus, is deposited on the basal structure and differs dependently on the family of injectisome.

![Figure 1](image_url): Illustration of the *Salmonella typhimurium* T3SS needle complexes. (A) Electron micrographs of purified needle complexes, after negatively staining. (B) Cross-picture of the injectisome, which is clustered to extracellular needle, outer rings on bacterial outer membrane, spanning neck from outer through periplasm to inner membrane and embedded inner ring on inner membrane. (C) Different views of surface rendered needle complex (adapted from Galán (16)).

Many pathogenic bacteria in plants (*Xanthomonas* spp., *Ralstonia solanacearum* and *Pseudomonas syringae*), human and animals (*Yersinia* spp., *Salmonella* spp., *Shigella* spp., *Chlamydia* spp., *Vibrio* spp. and *Pseudomonas aeruginosa*) emphasize a similar and sequential mechanism to transport their effector proteins to the target host cells, that trigger disease symptoms (6,17). As other Gram-negative bacteria, *Yersinia pseudotuberculosis* shares a virulence plasmid that encodes for T3SS (18,19). The *Yersinia* T3SS is coded by 70-kb PYV plasmid (20). The *Yersinia* secretion apparatus is built up by 29 *Yersinia* secretion components (Ysc) with some low calcium response (Lcr) proteins (21), *Yersinia* outer proteins (Yop) with 2 translocator proteins and 6 effector protein (22), and specific
Yops chaperones (Syc). The Yops sequences are usually included an N-terminal secretion signal domain and C-terminal catalytic domain (23). The secretion apparatus is build up by numbers of protein families as illustrated in Figure 2 from *Yersinia* genus. The nomenclature of different protein families, which are involved in assembly of T3SS, is summarized in Table 1.

![Figure 2: Schematic diagram of T3SS in *Yersinia* spp.](image)

**Table 1: Nomenclature of different protein families involved in T3SS (adapted from (10,11,24-27))**

<table>
<thead>
<tr>
<th>Yersinia spp.</th>
<th>Shigella flexneri</th>
<th>Salmonella spp. (SPI-1)</th>
<th>Salmonella spp. (SPI-2)</th>
<th>Pseudomonas aeruginosa</th>
<th>Common*</th>
</tr>
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<tbody>
<tr>
<td><strong>Translocator</strong></td>
<td>YopB/D</td>
<td>IpaB/C</td>
<td>SipB/C</td>
<td>SseD/C</td>
<td>PopB/D</td>
</tr>
<tr>
<td><strong>Needle tip</strong></td>
<td>LcrV</td>
<td>IpaD</td>
<td>SipD</td>
<td>SseB</td>
<td>PcrV</td>
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<tr>
<td><strong>Needle subunit</strong></td>
<td>YscF</td>
<td>MxiH</td>
<td>PrgI</td>
<td>SsaG</td>
<td>PscF</td>
</tr>
<tr>
<td><strong>Secretin apparatus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secretin</td>
<td>YscC</td>
<td>MxiD</td>
<td>InvG</td>
<td>SsaC</td>
<td>PscC</td>
</tr>
<tr>
<td>MS ring</td>
<td>YscJ</td>
<td>MxiJ</td>
<td>PrgK</td>
<td>SsaJ</td>
<td>PscJ</td>
</tr>
<tr>
<td>Inner rod</td>
<td>YscI</td>
<td>MxiI</td>
<td>PrgJ</td>
<td>SsaI</td>
<td>PscI</td>
</tr>
<tr>
<td><strong>Switch</strong></td>
<td>YscU</td>
<td>Spa40</td>
<td>SpaS</td>
<td>SsaU</td>
<td>PscU</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(SpaS)</td>
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<tr>
<td><strong>Needle ruler</strong></td>
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* The suggested common nomenclature for all of species.
** The substrate specificity switch protein, with FlhB homolog in flagella.
*** The molecular ruler, with FliK homolog in flagella.
An activated T3SS mediated protein translocation is generated by a complicated mechanism, involving more than 25 proteins in pathogenic Gram-negative bacteria (28). The hierarchy of the assembly process is unclear. To assemble an activated T3SS in Yersinia, the mechanism starts from the outer membrane of the pathogenic bacteria and follows the sequential scenario at 37°C in calcium-depleted medium (11,29) (Figure 3). This process demands three distinct phases with specific requirement and timing, regulated by different “switch proteins”: formation of the basal structure and needle of the injectisome; exporting of tip proteins and blocking T3SS; localization of the translocator proteins on the tip of needle and secretion of the outer proteins after contact with the host cell membrane (11,25). The basal structure is assembled from the outer membrane to the inner membrane of the bacteria (30). The early substrates required for the needle growth follows a sequentially process for a functional injectisome (6). The needle is growing until the first substrate specificity is switched to arrest the needle prolongation (31). To complete the needle structure, a second process is launched in which export of the tip needle proteins to the exposed surface of the injectisome is accomplished (32,33). The secretion apparatus is blocked until a specific signal triggers its activity. The trigger signal can be the direct contact with the host cell (34). After host cell contact, the translocator forms a pore on its membrane and the effector proteins are exported directly to the intracellular milieu through the needle complex in one-step injection process (9,35).
Figure 3: Schematic diagram illustrating the assembly phases and translocation of effector proteins by a one-step injection model of T3SS. The early substrates form the basal structure and the needle body in (A) and (B). The 1st substrate specificity switch protein is activated after the needle has reached its required length and the middle substrates (including the needle tip forming proteins and gate-keeper proteins) are immobilized to their functional positions in (C). While the bacteria contact with the host cells, the 2nd substrate specificity switch protein triggers the translocator proteins to form a pore on host cellular surface, followed by direct injecting the effector proteins to intracellular milieu through the needle (D) (adapt from Deane (25)).

In addition, there is an alternative 2-step translocation model allowing T3SS to translocate the effector proteins in vivo, of which the needle complex is functional as a target-cell sensor (36) (Figure 4). This model is based on the surface-localized of the translocators (IpaB, IpaC, and IpaD in Shigella flexneri (37), YopD in Yersinia pseudotuberculosis (38)) and effectors (YopEin Yersinia pseudotuberculosis (38)) before host cell contact. The first step of the model takes place after the needle complex is completed and regulated by “switch protein”. The translocators and effectors will be secreted by T3SS across the bacterial envelope to the bacterial surface before host cell contact (38) and released after reaching eukaryotic cells (36). The translocators will form a pore in the target cell plasma membrane and mediate the translocation of the effectors into the target cell cytoplasm. This model is similar to AB-toxin export mechanism.
**Figure 4:** Illustration of 2-step injection model found in *Yersinia pseudotuberculosis* and *Shigella flexneri*. The translocator (t) and effector (e) proteins are secreted through the needle complex localized on bacterial surface. When the bacteria contact the host cell by the T3SS, the translocator proteins are exported and form a pore on the cellular membrane. The toxin proteins will be translocated into the target cell cytoplasm (adapt from Edgren (36)).

*In vivo*, calcium concentration, temperature, and pH of the growth media are very important to regulate Yops secretion from *Yersinia* spp. Several previous studies have been made for calcium effect; including T3SS mediated secretion, translocation, and target cell induced expression of effector proteins. It has been shown that presence of 2.5 mM calcium in the growth medium blocked Yops secretion but bacteria still grew, while removal of calcium induced massive Yops secretion and prohibited bacterial proliferation (39,40). Depletion of calcium in the growth medium and simultaneously changing the temperature from 26°C to 37°C induces *Yersinia* T3SS and mimicking target cell contact (23). Temperature dependence of T3SS can be explained by the temperature dependence of the virulence plasmid. Indeed, growth of injectisome may be terminated by increasing expression of plasmid-encoded T3SS (41). During infection of *Yersinia pseudotuberculosis* in mice, plasmid copy number increases (42), indicating that gain of plasmid copy number is indispensable for T3SS function and virulence. Furthermore, pH of the growth media is able to control the secretion. In *Salmonella typhimurium* SPI-2, it has been show that switching the pH of growth media to pH 7.2 after growth at pH 5.0 caused effects to dissociation and degradation of regulatory complex proteins and effector translocation (43). On the other hand, switching growth media from pH 7.0 to 5.0 induces protein secretion (44), demonstrating that low pH might be a physiological stimulus for secretion of effector protein *in vivo*. Despite the massive research on the T3SS have been
published, the molecular mechanisms underlying the calcium effect, temperature and pH requirements are still ambiguous in the T3SS.

1.3. Needle length regulator protein YscP

In T3SS, the length of needle is a considerable factor, which is diverse for different bacterial species or even bacterial strains (45, 46). Indeed, the needle length was shown to be 80 nm in Salmonella typhimurium SPI-1 (47) or 150 nm Salmonella typhimurium SPI-2 (48), 45 nm in Shigella flexneri (Tamano, 2000 #124). In Yersinia spp., it was found to be 58 nm in Yersinia enterocolitica E40 (31) and is suggested 45 nm in Yersinia pestis (31). Thus, needle length seems to be evolutionary controlled for structural-functional implications (31, 34). Different mechanisms have been hardly studied, in effort to explain how the length of needle is controlled (Figure 5).

The first mechanism is the cup model, which is built based on the similarity between T3SS and flagella (49). In this model, the flagellar basal body is considered as a “cup” containing the hook subunits. The hook subunits will be secreted during the flagellar assembly until the “cup” is emptied. When the basal body is vacant, a substrate specificity switch protein FliK will be secreted.

The second model is about needle regulation by timing of the substrate specificity switch (50). Based on results from wild-type and deletion mutation of invJ (Salmonella typhimurium SPI-1), the external structure of these 2 injectisomes are comparable but no inner rod and over-expression of needle subunits were found in the mutant (11, 50, 51). Similar results were also found in mutation of spa32 in Shigella flexneri (52, 53), in which the needle became extremely long (5 μm) and lacked of the inner rod component.
B. Needle length control by timing of substrate switching: InvJ stabilizes the socket substructure (1), which is necessary for the assembly of the inner rod composed by PrgJ, while the needle assembles simultaneously (2, 3). Termination of the inner rod results in conformational changes that lead to substrate switching (4). Late substrates can then be exported (5).

**Figure 5**: Schematic diagram illustration for different regulation mechanisms of the needle and the hook (adapted from Cornelis (11)).
The most accepted model is “molecular ruler”, found in *Yersinia* spp. In this hypothesis, the length of needle is determined by “length” of a particular protein, named YscP (6,31,49,54). YscP is orthologue of FliK in flagellar, Spa32 in *Shigella flexneri*, InvJ in *Salmonella typhimurium* and PscP in *Pseudomonas aeruginosa* (55-57). YscP was firstly found as a secreted protein in calcium depleted media (58) and exposed on bacterial surface prior the secretion (59). If the YscP sequence is deleted or inserted, the injectisome is shorter or longer, respectively, indicating a linear correlation of needle’s length with the size of YscP (60-62). Indeed, needle length in *Yersinia enterocolitica* E40 is around 58 nm with 515-residues YscP while it is expected around 45 nm in *Yersinia pestis* or *Yersinia pseudotuberculosis* since their YscP includes 455 residues (31). Furthermore, YscP secretion is required in needle regulation but is not essential for Yops secretion (60). Interestingly, only one molecule of YscP is enough to adjust the needle length (61). The molecular ruler model is also discussed in the flagellar of *Salmonella enterica*, where FliK is an internal ruler (63,64). In conclusion, different mechanisms have been proposed for different obtained results in the T3SS field.

A systematic deletion analysis of YscP in *Yersinia enterocolitica* supports that the protein contains different domains with specific functions (54,60), including two distinct secretion signals (residues 1 to 35 and residues 97 to 137) and a substrate specificity switch domain located between residue 385 and 500 (45). The substrate specificity switch domain is suggested to include residues 341 to 441 in the YscP of *Yersinia pseudotuberculosis* (65). YscP is not only a molecular ruler determining needle length but also an important secretion component. Fusion of YscP with other globular proteins as ubiquitin or GST at C-terminal domain was established to terminate its secretion and blocked secretion of other effector proteins (66). An YscP orthologue with similar functions is FliK, found in the flagella and T3SS of diverse bacterial species (55). The YscP/FliK family members share little sequence similarity although their function are comparable. Both PscP (*Pseudomonas aeruginosa*) and FliK have “ball-and-chain” architecture consisting of a long intrinsically disordered, less conserved N-terminal domain and a helical, folded and conserved C-terminal domain (55,56) (Figure 6). They also contain a PxLG sequence, which is function as a stabilizer for their C-terminal domain.
Figure 6: Similarity of YscP and its homologs. (A) Schematic diagram for MAFT sequence alignment of YscP\textsubscript{en} from \textit{Yersinia enterocolitica} and YscP\textsubscript{pseu} from \textit{Yersinia pseudotuberculosis} (67). YscP\textsubscript{pseu} can be considered as a deletion mutant of YscP\textsubscript{en} from residue 290 to 350. The T3SS switch domain is coloured in red and located at the same position for both of the strain. The secreted signals are coloured green on YscP\textsubscript{en} (54, 60). (B) The YscP homologues FliK (blue) and PscP (green) have a conserved “ball-and-chain” architecture with a flexible N-terminal segment and a folded C-terminal domain. The structural alignment was created using the DALI server (68) based on the NMR structure of FliK from \textit{Salmonella typhimurium} (PDB code: 2RRL) (55) and the crystal structure of PscP (green) from \textit{Pseudomonas aeruginosa} (PDB code: 5CUK) (56).

1.4. Secretion apparatus protein YscU

As described above, the “switch proteins” have an important role to change the secretion states of pathogenic T3SS. The first “substrate specificity switch protein” (from early to late substrates) must only function when the length of needle has matched the specific structures at the bacterial and host-cell surface.

In \textit{Yersinia}, the switch protein YscU is sequence related to the flagellar protein family FlhB. YscU also shares sequence similarity to Spa40 in \textit{Shigella} spp., SpaS in \textit{Salmonella} spp. (SPI-1), EscU in pathogenic \textit{E. coli} and PscU in \textit{Pseudomonas aeruginosa}. YscU is a component of \textit{Yersinia} secretion basal structure, which is composed of 354 residues and separated into a four-helix trans-membrane N-terminal domain and a cytoplasmic C-terminal domain YscUC. The large cytoplasmic terminal domain YscUC, which is the most intensively studied protein in this thesis, consists of NPTH motif (from residue 263 to 266), explaining for YscUC auto-proteolysis into YscUC\textsubscript{N} (6.3 kDa, from residues 211 to 263) and YscUC\textsubscript{C} (10.5 kDa, from residues 264 to 354) (69, 70). Structural alignment of crystal wild-type YscUC (PDB: 2JLI) and non-cleavable mutant YscUC\textsubscript{N263A} (PDB: 2JLH) reveals the similarity between them (Figure 7).
Figure 7: Illustration of position and structure of *Yersinia* YscU. (A) Schematic diagram of YscU embedded in the bacterial membrane. The cytoplasmatic domain YscUC is consisting of residues 211 to 354. YscUCN is coloured pink while YscUCN is red. Crystal structure of YscUC (PDB cod: 2JLI) is from residue 240 to 340 (69). (B) Structural alignment of wild-type YscUC (red) (PDB cod: 2JLI) and the non-cleavable mutant YscUC(N263A) (blue) (PDB code: 2JLH) indicates the structural similarity before and after proteolysis.

The essential role of YscU/FlhB auto-proteolysis in T3SS has been deeply studied. A non-cleavable mutant EscU_{N262A} in Enteropathogenic *E. coli* is unable to secrete and translocate effector proteins (71). In *Salmonella* SPI-1, SpaS auto-proteolysis induces conformation changes before embedding to the needle structure but is not a signal for substrate specificity switch or needle length regulation (72). Deletion mutation of Spa40_{ANPTH} in *Shigella flexneri* leads to an incomplete needle and non-interaction with needle length regulator protein Spa32 (73). The mutant FlhB_{N269A} in *Salmonella* flagellar causes alternative cleavage, resulting in a polyhook phenotype (74,75). In all of YscU/FlhB proteins, it is hypothesized that the auto-cleavage forms an interaction interface with other inner membrane proteins, with a following effect on T3SS assembly and translocator of the effector proteins (71,72,76,77).

Auto-proteolysis of *Yersinia* YscU is crucial for a proper T3SS function and Yops secretion (69), indicating by incomplete Yops synthesis and secretion when the full-*yscU* gene or the NPTH coding sequence is deleted. Other hand, mutation at Pro264 (NPTH motif) reduces cleavage but secreted Yops are much less than wild type in calcium depleted medium and more than in presence of calcium (78). Besides, replacing residue N263 by alanine is able to prohibit cleavage and secreted Yops amount is decreased compared
to wild type in calcium-depleted media. Interestingly, this mutant releases equal amount of effector proteins in both presence and absence of calcium in medium (78). It is supposed that calcium regulates secretion and translocation by controlling YscU proteolysis in Yersinia. (78-80).

### 1.5. Inner rod protein YscI

In 2001, structural analysis of Shigella needle complex was visualized deeply at the first time to its central canal, which is 2-3 nm diameter (81). In this model, the hydrophilic protein MxiI was introduced for the first time as a periplasmic component, which is believed to have a functional equivalent of the flagellar hook cap protein FlgD (81,82). The unfolded MxiI forms a complex with MxiC, a gate-keeper protein, to plug the T3SS entry gate (83,84).

In 2004, Marlovits and co-workers reported the central structure of Salmonella typhimurium T3SS at 17 Å resolution, of which a socket-like structure was extended from the basal plate into the hollow chamber (85). This socket is established by the so-called inner-rod protein PrgJ which is largely unfolded and self-polymerized to assemble the inner rod (84). Later, Galán indicated that PrgJ was a secreted protein, substrate specificity switch and needle-length regulator, demonstrated by the abnormal long needle with single mutation on PrgJ C-terminal domain (50,86). In Enteropathogenic E. coli, Finlay also found that the inner rod protein EscI was involved in substrate specificity switch by binding to both cleavage and uncleavage EscU (YscU homolog) (87).

In agreement with previous models in Salmonella and E. coli, Lloyd and co-worker demonstrated that the inner rod protein YscI was an exported component, which was controlled by both YscU and YscP in Yersinia pseudotuberculosis (88). This result is in agreement with findings in Salmonella and E. coli which proposes a potential binding of YscI to YscU or/and YscP. Only few structural studies of T3SS inner rod proteins are exist due to their self-polymerization at the concentrations required for crystallography and nuclear magnetic resonance spectroscopy (84). Both PrgJ and MxiC are monomeric and partially folded at concentration below 0.1 mM (84), whereas Pseudomonas aeruginosa PscI forms a helical oligomer (89). Sequence alignment of YscI and its homologs (Figure 8) demonstrates a low similarity, meaning that YscI structure and function have to be studied independently for different bacterial species.
Figure 8: YscI is predicted to have high α helical content. (A) Sequential alignment of YscI homologs. MAFFT sequence alignment (67) of YscI (Yersinia pseudotuberculosis) and the homologues PscI (Pseudomonas aeruginosa), EscI (Enteropathogenic E. coli), MxiI (Shigella flexneri) and PrgJ (Salmonella typhimurium). The amino acids are numbered according to their positions in YscI of Yersinia pseudotuberculosis. Conserved residues are highlighted in red and similar residues are shown in yellow. The secondary structure of YscI was predicted using DSSP and PSIPRED (90, 91) and is indicated by the letter “h”. (B) Predicted model of YscI using SWISS-MODEL (92-94) with apolipoprotein A-IV, which has 18.75% sequence similarity to YscI. The conserved residues are highlighted as ball and stick, when the similar residues are marked yellow.

1.6. YscP and YscU interaction

FliK and FlhB in Salmonella typhimurium were firstly introduced as conjugated proteins for their determination of flagellar assembly (95, 96). The polyhook exhibited by a null mutant fliK was suppressed by single mutations, so called suppressor mutants, in cytoplasmic FlhB, which were characterized by genetic and sequence analysis close to the 3’-end of the flhB gene. These genetic experiments suggest a direct interaction between FlhB and FliK (97). By using surface plasmon resonance spectroscopy, binding affinities (Kd values) of FliK and both wild-type and non-cleavage FlhB are calculated between 1-10.5 μM (98, 99).

In Yersinia pseudotuberculosis, YscP and YscU co-ordinately regulate the substrate specificity of the Yersinia T3SS. It has been observed that an yscP null-mutant was unable to efficiently secrete effector Yops although the amount of the exposed needle component YscF to the bacterial cell surface was detected (100). Based on suppressor mutant in FlhB, several site-direct
Mutagenesis were found to be in the cytoplasmic domain of YscU such as A268F, Y287G, V292T, and Y317D; that were presumed to diminish the level of YscF secretion but raising the level of Yops secretion. Consequently, A268F and V292T mutants restored Yops secretion to wild-type YscU. The Y287G mutant released intermediate amount of Yops while the Y317D mutant could not suppress the secretion (100). These data postulate a direct or non-direct binding of YscP and YscU in Yersinia. However, these mutated residues are localized in YscU core-domain (Figure 9), which have low solvent accessible surface and a destabilizing effect.

**Figure 9:** Illustration of the structural location of suppressor mutants on the crystal structure of YscUC (PDB cod: 2JLI). A268 is coloured green, Y287 is coloured yellow, V292 is coloured magenta and Y317 is coloured as blue. These amino acids are all shown as ball and stick.
2. Materials and methods

2.1. Circular Dichroism (CD) spectroscopy

Proteins are built-up by different amino acids divided in three different types such as polar, nonpolar and charged residues. All amino acids with the exception of glycine have chiral α-carbons, which make them optically active. This is manifested as a difference in the interaction with polarized light. Furthermore, the secondary structure elements are optically active, for instance α-helices are predominantly right handed. Circular dichroism is a type of absorbance spectroscopy where left- and right-handed circularly polarized light are generated. The experimental observation in CD spectroscopy is the differences between the absorption (ΔA) for left, $A_L(\lambda)$, and right-handed, $A_R(\lambda)$, circularly polarized lights and recorded at a certain wavelengths (101-103) which characterizes for protein secondary structure (104). CD is a rapid, simple and non-destructive analytical technique (102) and often used to estimate the secondary structure contents in proteins. The observable signal in a CD measurement is provided by the equation below based on Lambert-Beer law (105).

$$\Delta A(\lambda) = A_L(\lambda) - A_R(\lambda) = \left[ \varepsilon_L(\lambda) - \varepsilon_R(\lambda) \right] = \Delta \varepsilon^* l^* c$$

Here $A$ is absorbance, $\varepsilon(\lambda)$ is the extinction coefficient for “L” left- and “R” right-handed circularly polarized light, $c$ is protein concentration and $l$ is cuvette path length (103).

Specific ranges of wavelength visualize different levels of protein structure. The near-UV (from 260 nm to 330 nm) CD signal arises from aromatic sidechains and disulphide bonds, reflecting the tertiary structure of proteins (106). Near-UV CD can also report on protein-metal interactions (107). The far-UV (from 190 to 260 nm) CD signals has contributions predominantly from the peptide bonds between the chiral amino acids, and is the most employed wavelength range (102,103). The peptide backbone illustrates the secondary structure of proteins, including α helixes, β sheet and random coil (90). Since CD spectrum of each type of peptide backbone overlays broadly on each other (Figure 10), it requests the spectral deconvolution to pure α helixes, β sheet and random coil spectra or to compare with database built from known protein structures (108).
Figure 10: Reference far-UV CD spectra for different types of pure secondary structure (adapted from Greenfield (109)).

CD spectra give less structural information or aggregation state of a specific protein compared to nuclear magnetic resonance spectroscopy and crystallography. For example, this technique cannot be used to identify protein-ligand binding sites or 3D structural changes before and after adding ligands. However, CD has some advantages in requirement of a low protein concentration in small volume in biological relevant buffers, and simple, quick operation procedures. Therefore, it is applicable in determination the small changes in protein structures following for instance perturbations with pH, temperature and ligand bindings (110).

In this thesis, far-UV CD (from 190 to 260 nm) which is sensitive to the secondary structure is applied to determine the biophysical characteristics of the proteins of interested. The signals at wavelength 220 nm, which is specific for α-helical content of a protein, will be utilized to examine thermal stability, ligand binding (calcium) and pH effects of proteins in the T3SS.
2.1.1. pH titration

In this experiment, protein samples in 1 cm path length cuvette with stirrer were titrated with either HCl (for acidic pHs) or NaOH (for alkaline pHs). The protein solution at each pH value was incubated 15 min before measurement to archive the equilibrium. The signal was recorded at 220 nm as a function of pH and fitted by the equation [1] (111,112).

\[ CD_{220nm} = \frac{CD_B + CD_A \times 10^{n(pK_a + pH)}}{1 + 10^{n(pK_a + pH)}} \]  

[1]

With \( CD_B \), \( CD_A \) are respectively CD_{220nm} signal before and after the transition, \( n \) is the number of protons involved to the transition, \( pK_a \) is the dissociation constant of amino acids which the involved protons belong to.

2.1.2. Calcium titration

When a ligand (or metal ion) binds to protein, it may cause secondary and/or tertiary structure changes of proteins and consequently alter the CD spectra of the studied protein (113). This thesis takes advantages of CD spectroscopy as quick and convenient technique to clarify if YscU is interacting with calcium. According to previous findings that Ca at 2.5mM can be used to affect effector secretion levels (39,40), the binding constant was therefore believed to be in the mM range, thus, the calcium concentration range will be from 0 to 15 mM to complete the binding process. The YscU-Ca complex will be assembled by the equilibrium below:

\[ YscU + Ca^{2+} \rightarrow [YscU-Ca^{2+}] \]

The measured Cd signal at 220 nm (CD_{220nm}) is composed by signals from both calcium bound protein and free protein according to:

\[ CD_{measured} = Pb \times Ib + Pf \times If \]

With \( Pb \), \( Pf \) is the fraction of bound and unbound protein, respectively, in which \( Pb + Pf = 1 \). \( Ib \), \( If \) is the CD_{220nm} of fully bound protein and unbound protein that can be examined as in Figure 11. Thus, the free fraction at each calcium concentration and dissociation constant are calculated as in equation 2 (114).
\[ P_b = \frac{CD_{measured} - I_f}{I_b - I_f} \]

\[ K_d = \frac{[Ca^{2+}] [YscU_C]}{[YscU_C - Ca^{2+}]} = \frac{[Ca^{2+}] P_f}{P_b} = \frac{[Ca^{2+}] 1-P_b}{P_b} \Rightarrow P_b = \frac{[Ca^{2+}]}{K_d + [Ca^{2+}]} \]

The calculated bound fraction is plotted against calcium concentration in order to estimate \( K_d \) value which can be used to evaluate the binding affinity.

**Figure 11:** Calcium titration curve with illustrated \( I_b, I_f \) (left) and fitted curves with bound fraction against calcium concentration (right)

### 2.1.3. Thermal unfolding experiments

The *Yersinia* T3SS is temperature susceptible in calcium-depleted media (23). Altering temperature from 26 to 37 °C with a simultaneous removal of Ca^{2+} is able to mimic the host cell contact and triggers Yops secretion. This effect relates to the substrate specificity, which may be YscU, during assembly of injectisome. In addition, thermal stability can be measured by the accumulation of small changes throughout the backbone conformation of a protein (115). We decided to apply CD to study the thermal stability of wild-type and variants of YscU to understand the temperature effects on secretion.

Experiments for determination thermal stability of the proteins of interest were performed at the same sample conditions for the up and down temperature scans to evaluate the reversibility of thermal transitions (116). The signal dependence on temperature scan rate was also investigated to confirm if the thermal unfolding was at the thermodynamic equilibrium. Data at 220 nm was fitted to two-state equation for every transition to elucidate melting temperature \( T_m \).
Figure 12: The temperature induced unfolding curve based on CD signal

The measured signal was analysed similarly as in denaturant unfolding with the assumption of a two-state transition (117).

Native ↔ Unfold

At equilibrium $K_{obs} = P_U/P_F$, with $P_U$ and $P_F$ are the fraction of unfolded and folded proteins in the sample, respectively.

The signals corresponding to the unfolded ($I_U$) and folded ($I_F$) states are temperature dependent (as illustrated in Figure 12) according to:

$$I_F = m_F \cdot T + e_F$$
$$I_U = m_U \cdot T + e_U$$

Where $m_F$ and $m_U$ are the slopes (temperature dependencies) of the folded and unfolded signals, $e_F$ and $e_U$ corresponds to the folded and unfolded state signals at zero Kelvin, and $T$ is the studied temperature (K).

The data was analysed by applying nonlinear fitting routines as equation [3] and van’t Hoff equation (118,119)

$$K_{obs} = \exp\left(-\frac{\Delta H}{R \cdot T} + \frac{\Delta H}{R \cdot T_m}\right)$$

$$CD_{220nm} = \frac{I_F + I_U \cdot K_{obs}}{1 + K_{obs}}$$
2.2. **Nuclear Magnetic Resonance (NMR) spectroscopy**

Nuclear magnetic resonance spectroscopy (NMR) has established to be a crucial technique in studying structural biology for proteins, nucleic acids and carbohydrates. In proteins, it is capable to examine directly 3D-structure, interaction and dynamic in atomic resolution level \(120,121\) without destructing the samples by varied type of experiments. Selective isotopic labelling for full-length or selected domains of a protein, that is successfully engaged in NMR, helps to simplify the obtained data compared to crystallography \(122-125\). By using NMR, the protein is not only studied as purified ingredient in buffer condition but also in living cell or tissue based on new developed in-cell and solid state NMR spectroscopy, which assists to understand the correlation of *in vivo* and *in vitro* mechanism \(126,127\). Other advantages of NMR are its ability to be hyphenated with an auto-sampler in drug discovery, or with separation techniques to improve the sample purity \(128,129\). However, traditional NMR has also limitations that needs to be overcome by alternative spectroscopy. First of all, the protein concentration in the samples should be high (usually from 100 µM) that potentially results in aggregation and insolubility problems. NMR is also usable for small size proteins (around 250 residues \(130\)) of which signals are well dispersed and less line broadening. The proteins with high number of residues seem to be challenged by using basic NMR, however, the dynamic and structural studies of macromolecules up to mega-Dalton is accessible nowadays with the advance multidimensional NMR in high magnetic field \(131,132\) and cryogenic system \(133\). As a conclusion, NMR becomes a powerful technique to explore protein. In the limitation of the thesis, the basic concepts of NMR and the 2D heteronuclear single quantum coherence spectroscopy (HSQC), which were utilized as the principal tools, will be briefly introduced.

### 2.2.1. NMR signal

In my thesis NMR, spectroscopy has been used as one of biophysical and biochemical techniques to understand the function of the YscU protein. Theory of NMR will be described as following. Atoms are made of electrons and nuclei. Every atomic nucleus consists of protons and neutrons and has five properties; mass, electric charge, energy, magnetism and spin \(134\). The nuclear spin, which is an intrinsic form of angular momentum, is defined by the quantum number \(I\). I can adapt integer or half integer values (for example 0, ½, 1, 3/2, ..., 6). The nuclear magnetism is defined by the magnetic quantum number \(m\) and can take values in the interval \([I, I-1, ..., -I+1, -I]\), resulting in \((2I+1)\) possible states, however, if a nucleus has \(I=0\) (for example \(^{16}\)O, \(^{12}\)C), it is “invisible” in NMR. In biochemistry, many nuclei are
NMR active such as $^1$H, $^{13}$C, $^{15}$N and $^{31}$P all of which has I=1/2 with observable signals.

When a sample is placed in an external magnetic field, $B_0$, the nuclear energy states are separated with small energetic difference, $\Delta E$, between them and the spins are aligned along z-axis, in parallel to the applied magnetic field (Figure 13). This splitting of energy is based on the Zeeman effect, which constitutes the basis of NMR spectroscopy (135). $\Delta E$ is proportional to the strength of $B_0$ and the gyromagnetic constant $\gamma$, which is a nucleus specific constant for nuclei with I≠0. When I=1/2, $\Delta E$ is the difference between energy state $\alpha$ ($m=1/2$) and $\beta$ ($m=-1/2$) (Figure 13), where the energy of the two states are given by $E=-m*\gamma*h*B_0$, with $h$ is Planks constant divided by $2\pi$. However, the nuclear spins populate the two energy states differently according to the Boltzmann distribution, more spins occupy the lower energy state causing a bulk magnetization $M_z$ aligned with external magnetic field when the nuclear system is in equilibrium. This longitudinal magnetization should be transformed to transverse magnetization to be detected. Therefore, it is tilted from the z-axis to the xy-plane by application of a radio-frequency pulse (RF). The magnetization in the transverse plane will now rotate with the so-called Larmor frequency $\omega=-\gamma*B_0$. The new rotating magnetization in the xy-plane will have a component that will oscillate along the x-axis with a frequency of $\omega_0$. Furthermore, the precession magnetization will induce an oscillating voltage in the receiver coil along x and y-axis which is displayed as free induction decay (FID). The Fourier transform, which is a mathematical operation, is employed to transform the precession magnetization from the time domain to the frequency domain. The signal in the frequency domain will be processed by apodization functions to constitute an NMR spectrum.

Figure 13: Illustration of NMR principles. (A) The energy of a nucleus with spin $m=1/2$ is splitted to 2 new states $\alpha$ and $\beta$ when an external magnetic field is applied. Stronger applied magnetic fields generates a larger energy difference. (B) Vector illustration of 90° pulse to longitudinal $M_z$ to detectable transverse $M_y$. 

2.2.2. *The chemical shift*

In protein NMR, the resonance frequency of a nucleus is considered as a marker and modulated by its surrounding electrons. The electrons surrounding a nucleus are distinct and crucially dependent on the protein structure. In the external magnetic field, these electrons induce a local magnetic field that is unique for each nucleus, the so-called shielding effect. The difference between nuclear magnetic resonance frequencies of the same kind of nucleus (\(^1\text{H}\), \(\text{^13C}\), \(\text{^15N}\) or \(\text{^31P}\)) is considered as chemical shift, which is usually very small as part per million. To visualize the chemical shift as an interpretable signal, the chemical shift of a nucleus of interest is based on the comparison to the resonance frequency of a reference or standard nucleus. The chemical shift of a nucleus can be calculated as following (136)

\[
\delta = \frac{\nu_{\text{sample}} - \nu_{\text{reference}}}{\nu_{\text{reference}}} \times 10^6 \text{ (ppm)}
\]

With \(\nu_{\text{sample}}\) and \(\nu_{\text{reference}}\) are the absolute nuclear resonance frequency of the sample and reference compound, respectively.

In 1D NMR, the reference can be 4,4-*dimethyl-4-silapentane-1-sulfonic acid* (DDS) or *tetramethylsilane* (TMS) with chemical shifts are zero at certain pH, temperature and solvent conditions (137). By using chemical shifts, the external magnetic field strength will not influence the absolute value of obtained signals but improve the spectral resolution when it is increased. In biochemistry, chemical shift of a residue in an unknown protein can be employed to estimate the secondary structural components by using the chemical shift index library, which is built from chemical shifts of assigned residues of known structural proteins (137, 138).

2.2.3. *NMR relaxation*

In NMR spectroscopy, relaxation is the process where the magnetization returns to its equilibrium, meaning that its spin populations distribute accordingly to Boltzmann equation and no transverse magnetization presents in the system (139). This process is defined by a time constant (T) or a rate constant (\(R = 1/T\)) (140) due to the spin itself and other surrounding spins. In practical level, the rate of relaxation is affected by the physical properties of a molecule (size and folding state) and external variables (viscosity and temperature). Relaxation includes two types of relaxation: longitudinal relaxation \(R_1\) and transverse relaxation \(R_2\) (141).
The longitudinal (or spin-lattice or z-direction) relaxation is defined as $R_1$ or $T_1$ parameter and is sensitive to very fast molecular tumbling in ps-ns time-scale. It is the process when $M_z$ magnetization returns to its thermal equilibrium at Boltzmann population of the excited energetic states in presence of $B_0$. Upon $T_1$ relaxation, spin energy will be exchanged by the environment (lattice). $T_1$ can be experimentally determined through inversion recovery experiments, in which the delay time $\tau$ between $180^\circ$ and $90^\circ$ pulse before acquisition is varied. When $\tau>T_1$, the magnetization $M_z$ is fully recovered and signal intensities are maximum.

The transverse (or spin-spin or $xy$-plane) relaxation is denoted as $R_2$ or $T_2$, describes the transverse magnetization on $xy$-plane (observable $M_x$ and $M_y$) decaying to zero. This relaxation does not cause energy exchange between nuclear system and environment, thus energy of the system is conserved. In practice, the transverse relaxation is predominantly caused by slight differences of external magnetic field $B_0$ which is experienced by every nucleus in the same sample. Consequently, some of the same nuclei will have faster precession while other have slower precession, resulting a loss of phase coherence.

Both $T_1$ and $T_2$ relaxation are dependent on intra- and extra-molecular interactions, including dipole-dipole relaxation, chemical shift anisotropy, spin-rotation relaxation, scalar coupling, electric quadrupole relaxation, interactions with unpaired electrons in paramagnetic compounds, etc (140). In protein and nucleic acid NMR, dipole-dipole relaxation and chemical shift anisotropy have important contributions to the relaxation process. The dipole-dipole relaxation time, which is proportional to correlation time $\tau_C$ in aqueous solution, has major role in $T_1$ relaxation. The smaller proteins have faster tumbling rate, shorter $\tau_C$, longer relaxation time and sharper peaks as in comparison with larger proteins. Regarding $T_2$ relaxation, chemical shift anisotropy is formed by shielding effect of surrounding electron density that causes locally miscellaneous magnetic field to nuclei. The dipole-dipole (dipolar) relaxation is resulted from dipolar interactions that depends on both of distance, angle, and relative motion between two nuclei (or two dipoles in presence of electro-magnetic field) and gyromagnetic constant for each nucleus (141).

From a practical standpoint in this thesis, the effects of $R_2$ relaxation has been utilized to probe the dissociation kinetics of YscU. Since YscUc dissociates into a folded CN fragment and an oligomeric CC fragment, observation of methyl protons in YscUc that are situated in the CC fragment are suitable markers to follow this event. We employed the $R_2$ relaxation effect that the oligomeric form of the CC fragment will have a large size
making the signals invisible to NMR. Hence, the intensity of the mentioned methyl groups will decay because of dissociation and oligomerization of the CC fragment. The dissociation process can be quantified by fitting the rates of signal reduction to single exponential decay function.

2.2.4. Proton nuclear magnetic resonance spectroscopy

Proton NMR or 1D-NMR, or $^1$H-NMR is the application of NMR to nuclei of proton $^1$H (122). A protein $^1$H-NMR spectrum is difficult to assign and interpret, however it supports information of protein folding and binding affinity with other ligands (142). An unassigned protein can be considered as a folded protein if the peaks are sharp and well dispersed in the range in both amide region (6.5 to 8 ppm) and methyl region (-1 to 1 ppm) in its $^1$H-NMR spectrum. Otherwise, if the signals have non-equal intensities and a poor dispersion, the protein can be in a globular molten structure or unfolded state (143). Furthermore, binding affinity of a ligand to a protein $P + L \leftrightarrow PL$ can be calculated by equation [4] using chemical shift variations from an individual peak upon titration in methyl region (144)

$$\Delta \delta \text{ (ppm)} = \frac{\Delta \delta_{\text{max}}}{2} \left(1 + x + \frac{K_d}{P}\right) - \sqrt{\left(1 + x + \frac{K_d}{P}\right)^2 - 4x}$$  [4]

With $P$ is concentration of protein, $x$ is molar ratio of ligand:protein, $\Delta \delta_{\text{max}}$ is the perturbation of the chemical shift when the binding is saturated, $K_d$ is dissociation constant analyzed from the peak.

2.2.5. Heteronuclear single quantum coherence spectroscopy (HSQC)

HSQC is widely used experiment resulting in 2D- spectra with one axis for proton ($^1$H) and the other for a different nucleus which can be $^{13}$C or $^{15}$N. The natural abundance of $^{13}$C or $^{15}$N is low, therefore it requires isotopically labelling of an interested protein by expression cells in $^{13}$C or $^{15}$N-labelled media. In protein NMR, a HSQC spectrum includes chemical shifts (peaks) from protons, which are attached to a nitrogen ($^1$H-$^{15}$N HSQC) or aliphatic carbon ($^1$H-$^{13}$C HSQC) in the peptide bond (145). In this thesis, we use $^1$H-$^{15}$N HSQC to evaluate protein structure and interaction. A HSQC spectrum of an isolated protein can reveal if a protein is a folded monomer with dispersed peaks, otherwise unfolded protein with broadened and not well-dispersed peaks. Protein-protein or protein-ligand binding interfaces can also be identified by spectral comparison before and after adding the non-labelled
protein/ligand to isotopically labelled protein. The binding surface will be localized on interested protein by residues with high chemical shift perturbations. In this thesis, HSQC of $^{15}$N-labelled YscUc was monitored to determine its dissociation kinetic, protein thermal unfolding and its binding surface to YscP.

2.3. Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry (ITC) is one of the most quantitative techniques that can be used to quantify the thermodynamic parameters of biological macromolecular interaction (146). In addition, the technique is non-destructive and the samples can be reused for other purposes after the ITC experiment. ITC data can complement dissection of interaction surfaces studied by NMR by adding energetic values (147). The calorimeter includes a sample cell and a reference cell in an adiabatic jacket, which preserves a fixed, small temperature difference between two of the cells (almost zero) (148). The experiment is performed by titration of a precise volume of a titrant to the sample cell filled by protein of the calorimeter at a stable temperature (149). The heat absorbed or released of the sample cell after each adding aliquot is recorded in relation to the reference cell, which contains buffer. The heat changes upon titration are converted to electric power (micro calories per second) which is required to keep the constant temperature difference of two cells (149). Therefore, ITC determines directly the heat associated with binding of titrant-protein at equilibrium and deduces the value of binding constant, stoichiometry, enthalpy and entropy (146). However, the measured heat is a universal signal, which complicates the interpretation of the results (148). As NMR, ITC demands large amount of samples, in which concentration of protein in protein-protein interaction can leads to aggregation or precipitation. Despite the disadvantages of ITC, it is a powerful technique widely used for characterization thermodynamics of binding or kinetic studies. In this thesis, ITC is used as a tool to study binding of YscUc to YscI at 25°C.

2.4. Membrane mimicking media

*Yersinia pseudotuberculosis*, as well as other Gram-negative bacteria, has a cell envelope composed by an outer membrane, an intermediate periplasm and an inner membrane (150). The outer membrane, which is unique to different Gram-negative organisms, is composed of lipopolysaccharide with inserted proteins and lipids (151). The aqueous periplasm consists of peptidoglycan (152) and functions to maintaining the shape of a bacterial cell. The inner membrane is built from phospholipid bilayer and membrane proteins (152), which supports biosynthesis and interactions of membrane
lipids and embedded proteins at the cytoplasmic surface (153,154). Nowadays, different membrane mimicked media, including bilayer sheets, micelles and liposomes (Figure 14), have been synthesized based on derived phospholipids to explore the membrane protein dynamics and interactions.

In Gram-negative bacterial membrane, phospholipids is the most abundant lipids with glycerophospholipids are predominant lipids (154). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA) are often found in the inner membrane bilayer (155). Lipid bilayers formed by phospholipids are based on their amphiphilic character. A phospholipid has a hydrophobic tail group and a hydrophilic head group, whose charge can be zwitterionic, negative or neutral dependently on the pH.

In this study, the polypeptide YscU_CN was firstly structurally characterized by CD using liposomes (lipid vesicles) as membrane mimicking media. The Yersinia inner membrane is negatively charged, thus, the working liposome was mixed by the negative charged 1,2-dimyristoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DMPG) and zwitterionic charged 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (Figure 14) in varied molar ratio. The NMR based model of the unfolded YscU_CN was achieved in sodium dodecyl sulfate micelles, suggesting the potential interaction of this linker and bacterial inner membrane.

**Figure 14:** Membrane mimicking media. (A) Schematic diagram of different membrane mimicking media (adapted from Bitounis (156)), in which liposome is studied. (B) Illustration for phospholipid structure. (C) Structure of DMPC and DMPG (adapted from Avanti Polar Lipids, Inc.)
3. Summary of papers

3.1. Paper I - Auto-proteolysis and intramolecular dissociation of Yersinia YscU precedes secretion of its C-terminal polypeptide YscUc

Summary

YscU is auto-cleaved at the conserved NPTH motif leaving the protein as a heterodimer. The role of the cleavage event is previously postulated to generate a protein-protein interaction surface that is primed for interaction with down-stream targets (69). In this paper, we present an alternative hypothesis where the role of cleavage is to enable dissociation of YscU followed by secretion of its C-terminal polypeptide YscUcc. The secretion of YscUcc is paralleled with secretion of effector proteins (Yop).

Aim of the study

In vivo, type III translocation and secretion of effector proteins are triggered by target cell contact through means of the substrate specificity switch proteins. Substrate switching can be mimicked in vitro by changing growth conditions of bacteria. Here we show that supplement calcium, changing to pH 7.0 or shifting temperature to 37 °C may induced structural changes of substrate switching protein YscU. These structural changes are likely involved in the switch from early to late substrate exporting, and consequently the ability of the type III injectisome assembly and toxin protein export. The advantages of CD spectroscopy was utilized to study calcium binding to the cytoplasmic domain YscU, and dependence of protein's thermal stability on calcium and pH. To understand the temperature stimulation of the secretion mechanism, dissociation kinetics of YscUc and non-stable variants found from secondary suppression mutations were validated with 1H-NMR.

Results

Far UV-CD revealed that YscUc was a folded protein at pH 7.4 (Figure 15) as indicated with the characteristic CD bands at 208 and 220 nm. Addition of 2.5 mM CaCl₂ (hereafter referred to as Ca) did not perturb the secondary structure of YscUc while changing pH from 7.0 to 6.0 was followed by a decrease of the CD signal at 220 nm suggesting local reorientations of protein helices. The CD spectrum of the non-cleavable YscUc(P264A) was similar to wild-type but the helical content was increased, as suggested from a more pronounced CD band at 220nm.
Figure 15: Near UV-CD spectra of YscUc at pH 7.4 (black), pH 6.0 (red) or YscUc(P264A) at pH 7.4 (green) in absence of calcium and YscUc in pH 7.4 with CaCl₂ 2.5 mM (blue) at 20°C

In case of pH 7.4 in the absence of Ca (Figure 16A), three transitions were observed during heating YscUc at defined temperatures where only the third one was reversible during a reverse temperature scan. The first (37°C) and the second transition (58°C) were irreversible for both upscan and downscan. By comparison of assigned ¹H-¹⁵N HSQC spectra of YscUc at 20°C before and after 10 minutes incubation at 60°C, pH 7.4, (Figure 16B), we found that the second transition of the CD unfolding profile was dependent on the dissociation of YscUc. In vitro the dissociation process results in a soluble and unfolded YscUcN fragment and an oligomeric assembly of YscUcc. In addition, the CD signal at 220 nm after the heating/cooling experiment suggested that stable α-helical structure was present in the C-terminal YscUcc (Figure 16C). In vivo, it has been shown that YscUcc is released from the membrane anchored YscU after dissociation (78).

Figure 16: Dissociation of YscUc. (A) Thermal unfolding of YscUc at pH 7.4. The upscan indicates for the increasing temperature scanning and the downscan indicates for the decreasing temperature scanning of the same sample. (B) ¹H-¹⁵N HSQC spectra of YscUc at 20°C, pH 7.4, before (blue) and after (red) incubation at 60°C for 10 minutes. (C) Near UV-CD of YscUc at pH 7.4 before (blue) and after (red) heating/cooling cycle in (A).
To access how calcium and pH can regulate the substrate switch in T3SS, we monitored the thermal stability of YscUc at different pH values in the presence and absence of Ca and analysed the CD signal at 220 nm. To establish the influence of pH on thermal unfolding of YscUc, thermal denaturation curve for YscUc was probed at pH 6.0 (Figure 17A). Empirically, the C-terminal auto-proteolytic product of YscUc should be insoluble due to its pI 5.5. The thermal upscan displayed two-state transition which were found to be irreversible in the downscan. Furthermore, the third transition observed at pH 7.4 was absent at pH 6.0. The melting temperatures for the observed transitions were comparable for pH 7.4 and pH 6.0. Since precipitation was observed in the cuvette for the experiment conducted at pH 6.0, it appeared that dissociated YscUcc was precipitated and therefore could not be secreted in vivo. In fact, no secreted YscUcc in supernatant of growth medium was found at pH 6.0. To further characterize the pH effect on T3SS and YscU structure, a CD-based pH titration was performed. The data in the range 4-7.5 was selected for fitting by using equation [1], in which the fitted pKa interprets for amino acids related to pH transition (Figure 17 B). The fitting provided a pKa value of 6.17 with three involved protons that might belong to Histidine (theoretical pKa value is 6.10). From the YscUc sequence, there are three histidines in C-terminal fragment and one in N terminal fragment. We concluded empirically that histidines of YscUcc were responsible for the observed pH dependencies.

Figure 17: Thermal unfolding of YscUc at pH 6.0 (A). The upscans and downscans are indicated. (B) pH titration of YscUc followed by the CD signals at 220 nm.

In the next step, the non-cleavable mutant YscUc(P264A) was subjected to thermal unfolding to address if dissociation of YscUc has biological relevance for Yops secretion. The thermogram of this mutant was significantly perturbed compared to wild-type and only one irreversible transition at 58°C was observed (Figure 18 A). The heating/cooling procedure also induced a partial unfolded product which could be quantified by using far UV-CD. To
investigate the biological relevance further, we tested effects of 2.5 mM Ca to the YscUc dissociation in vitro by using thermal unfolding experiments. Interestingly, thermal unfolding of wild-type YscUc in the presence of Ca was notably similar to variant P264A in absence of Ca (Figure 18B). The calcium titration curve fitted by equation [2] revealed the dissociation constant $K_d$ was 800 µM (Figure 18C). Hence, adding calcium mimics the effect of a mutation that inhibits YscUc auto-proteolysis.

**Figure 18:** Thermal unfolding of YscUc(P264A) at pH 7.4 (A), and YscUc at pH 7.4 with CaCl$_2$ 2.5 mM (B), monitored by CD at 220 nm. The upscan indicates for the increasing temperature scanning and the downscan indicates for the decreasing temperature scanning of the same sample. (C) Calcium titration by using CaCl$_2$ as titrant of YscUc at 20°C, monitored by CD at 220 nm. Solid line is correspondent to fit of the data by equation [2].

We attempted to further characterize the selectivity of calcium binding by using other divalent cations as MgCl$_2$, BaCl$_2$ and SrCl$_2$ (Figure 19). Ion titrations to YscUc resulted in µM range $K_d$ values ($K_d$ 126 µM, 900 µM and 858 µM for Mg$^{2+}$, Ba$^{2+}$ and Sr$^{2+}$ respectively) by fitting to a one site binding model (equation [2]). Thermal unfolding of YscUc in presence of these alkaline ions were similar with calcium thermal denaturation where only one transition was recorded. These findings indicated that YscU interacted non-selectively with divalent cations. Therefore, the regulation of calcium in T3SS may involve also other unknown proteins. In summary, both calcium and low pH altered dissociation of YscUCC from the YscUCN polypeptide. The thermal unfolding parameters from thermograms in Figure 17, 18 were fitted by equation [3] and summarized in table 2.
Figure 19: Divalent cation titration by using MgCl$_2$, BaCl$_2$ or SrCl$_2$ as titrant of YscU$_C$ at 20°C (A), monitored by CD at 220 nm; solid lines is correspondent to fit of the data by equation [2]. Thermal unfolding of YscU$_C$ at pH 7.4 with MgCl$_2$, BaCl$_2$ or SrCl$_2$ 2.5 mM (B), monitored by CD at 220 nm. Samples with MgCl$_2$, BaCl$_2$ or SrCl$_2$ are coloured as black, green or pink, respectively.

Table 2: Summary of thermal unfolding parameter on working proteins

<table>
<thead>
<tr>
<th>Parameter</th>
<th>YscU$_C$ pH 6.0</th>
<th>YscU$_C$(P26A) pH 7.4</th>
<th>YscU$_C$ pH 7.4 2.5 mM Ca$^{2+}$</th>
<th>YscU$_C$ pH 7.4 2.5 mM Mg$^{2+}$</th>
<th>YscU$_C$ pH 7.4 2.5 mM Ba$^{2+}$</th>
<th>YscU$_C$ pH 7.4 2.5 mM Sr$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta H_{Tm}$ kJ mol$^{-1}$</td>
<td>534</td>
<td>385</td>
<td>426</td>
<td>427</td>
<td>336</td>
<td>430</td>
</tr>
<tr>
<td>$T_m$, °C</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>57</td>
<td>56</td>
</tr>
</tbody>
</table>

The results described above demonstrate the crucial role of YscUCC dissociation from remainder of YscU for function of the T3SS. It has previously been shown that a null mutation of the yscP product results in a non-secreting phenotype. This phenotype can be partially reverted by introduction of specific mutations in the YscU gene. These gain of function mutations are formally denoted as secondary suppressor mutations. The specific mutations were previously identified from genetic experiments based on protein homologs in flagella (96). Previous data have been shown that substitutions of the particular residues in YscUCC could suppress the Yops secretion phenotype of the Yersinia ΔyscP mutant (53,96,100). Interestingly, these residues are either partially or fully buried in protein structure. Mutation at buried positions are expected to act as destabilizing. In yscP suppressor YscU variants, it turned out that mutation of the buried amino acid on YscUcc did not reorder protein structure but decreased protein stability compared to wild type. We focused on the suppressor mutant YscUCC(V292T) due to its high purification yield to examine the protein stability (Figure 20). It has been noticed that a temperature shift from 26 to 37 °C
together with removal of Ca (by adding chelating agent EGTA, for instance) induces Yops secretion (39), thus both wild-type and mutant of YscUC would be checked stability at 37 °C by following the signal intensity of $^1$H-NMR dependence on incubation time. These observed methyl signals in YscUC spectra were broadened beyond detection, that was due to dissociation and formation of the YscUC oligomer. Protein stability was reported as dissociation lifetime ($\tau_{\text{diss}}$) in this paper. We found that the dissociation kinetics of YscU(V292T) at 30 °C was very similar to the wild-type at 37 °C. Furthermore, the wild-type displayed virtually no dissociation at 30 °C. Hence the stability of the wild-type protein was strong enough to prevent the dissociation process at 30 °C. From these findings, the model with dissociation of YscUC should result in a scenario where a strain carrying the YscU(V292T) substitution should secrete Yops at 30°C whereas the wild-type strain would not secrete effector proteins at this temperature. This postulate was subsequently confirmed with secretion experiments at 30 °C while YscU(V292T) or YscU(A268F) was able to secrete Yops when no effector protein was found for YscU. It should be noticed that suppressor mutations retain calcium and pH-regulated manner. Taken together, we have indicated a strong correlation in vivo and in vitro for the fundamental required dissociation of YscUC from full length YscU.

**Figure 20:** Primary $^1$H-NMR signal dependence on incubation time for quantification of dissociation kinetics (A). Signal at 0, 145 or 882 minute incubation time is shown in black, red and green, respectively. Dissociation kinetics quantified as dissociation life-times ($\tau_{\text{diss}}$) of YscUC (black) and V292T (red) at pH 7.4 followed with NMR-spectroscopy at 37°C (B) and 30°C (C) respectively. Solid lines is correspondent to fit of the signal to single exponential decays.

**Conclusion**

*Yersinia* YscU and its homologs in other bacteria have a conserved NPTH motif, which is responsible for auto-proteolysis of the protein. It has been hypothesized that the auto-cleavage results in exposure a new binding surface of YscU to the other T3SS proteins therefore it has function as a
substrate specificity switch (69). In this paper, we suggested an alternative mechanism, in which dissociation YscUcc from the full-length YscU, not auto-cleavage, had an essential role in substrate switching.

In vivo, we showed that Yops secretion was regulated effectively by adding 1 mM calcium or pH below 6.5. In vitro analysis, Ca binding to YscUc with \( K_d \) 800 \( \mu \)M, which interferes protein dissociation, is in agreement with the obtained results in vivo. In addition, other divalent cations as Mg\(^{2+}\), Sr\(^{2+}\), Ba\(^{2+}\) also interact with YscUc at similar extent illustrating for the indiscriminative binding of YscUc to the alkaline earth metal ions, therefore calcium regulation of T3SS can likely not be simplified based only on YscU-calcium interaction. Regarding regulation of pH in Yops secretion, we suggest that histidines of YscUcc are responsible for the pH effect. However, the NPTH histidine is not suitable for mutation due to its functional role in protein auto-cleavage. Thus, pH is potentially an extracellular regulation for T3SS. Regulation of temperature on T3SS was also included in this study based on the yscU suppressor mutants, which can recover Yops secretion with a null-mutation “needle-length regulator” protein yscP. These point mutations were found to be located in YscUcc, which might induce structural perturbation and dissociation of YscU. Indeed, bacterial strains carried the suppressor mutants showed Yops secretion at 30°C while wild-type strain could not secrete Yops in absence of calcium. Dissociation kinetic of wild-type and suppressor mutant YscUc(V292T) quantified by \(^1\)H-NMR demonstrated that dissociation of YscUc(V292T) at 30°C was as fast as wild-type at 37°C. As conclusion, these findings reveal for the remarkable importance of YscU dissociation and YscUcc secretion.
3.2. **Paper II** – Negatively charged lipid membranes promote a disorder-order transition in the *Yersinia* YscU protein.

**Summary**

YscU and homologues in other bacteria are two-domain proteins consisting of one transmembrane domain and one soluble domain (denoted YscUc in *Yersinia*) that are separated by a linker sequence. The prevailing concept is that the linker is flexible inside bacteria and the soluble domain is can freely diffuse within the limits defined by the length of the linker. In this study, we showed that the linker sequence in *Yersinia* could bind to the negatively charged membrane surfaces. We suggest that the linker anchors the soluble YscUc domain to the negatively charged inner membrane through electrostatic interactions formed by helical segments that are induced by the membrane interaction. Functional analysis demonstrated the relevance of the interaction in vivo. The findings add a novel concept to the structural biology of the secretion switch protein YscU in *Yersinia*.

**Aim of the study**

The polypeptide YscUcn studied by us includes the linker sequence that connects the soluble domain with the transmembrane spanning domain. YscUcn was found to be unfolded in solution by NMR as indicated in Paper I. On the other hand, crystal structure of YscUc showed that YscUcn (200-263) contained a short α helix and a β sheet. The disagreement between NMR and crystal structure can be interpreted by different physical-chemical phases of collected samples, but indicates that part of the YscUcn polypeptide may have helical propensity (157,158). We then aligned a number of protein sequences from YscU/HllB homologs from other organisms. Interestingly, we found that positively charged residues arranged with a pattern of amphipathic helical periodicity in YscUcn cross species. It should be noticed that YscU is a membrane protein that places the linker sequence in close proximity to the negatively charged inner membrane of Gram-negative *Yersinia*. Therefore, the membrane may interact with YscUcn polypeptide and facilitate α helical conformation for this linker. This paper investigated for the first time the membrane induced structural changes of YscUcn by using membrane mimicking liposomes and CD spectroscopy. A NMR based structure was determined in complex with sodium dodecyl sulfate micelle and the structural positions conserved positively charged amino acids were examined. Correlation of biological aspects in vitro was tested by replacement of these amino acids by alanine.
Results

First of all, we attempted to isolate wild-type YscUΔN by heating YscUΔC at 80°C, oligomer YscUΔCC was formed and precipitated, YscUΔN in supernatant was collected for further studies. In the next step, interaction of this polypeptide with membrane models was investigated with CD and NMR spectroscopy. Far UV-CD of purified YscUΔN showed that it predominantly populated a random coil structure. Adding zwitterionic liposome DMPC did not disturb the peptide secondary structure. This observation is in line with our model since YscUΔN is positively charged at pH 7.4. Titration of negatively charged liposomes composed of DMPG/DMPC mixtures gave rise to changes in CD signal, especially at 208 and 220 nm (Figure 21A). The new peaks were more pronounced when increasing the proportion of anionic DMPG incorporated into neutral DMPC vesicles. Those findings indicate that the peptide-vesicle interaction is driven by electrostatics and in accompanying with a disorder-order transition in YscUΔN.

To further characterize binding of YscUΔN to a more realistic inner membrane, liposomes were generated from a E.coli lipid extract. In order to enable high-resolution structural analysis by NMR, we also studied the interaction between YscUΔN and SDS micelles. The lipid extract was able to induce disorder-to-order transition which was confirmed by an increasing of the helical content of protein structure. However, this enhancement was less pronounced in comparison with SDS micelles. In HSQC spectra for 15N-labeled YscUΔN, presence of SDS micelles induced a remarkable signal dispersion of YscUΔN that suggests a large structural transition of protein in the samples. The atomic details of the structural changes were investigated by assignment of backbone resonances and chemical shift analysis, resulting in folded YscUΔN with three α helices S[0]-K215, K218-E29, and I234-Q246. Positions of the three helices in micelle media were investigated by performance of paramagnetic relaxation enhancements (PRE) with doxyl-5-stereate or Mn²⁺. Doxyl-5-stereate experiment denoted that helix 1 and 3 were buried with enhanced PRE, while adding Mn²⁺ caused stronger PRE for helix 2 and weaker for helix 1 and 3. Both of the results confirmed that residues in helix 1 and 3 were protected inside micelles and residues in helix 2 stayed closely to micelle surface with highly solvent accessible.

The biological relevance of the membrane binding by YscUΔN was examined by performing the Yops secretion assay for ΔyscU strains with single mutations introduced into yscU. In a first proof of concept, all positively charged aminoacid residues in helix 1 and helix 2 were replaced with alanine. The residues were selected to be K212, K215, K218, K222, R223 and K226. The membrane interacting capacity of the mutant was strongly
impaired. Further, we observed no secreted Yop in the growth media but they were produced at the same level as wild type in the cell pellet. Thus, we come to conclusion that positively charged residues of YscUCN are essential for the function of the T3SS due to their role in membrane interaction.

To test the importance of each positively charred residue in YscU, we performed the Yops secretion assay for every single mutant. Yopssecretion level of K212A was maintained at wild-type despite the fact that it is the most conserved residue found in different bacterial YscUCN sequences. Strain of K222A or K226 secreted about 25% Yops lower than wild-type. Strain of K215A, K218A or R223A displayed the lowest Yops secretion levels with only 50% compared to wild type. Thus, YscUCN with K218A or R223A was selected for further characterization in vitro. Some strains with negatively charged residues as E213, E220 and E224 were also mutated to alanine as positive controls of membrane binding in the Yops assay. The mutation E213A did not affect to export effector proteins but the other glutamic acid mutants significantly attenuated Yops secretion. These mutant behaviours could not be explained by deletion of the membrane interaction. Therefore, E220A together with K218A, R223A and 6 alanine replaced mutants were purified and characterized for their binding capacity to membrane mimicking vesicle. The CD signal at 220 nm of protein samples, which is indicative of helical content of secondary structure of protein, was acquired in absence or presence of vesicles with different negatively charged densities (Figure 21 B). YscUCN and its variant with 6 alanine substitutions displayed the highest and lowest binding capacity to negatively charged vesicles, respectively. The positively charged mutants K218A and R223A showed intermediate binding capacity to vesicles. The negatively charged mutants E220A could bind to vesicles as strong as wild-type YscUCN, indicating that membrane interaction is depended on the positive charged residues. The elimination of Yops secretion in case of E220A could potentially be explained by alternation binding surface of other T3SS protein to YscU. In combination of Yops secretion in vivo and mutation experiment in vitro, we speculate that membrane interaction by reserved positive amino acids is functional correlated with effector secretion.
Figure 21: Disorder-to-order transition of YscU<sub>CN</sub> variants upon titration by anion phospholipid vesicles. (A) Far UV-CD probes secondary structural changes of YscU<sub>CN</sub> in response to anionic charge density. (B) Interaction capacity of YscU<sub>CN</sub> mutants to vesicles with different negatively charged densities, with the molar ratio of DMPG and DMPC in 2 mM is varied. The interaction was monitored by following the CD signal at 220 nm. Wild-type YscU<sub>CN</sub> is denoted as CN<sub>WT</sub>, its variants were denoted as CN<sub>x</sub> with x is represented mutated residue number, CN<sub>6</sub> is variant with 6 alanine substitution.

Conclusion

In this paper, we have found that the linker peptide in YscU is able to bind to the bacterial inner membrane. This interaction is driven by electrostatics between positively charged residues and negatively charged inner membrane. A structure calculation from NMR with YscU<sub>CN</sub> in complex with SDS micelles demonstrated the formation of three α-helices in YscU<sub>CN</sub> upon membrane interaction. In vivo, Yops secretion assay confirms the importance of the interaction by single point mutation at positively charged residues. However, we were unable to distinguish whether the binding model was following conformational selection or induced fit pathway. Testing binding capacity of different mutants suggested from NMR has confirmed the in vivo and in vitro correlation of the observed data. The highly conserved residues and interaction with E. coli membrane extract of YscU<sub>CN</sub> indicates that our model can be relevant not only in Yersinia but also other Gram negative pathogenic bacteria that utilize the T3SS.
3.3. Paper III - Characterization of the ruler protein interaction interface on the substrate specificity switch protein in the Yersinia type III secretion system

Summary

In T3SS, the most favored model is that auto-proteolysis has a structural role and has involved to generate a binding surface for other T3SS proteins. In this model, YscU and related proteins in other bacteria are auto-cleaved during their folding process and consequently no uncleaved protein is present inside living bacteria. In this manuscript, we have identified the binding interface for YscP on YscU. The binding interface was identified with NMR spectroscopy and was found to be centered on the C-terminal α-helix in YscU. The interaction was found to be biological significance since disruption of YscU/YscP binding by mutation of the binding interface significantly reduced the Yops secretion capacity. By studying a slowly self-cleaving YscU variant (P264A) we found that YscP eliminated the rate-constant of auto-cleavage. On basis of our experimental data, we propose a model where YscP binding to YscU inhibits auto-cleavage; consequently release of YscP is a trigger signal for YscU auto-cleavage. Therefore, we suggest that YscU auto-cleavage has a regulatory rather than structural role. This new model indicates that there should be non-cleaved full-length YscU inside living bacteria and we have recently achieve this observation in Yersinia cells (unpublished data).

Aim of the study

The direct interactions between the YscP/YscU homologues in both Pseudomonas aeruginosa (56) and Salmonella typhimurium (98) have been established recently. In this manuscript, we attempt to identify the YscP binding interface on YscU and to characterize the structural properties of YscP. The YscP structure was determined by applying both CD and NMR spectroscopy for full-length and various truncated YscP variants. In addition, we set out experiments to explore a potential physical interaction between the YscU and the inner rod protein YscI by making use of isothermal titration calorimetry.

Results

YscP is a large (455 residues) and partially folded protein, which imposes significant challenges for its structural characterization by using NMR or crystallography. In order to generate suitable constructs for analysis of YscP, we combined sequence alignments and a limited proteolysis experiment,
resulting in several protein sequences. By analyzing the constructs illustrated in Figure 22, we found that YscP consisted of a large N-terminal disordered segment and a small C-terminal folded domain. This domain architecture has previously been observed for the PscP protein in *Pseudomonas aeruginosa* with a ball-and-chain arrangement (56). Our data displayed that this arrangement was a conserved feature in the YscP family of proteins. However, purified YscP variants were aggregated with high number of involved molecules; therefore, no high-resolution structures of them were reported. In the next step, binding between YscP and YscU was quantified with NMR spectroscopy using both 1D and 2D experiments. From comparative analysis of the different YscP constructs, we found that the binding activity toward YscU was confined to the YscP segment composed of residues 203-341.

![Figure 22](image.png)

**Figure 22**: Schematic illustration of different YscP deletion mutants used in this paper. YscP is a largely unfolded protein with a short folded domain consisting of residues 342 to 442.

The binding affinity (*Kd*) of YscU and YscP was quantified by following methyl resonances in 1H-NMR spectra at methyl region when titration silent NMR YscP to YscUc. Based on the changes of the peaks, the *Kd* value was calculated to be 430 μM based on equation [4]. We then attempt to identify the YscP binding surface on YscU by titration of unlabeled YscP to 15N-labelled YscUc, and monitor changes in YscU resonances in HSQC experiments. The experiments were performed at 37 °C to mimic biological temperature (Figure 23). Plotting the residues with high chemical shift perturbations and high solvent accessible on the YscUc crystal structure showed that the binding surface is centered on the last α helix of YscUc. In order to address the functional role of the interaction, binding interface residues were selected to be replaced as following: L280N, E332A, A335N, E336A. The *Yersinia ΔyscU* strain was replaced with plasmids carried mutated yscU in Yops secretion assays and HELA cell cytotoxicity experiments. Mutation of E336A had no effect on Yops secretion, while E332A had no effect on both Yops secretion and HELA cell. yscU(L280A) had intermediate effect on Yops secretion and HELA cell. Notably, yscU(A335N) significantly lowered Yops secretion and no cytotoxicity of HELA cells was
detected in this experiment. We showed with NMR spectroscopy that the A335N variant is a well-folded protein, hence the functional effect of the mutation is strictly confined to disruption of the YscP binding activity. Taking together, residue A335 on YscU is concluded as a key residue of T3SS due to its function in YscU-YscP interaction.

Figure 23: Identification of the YscP binding interface on YscUC. (A) $^1$H-$^15$N HSQC of $^15$N-labelled YscUC in absence (blue) and presence (red) of YscP at a molar ratio 1:4. The arrows indicate peaks with large chemical shift perturbations caused by adding YscP. (B) Residues with large chemical shift perturbations are plotted on the YscUC crystal structure as blue ball and stick. The green residue A335 with high influence on Yops secretion is shown with ball and stick.

To test if the binding of YscP can influence the rate of YscU auto-cleavage we developed a protocol based on Western blots. Since auto-cleavage of wild-type YscUC takes place during purification, the Western blot assay displayed cleaved YscUC as two bands. In order to follow the process of auto-cleavage we turned to the slowly auto-cleaving mutant P264A. The non-cleavable mutant YscUC(P264A) and YscUC(P264A/A335N) were incubated with varied molar ratio of YscP at 37°C for 0, 1 and 2 days. The obtained samples were diluted and applied to SDS-PAGE, followed by Western blot assay with YscUC as primary antibody. Analysis of band intensities from YscUC(P264A) indicated that YscP binding reduced the rate-constant of auto-proteolysis. The YscUC(P264A/A335N) with the disrupted YscP binding capacity was used a control and we found that the inhibitory effect of YscP on auto-cleavage of this variant was diminished. All of these data strongly suggest that YscP binding can inhibit the rate of YscU auto-cleavage and consequently its regulatory role in the T3SS.

In addition, YscP and YscU coordinate to regulate secretion of the inner rod protein YscI in T3SS assembly. YscI is a structural protein and has tendency to aggregate. Indeed, we showed that YscI was a protein with high
helical content and aggregated at $\mu$M concentrations. Therefore no further structural research was performed for YscI. Isothermal titration calorimetry showed that YscI was binding to YscUc with a $K_d$ 3.8 $\mu$M at 1:1 stoichiometry. NMR spectroscopy showed that the YscI binding surface on YscU was distinct from the YscP binding surface.

**Conclusion**

In this manuscript, the structure of *Yersinia* YscP was found as a partially unfolded protein. Despite of similar function of its homologs, the YscP folded domain was found to be aggregated while its homologs are monomeric and accessible for structural characterization. We found that YscP interacted with YscU on a particular surface located on the last helix with high solvent accessible and inhibits YscU auto-cleavage. In addition, the inner rod protein YscI interacts with YscU with one to one ratio. YscU and YscP are considered to influence YscI secretion, which upregulate effector secretion. These findings hint for a complicated mechanism how the switch is triggered in assembly of T3SS.
3.4. **Paper IV** - Targeting dissociation of the substrate specificity switch protein YscU in the *Yersinia* type III secretion system with small molecules

**Summary**

From the results in papers I and III we have suggested a model where the functional role of YscU auto-proteolysis is to enable its dissociation and subsequent secretion of its C-terminal polypeptide (YscUcc). This model is inconsistent with the dominating paradigm in the field where the role of auto-proteolysis is purely structural and is proposed to generate a protein-protein interaction surface for downstream targets. Our model opens a new possibility for small molecule interference with the pathogenic process, namely inhibition of YscU dissociation through stabilization of the protein by binding of small-molecules. This research has indicated several classes of molecules that may bind to YscU. A subset of these molecules can hinder the dissociation event and inhibit secretion of effector proteins to some degree and hinder *Yersinia* induced cytotoxicity of HELA cells. Although the concept appears promising, more works are required to establish the idea.

**Aim of the study**

Several Gram-negative bacteria have share similar virulence plasmids that are coding for the T3SS. A possible way of interfering with bacterial virulence is to target the injectisome assembly. The T3SS YscU/FlhB family of proteins has a conserved NPTH motif that has important function as regulator for needle assembly and effector protein secretion. This motif facilitates YscU/FlhB auto-protelysis, which is hypothesized to enable dissociation of YscU in paper I. In this study, we are addressing whether small-molecule binding to YscU can hinder its dissociation and subsequently interfere with the pathogenic process. The fragment based screening method based on 1D-NMR spectroscopy was employed to identify small-molecules, which could bind to YscU. The influence of molecular binding on YscUc dissociation was evaluated by the NMR protocol published in paper I. Finally, the functional consequence of the small molecules was investigated by analysing effector protein secretion and also *Yersinia* induced HELA cell cytotoxicity.

**Results**

First of all, a compound library was scanned to identify YscUc binders by a fragment based screening. A spectrum from a mixture of different compounds was acquired as reference with T2 relaxation of NMR
experiments. A small volume of YscUc was added to NMR tube contained the references compounds and spectra of the molecules were compared before and after adding protein. We have screened through 928 compounds in 116 mixtures, observing two fragments with strong reduction (Figure 24A)), five molecules with moderate reduction, three molecules with weak reduction in the signal intensities of NMR spectra, meaning that these observed molecules were able to interact selectively with YscUc. Compounds was selected for further analysis on basis of their commercial availability and solubility in common buffer. These obtained molecules are illustrated in Figure 24B together with their abbreviations used in this study.

![Figure 24: Fragment based screening to identify small-molecules binding to YscUc. (A) Illustration of spectral changes of a “strong” binding compound before and after adding YscUc. (B) Summary of compounds used in this paper. 3A and 3B have significant changes in spectra after adding YscUc. 2A, 2B and 2C have moderate signal reductions in the spectra in presence of YscUc.](image)

We then evaluated the potentially structural alterations caused by these collected fragments to YscUc. Spectra of 15N-labelled YscUc samples were compared before and after adding of compounds. However, only minor changes in chemical shifts were observed in the spectra, thus no structural perturbation was identified for any of compounds at 800 μM. This result indicates that compounds interact to YscUc with a weak affinity of interaction or insignificant structural perturbations. However, the fragment bindings may alter YscUc stability rather than inducing structural changes. We have shown in paper I that dissociation of YscUc results in an unfolded YscUcN and folded oligomeric YscUcc; therefore, the signal intensity of residues in the C-terminal YscUcc polypeptide decay following the incubation at 37 °C. In this study, the dissociation kinetic of YscUc was probed in the presence of the identified small-molecules and reported as dissociation lifetime $\tau_{\text{diss}}$. We were able to identify two compounds denoted 2C or 3A which worked as dissociation inhibitor of YscU. $\tau_{\text{diss}}$ of YscUc was increased from $19.8 \pm 1.5$ hours to $35.6 \pm 5.7$ hours in presence of 2C or 3A.
In combination with the YscUc dissociation assay, we used the Yops secretion assay to determine effects of YscUc binding with other small-molecules in vivo. Both the levels of Yops remaining in bacterial cells after secretion and Yops secreted to the growth media supernatant were analysed at compound’s concentration of 400 μM. Compound 2B and 3B significantly decreased cellular and secreted Yops level. Compound 2A lowered at intermediate level of cellular and secreted Yops while compound 2C decreased small amount of secreted Yops and remained same amounts of Yops kept in cells. Fragment 3A with highest inhibition ability in vitro was found as having less effect to Yops level in both cellular and secreted levels. Therefore, 2C was a potential candidate for drug design and YscUc structure in presence of 2C would be studied further. Since no chemical shift perturbations was observed in NMR experiments, we attempted to utilize crystallography to establish the binding interface of 2C on YscUc(Figure 25). The fragment 2C promoted crystallization of YscUc as a dimer, of which 2C was detected at a hydrophobic pocket formed by last helixes of the YscUc monomers. This information means that 2C bound to the YscP binding surface which has been identified in Paper III. Notably, the linker region between the cytoplasmic C-terminal domain and a four-helix transmembrane domain was visible as an elongated α-helix, which is coincident with our model for membrane interaction in paper II. These findings suggest that targeting of YscU is a realistic strategy. However, we recognize that the biological effects related to the observed compounds likely are more complex than just inhibition of YscU dissociation/auto-proteolysis.

**Figure 25:** Crystal structure of YscUc in presence of 2C (methyl(5-methyl-2-phenyl-1,3-thiazolidin-4-yl)acetate). YscUc was crystallized as dimer, with YscUcN is coloured as pink and YscUcC is coloured as red. Compound 2C, which was located in pocket formed by the two monomers, is shown as green sticks and residue A335, which is responsible for YscP binding on YscUc, is shown as yellow spheres.
**Conclusion**

In this paper, the dissociation of a protein is for the first time considered as a potential therapeutic strategy. The conserved NPTH motif is found in the cytoplasmic domain of YscU and is responsible for protein auto-proteolysis, followed by dissociation. A fragment based screening method using NMR was employed in this paper to verify small organic molecules which could bind to YscUc. We found several compounds from the 928 molecular library, meaning that these compounds were binding selectively to YscUc. Dissociation assays and crystallography of YscUc in presence of a particular molecule named 2C indicated that this fragment could bind to YscUcat YscP binding interface and facilitate protein stability. However, the influence of the 2C and other observed compounds on Yops secretion and translocation were ambiguous that are needed to be optimized for future aspects.
4. Main observations and outlook

The main purpose of this thesis is to contribute to a better understanding of T3SS assembly and function. The subtract specificity switch protein YscU is a remarkable multi-functional protein which regulates T3SS function through specific aspects of its structure. Here we have utilized a combined biophysics and functional approaches in order to address the function of YscU. In the thesis, we have been part in discovering several new aspects of YscU function. The main findings are:

1. The evolutionary role of YscU auto-proteolysis is to enable dissociation of the protein with subsequent secretion of its C-terminal fragment YscUc.

2. Binding of YscP to a C-terminal binding surface on YscU may have a regulatory role for the rate-constant of YscU auto-proteolysis in vivo.

3. The linker sequence that separates the transmembrane and cytoplasmic domains can interact with the inner-membrane of Yersinia.

Our studies indicate that YscU linker separating the transmembrane domain and cytoplasmic domain undergoes a disorder-to-order transition upon interaction with negatively charged membrane models. In addition, YscU interacts with the needle-length regulator protein YscP with a binding surface centred on the last helix of YscU. Disruption of the binding surface with mutation resulted in significantly reduced Yops secretion and translocation to eukaryotic host cells. Due to aggregation of YscP deletion mutants, we could not determine its complete quaternary structure but the YscU binding site was validated to be at coiled N-terminal domain of YscP. Previous studies have indicated that YscP controls the length of the needle assembly indirectly by a secretion signal sequence located at its N-terminal domain. Our observation strongly indicates that YscP may adjust the injectisome assembly through binding to YscU. Further, we have demonstrated that YscU can interact with the inner rod protein YscI with low micro-molar affinity. We also found that the YscP and YscI binding interfaces on YscU were distinct and non-overlapping.

In terms of future, therapeutic discovery for small molecules that stabilize YscU and inhibit its dissociation would constitute a fundamentally new approach. Traditionally, antibiotics target to interfere with the active site of enzymes, targeting of YscU stability is beneficial since any variation in its stability causes perturbations in Yops secretion and translocation. We have made initial attempts in this direction and we have discovered small
molecules that can bind to YscUc. However, more works are required in order to establish if such molecules can reduce the pathogenic potential of *Yersinia*. Another future direction is to analyse the rate-constant and regulation of YscU auto-proteolysis *in vivo*. We have preliminary observations showing that YscU indeed is present in an un-cleaved state inside *Yersinia*. This result means that auto-cleavage of YscU is regulated. Summary of this thesis is illustrated in Figure 26.

**Figure 26:** Model of YscU structure, dissociation and its interaction with YscI and YscP *in vitro*.
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Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.

- Marie Curie