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Multiple functions of YopN in the *Yersinia pseudotuberculosis* type III secretion system

From regulation to *in vivo* infection

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Waking up

But not opening my eyes.

Because

When you turn one more page

In the first war ever known

Before all legends,

Mythologies,

And crimson,

You have a side

By the evil!

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Abstract

The type 3 secretion systems (T3SSs) are virulence mechanisms used by various Gram-negative bacteria to overcome the host immunity. They are often target-cell contact induced and activated. Activation results in targeting of virulence effector substrates into host cells. One class of secreted substrates, translocators, are required for the intracellular targeting of the second class, the virulence effectors, into host target cells. T3SSs are mainly regulated at 2 levels; a shift from environmental to host temperature results in low level induction of the system whereas target cell contact further induces and activates the system. In the *Yersinia* T3SS, YopN, one of the secreted substrates, is involved in the latter level of activation. Under non-inducing conditions, YopN complexes with TyeA, SycN and YscB and this complex suppresses the T3SS via an unknown mechanism. When the system is induced, the complex is believed to dissociate and YopN is secreted resulting in the activation of the system. Earlier studies indicated that YopN is not only secreted but also translocated into target cells in a T3SS dependent manner. TyeA, SycN and YscB bind to the C-terminal and N-terminal YopN respectively but so far the central region (CR) of YopN has not been characterized. In this study we have focused on the function of the YopN central region.

We therefore generated in-frame deletion mutants within the CR of YopN. One of these deletion mutants, aa 76-181, showed decreased early translocation of both YopE and YopH into infected host cells and also failed to efficiently block phagocytosis by macrophages. However, the $YopN_{\Delta 76\text{-}181}$ protein was expressed at lower levels compared to wt YopN and also showed a slightly deregulated phenotype when expressed from its native promoter and were as a consequence not possible to use in *in vivo* infection studies.

Therefore, we generated mutants that disrupted a putative coiled coil domain located at the very N-terminal of CR. Similar to $YopN_{\Delta 76\text{-}181}$, these substitution mutants were affected in the early translocation of effector proteins. Importantly, they were as stable as wt YopN when expressed from the native promoter. One of these mutants was unable to cause systemic infection in mice indicating that YopN indeed also has a direct role in virulence and is required for establishment of systemic infection *in vivo*.

Papers included in this thesis

Bamyaci S, Nordfelth R and Forsberg Å (2019) **Kinetics of Type III secretion in *Yersinia* and sub-cellular localization of the Yops under non-inducing conditions.** Manuscript

Bamyaci S*, Ekestubbe S*, Nordfelth R, Erttmann SF, Edgren T and Forsberg Å. (2018) **YopN is required for efficient effector translocation and virulence in *Yersinia pseudotuberculosis*.** Infect Immun 86:e00957-17.

Bamyaci S, Nordfelth R and Forsberg Å**. Identification of specific sequence motif of YopN of *Yersinia pseudotuberculosis* required for systemic infection.** (2019) Virulence, 10:1, 10-25

*Denotes equal contribution

List of abbreviations

aa	Amino acid
ABC	ATP-binding cassette
Ail	Attachment and invasion locus
Arg	Arginine
ATP	Adenosine triphosphate
Bla	Beta-lactamase
Bp	Base pairs
CB	Calcium blind
CBD	Chaperone binding domain
CCD	Coiled coil domain
Cop	*Chlamydia* outer protein
CR	Central region
DNA	Deoxyribonucleic acid
EPEC	Enteropathogenic *Escherichia coli*
Esc	*Escherichia* secretion
FAK	Focal adhesion kinase
GAP	GTPase activating protein
GTP	Guanosine triphosphate
HA	Hemagglutinin
IM	Inner membrane
Inv	Invasion
Ipa	Invasion plasmid antigen
IVIS	*In vivo* Imaging System
Kb	Kilo base
kDA	Kilo Dalton
Lcr	Low calcium response
MAPK	Mitogen activated protein kinase
MLNs	Mesenteric lymph nodes
MOI	Multiplicity of infection
mRNA	Messenger RNA
Mxi	Membrane expression of Ipa
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
OM	Outer membrane
p.i.	Post infection
Pcr	*Pseudomonas* calcium response
PMF	Proton motive force
PLC	Phospholipase C
PLD	Phospholipase D
Pop	*Pseudomonas* outer proteins
PPs	Peyer's patches
PTPase	Protein tyrosine phosphatase
RACK1	Receptor for activated C-kinase 1
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid

Sep	Secretion of *E.coli* proteins
Spa	Surface presentation of antigen
Ssa	Secretion system apparatus
SPI	*Salmonella* pathogenicity island
SS	Signal sequence
Syc	Specific Yop Chaperone
T3SA	Type 3 secretion apparatus
T3SS	Type 3 secretion system
TBD	TyeA binding domain
TnSS	Type n secretion system
Tat	Twin-arginine translocation
TyeA	Translocation of Yops into eukaryotic cells A
UTR	Untranslated region
Y2H	Yeast-two-hybrid
YadA	*Yersinia* adhesin A
Yop	*Yersinia* outer proteins
YopE-Bla	$YopH_{6-86}$-Bla
YopH-Bla	$YopE_{6-99}$-Bla
Ysc	*Yersinia* secretion

1. Introduction

Since the first emergence of life, living entities have developed mechanisms to sense their environment to better adapt to their respective niche. The growing number of these entities in time forced them to develop strategies to interact with each other as well. One of the most striking and important example to these interactions was proposed by the pioneering work of Margulis, where she introduced endosymbiosis as the mechanism of eukaryotic cell evolution (270). This theory has been developed since then and today it is widely believed that mitochondria and plastids developed from prokaryotic organisms that were internalized by an archean cell. After multiple levels of gene exchange between the host and the symbionts (198), they became indispensable to each other and formed the ancestor of what we today call 'a eukaryotic cell'.

The interaction between the living entities is still ongoing and today they are one of the most important aspects of human health and an important branch of biological research. In our bodies the number of commensal bacteria, most of which are "the good guys", exceeds the number of our own cells. They are found on our skin as well as within our body and are part of a mutual beneficial relationship, e.g. by helping us in digestion and to fight against the pathogenic bacteria, the bad guys.

1.1. Pathogenesis – different life styles of pathogens

The continuous interaction between the eukaryotes and pathogenic bacteria forced both sides to co-evolve, the host to protect itself against the pathogens and the pathogens to overcome the protective action taken by the host. Millions of years of co-evolution resulted in the immune system in higher eukaryotes which is the main defense strategy against pathogens. Today, we know that both animals and plants have highly regulated immune systems against any kind of invaders. On the other hand, pathogenic bacteria needed to evolve novel strategies to thwart immunity and survive. In the border line between the commensal and pathogenic bacteria, opportunistic pathogens lie. Normally,

opportunistic pathogens are not harmful and found in hosts without causing any infection or symptoms. However, as their name implies, if host immunity is impaired, they take the opportunity and act as pathogens by defeating the weakened immune system of the host organism.

Although, pathogenic bacteria in principle can infect any part of their hosts, they often have a preferred niche within a tissue. Some bacteria prefer to be taken up by the host cells and remain intra-cellular; others instead prevent uptake and are extra-cellular pathogens.

1.1.1. Intra-cellular pathogens

Some pathogenic bacteria such as *Salmonella*, *Shigella* and *Chlamydia* prefer to be taken up by their target cells. Some of these bacteria induce their uptake for successful establishment in the host but can also be extra-cellular at other times. *Salmonella* and *Shigella* are in this group. Others, including *Chlamydia*, are obligate intra-cellular pathogens and cannot replicate outside host cells. However, it is crucial for both groups to survive in the harsh intracellular environment and somehow establish a favorable niche for themselves within the host cell.

1.1.1.1. Phagocytosis and escaping phagosome

As basic text book knowledge, phagocytosis can be explained as 'eating of cells'. In a more scientific definition it is the ingestion of large particles by eukaryotic cells. Phagocytosis is a vital process in multicellular organisms working in both maintaining the tissue homeostasis and their fight against the pathogens (186). Although most eukaryotic cells can take up particles, some cell types in the immune system, such as monocytes, macrophages and neutrophils, are the professional phagocytes. Their main difference from the non-professional phagocytes is their ability to express special receptors for phagocytosis (252). Phagocytosis starts with the recognition of pathogens followed by their

ingestion into the early phagosome, vacuoles that keep the ingested material within. The switch into late phagosome is a result of fusion and separation of endocytic and secretory vesicles, respectively. After this stage, lysosomes coalesce with the phagosome, change their internal environment dramatically and form phagolysosome (309). Any of these stages can be the target of pathogens' strategy to overcome the host immunity.

One strategy against phagocytosis is physically escaping from the phagosome. One example of this is *Shigella*. It has long been known that *Shigella* can lyse the phagosome membrane by the help of the pore forming proteins IpaB and IpaC and that is an important part of *Shigella*'s pathogenic life cycle. (29, 130). In addition to *Shigella*, *Listeria* escapes the phagosome with the help of pore-forming listeriolysin O (278) and phospholipases PLC and PLD (119). Similarly, *Rickettsia* escapes phagosomes with the help of phospholipase A2 (319).

1.1.1.2. Surviving in phagosome

Once pathogens are ingested into the phagocytic vacuole, they encounter a very harsh environment. During maturation of phagolysosome, Mn^{+2} cations are removed to increase the acidity of the environment. Mn^{+2} is a cofactor of superoxide dismutase expressed by several unrelated pathogens and its removal results in the loss of the protective effect of the protein (147). Therefore, some pathogens block removal of Mn^{+2} to create a more friendly environment to themselves (309). Further survival strategies include blocking lysosome fusion with phagosome and keeping it as an early phagosome, evolving proteins that can stop detrimental enzymatic activities against them or effects of the oxidative environment within phagolysosome, neutralizing the acidity, using high-cation affinity proteins to prevent their export and using lipids of the host as nutrients (309).

1.1.2. Extra-cellular pathogens

A second group of pathogens prefers to stay outside the host cells. Unlike the intracellular pathogens, their virulence mechanisms are based on strategies to replicate in the extracellular environment and to block phagocytosis. This can, for instance, be achieved by depolymerizing host cytoskeleton, directly blocking phagocytosis or activating apoptotic pathways in the professional phagocytes. The genus *Yersinia*, *Pseudomonas* and *Vibrio* are examples of such microorganisms.

1.1.3. Antigenic variation

The never-ending competition between pathogens and hosts has resulted in not only the evolution of host cell manipulation by bacteria but also efficient mechanisms to escape from the host immune system. Antigens are the molecules that can induce an immune response in a host and used by immune system to recognize any invaders. This recognition creates an immune memory that can last for decades (sometimes even life-long). So, for a pathogen to persist in host or to re-infect the same host, it is crucial to overcome this memory. Antigenic variation is one such mechanism which increases pathogens' protein diversity and thus changes their exposed structures to hide from the acquired immunity of the host (79). These changes can stem from genetic factors where changes in the nucleotide sequence cause changes in amino acid sequence or expression levels; or epigenetic where the expression levels change by factors other than mutations in the DNA sequence. For example, a simple form of antigenic variation, phase variation, works by switching a specific gene's expression between ON and OFF. The number of different phenotypical combinations can be formulated as 2^n, where 'n' is the total number of genes regulated by phase variation (79).

1.1.4. Bacteria Host Interactions – Adhesins

One important early step in the establishment of infection is the direct interaction between bacteria and host cells. From the bacterial side, these interactions are mediated via sticky proteins called 'adhesins'. They are made up of proteins or polysaccharides and required to resist against the physical shear forces created by the host to shear off the pathogens (301, 330). In almost all cases, adhesins bind to receptors located at the host cell surface (330). However, in rare cases it has been observed that the pathogen inserts the receptor itself onto the target cell surface and binds to it (160). Most pathogens encode more than one adhesion molecule (330). As a result of their importance in bacterial virulence, recently adhesins are being investigated as potential targets in pharmaceutical research.

1.2. Impact of different environments on bacterial evolution and the specific role of secretion systems

The interaction of bacteria with their environment is a driving force in their evolution to survive and/or to improve adaptation. A bacterial cell always contains a huge variety of atoms, molecules and proteins at a given point; some of which are synthesized by the bacterium and others are needed to be taken up from the environment. The environment at this very moment has a big influence on the internal composition of the bacterium. Both presence and absence of other bacteria (either from same species or not), host cells, nutrient sources, bacteriophages, harmful agents are of importance. The micro-changes in these conditions might require major changes inside the bacterial cell for functional adaptation.

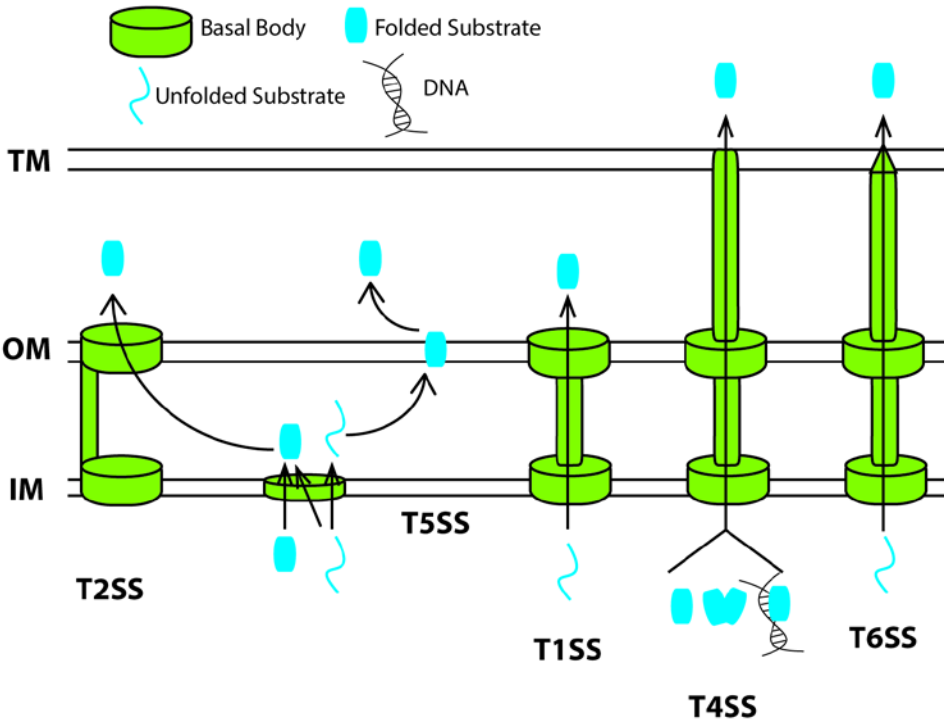

Figure 1. Most studied secretion systems in Gram-negative bacteria. Type 1-6 secretion systems are illustrated except T3SS which will be discussed in detail in Section 1.3. T2SS and T5SS export substrates only from periplasm whereas T1SS, T4SS and T6SS exports substrates from cytosol through both bacterial membranes (Outer membrane, OM; inner membrane, IM). In addition to the bacterial membranes, T4SS and T6SS can span the target cell membrane (TM) and forms a direct channel between bacterial and target cell cytosols.

1.2.1. Secretion Systems

Gram-negative bacteria have evolved a number of specific secretion systems to export proteins to the external environment including host cells. These systems require a high level of energy as ATP or proton motive force (PMF) (234) for their function and are therefore often tightly regulated to ensure that they are only expressed when needed to use the energy efficiently. Five of the six most

studied secretion systems that have evolved in Gram-negative bacteria are described in Figure 1.

Type 2 and 5 secretion systems (denoted T2SS and T5SS, respectively) can secrete proteins from periplasmic space to outside. Thus they require Sec (T2SS and T5SS) or twin-arginine translocation (Tat) (T2SS) systems for the transfer of substrates from cytosol to periplasm (121). After the transfer of proteins to the periplasm, T2SS and T5SS mediate the export of the proteins from the bacteria. In order to be secreted by T2SS, proteins must be folded (121). Secretion is through the secretin protein complex embedded into the outer membrane (OM) (154) which also extends to an (inner membrane) IM complex to form an interaction with cytoplasmic ATPase (165). Since T2SS is closely related to Type 4 pilus (26), it has been suggested that T2SS uses a similar mechanism where pseudopilus of T2SS retracts, the substrate interacts with secretin and the pseudopilus extends again pumping the substrate out like a piston (92, 165, 254). Most of the T2SS substrates are hydrolytic enzymes that function in nutrient acquisition. However, the system was also shown to secrete proteins directly related to virulence such as the famous toxic protein of *Vibrio cholerae*, Cholera toxin (224).

T5SS is different from the other systems and denoted as autotransporters. The translocation domains of T5SS proteins are highly homologues to each other whereas passenger domains vary greatly. These proteins are known to be directly related to the pathogenicity of the bacteria encoding them. Their functions involve bacterial motility, enzymatic activity, toxins, adhesins or maturation of other toxins (129).

The main difference between Type 1, Type 3, Type 4 and Type 6 secretion systems (T1SS, T3SS, T4SS and T6SS, respectively) and T2SS and T5SS is their ability to secrete cytoplasmic substrates without the requirement of a prior transport across IM (121). T1SS is very similar to ATP-binding cassette (ABC) transporters (121). The ABC transporters of T1SS carry a transmembrane domain within IM (135), and a cytoplasmic nucleotide binding domain. The

ABC transporters then interact with membrane fusion proteins (80) which is followed by an interaction with the substrate. As the final step, OM factor joins the complex (157). The substrates are secreted unfolded (28). Main function of T1SS substrates includes nutrient acquisition such as iron scavenging, together with more direct roles in virulence, such as haemolysins and leukotoxins and also antibacterial bacteriocins (157).

T4SSs are close relatives of conjugation systems evolutionarily. Unlike T1SS, after spanning both bacterial membranes T4SS can also form a channel across target cells membrane which can be both a bacterial or a eukaryotic cell (121). One important property of T4SSs is that they can secrete not only proteins but also complexes of protein-protein or protein-DNA (121). Translocations of all these different classes of substrates are of medical importance. DNA transfer by T4SSs is one of the most common ways of antibiotic resistance acquisition among bacteria. In addition, various effector proteins have been described to be translocated by T4SS into target cells (13).

T6SSs, also, span both bacterial membranes and the host membrane. Their main property lies in including other bacteria as target cells in addition to eukaryotic cells. Like other secretion systems, they also have an evolutionary relationship with another system, phage-tails (182). T6SS substrates mostly carry immunomodulatory roles inside the target cells (133).

T3SS is discussed in more detail in the next section (Section 1.3).

1.3. Type 3 Secretion Systems (T3SS)

T3SSs are one of the most important and widespread virulence mechanisms for Gram-negative bacteria targeting both animal and plant hosts and can for instance be found in *Yersinia*, *Salmonella*, *Shigella*, *E. coli*, *Pseudomonas*, *Vibrio*, *Chlamydia*, *Burkholderia*, *Xanthomonas*, *Rhizobium* and *Aeromonas*. Among animal and human pathogens T3SS has been more extensively studied in *Yersinia*, *Salmonella* and *Shigella*. Although it is mostly known as a virulence mechanism, some bacteria use T3SS to establish a symbiotic relationship with

their host such as the symbiosis between *Rhizobium* and legumes where *Rhizobium* requires T3SS dependent translocation of effectors (315).

T3SSs commonly secrete two classes of substrates, translocators and effectors, through its basal body which spans IM, periplasm and OM of bacteria (51). Effector proteins are destined to be translocated into the host target cells by the help of translocators in a single and/or 2 step mechanism (Section 1.3.7.5) (96, 114). The respective targets and functions of effector proteins reflect the pathogenic life style of the bacteria, such as intracellular/extracellular or pathogenic/symbiotic.

T3SSs from different bacteria can be clustered into different families. Among these, the Ysc-Yop family of *Yersinia* and *Pseudomonas*, Inv-Mxi-Spa family of *Shigella* and *Salmonella* pathogenicity island (SPI) 1 and Ssa-Esc family of *Salmonella* SPI 2 and EPEC have been most extensively studied (51).

1.3.1. Origin and relation to flagellar system

Flagella is used by bacteria for motility and can be found in both Gram-positive and Gram-negative bacteria. Structural and functional analysis of flagellar system and T3SS brought the idea that they are evolutionarily related (38, 120). However there is no consensus on which system evolved first, yet. Since, unlike T3SS, flagella can also be found in Gram-positive bacteria and origin of flagella is believed to be much earlier than eukaryotic cells (which are the targets of T3SSs), Nguyen et. al. proposed that flagella evolved first (222). Also, a need for motility very early in the bacterial evolution to reach nutrients and move to a more friendly niche further supported the 'flagella-first' hypothesis (271). In addition to these, in their study where they analyzed more than 1000 genomes for flagellar system and T3SS, Abby and Rocha argued that the evolution of T3SS from flagella occurred at least in 2 steps: first acquisition of a part of flagella which was followed by acquisition of an OM secretin (1).

On the other hand, another view proposes that flagellar and T3SS genes share a similar degree of antiquity. Thus, the two systems were suggested to share a

common ancestor and evolved independent of each other (120). Phylogenetic comparison of 16S rDNA and T3SS genes led to the discovery that the resulting trees are different from each other. As a result it was concluded that the evolution of the two systems did not follow each other and multiple horizontal gene transfers are responsible for spread of T3SS (120).

1.3.2. Assembly of T3SS

T3SS is composed of more than 20 proteins (51) and assembly of all these proteins at the correct time and place is very important for establishment of a functional system (Figure 2). The expressions of the genes giving rise to the basal body proteins are simultaneous and the assembly order is believed to be

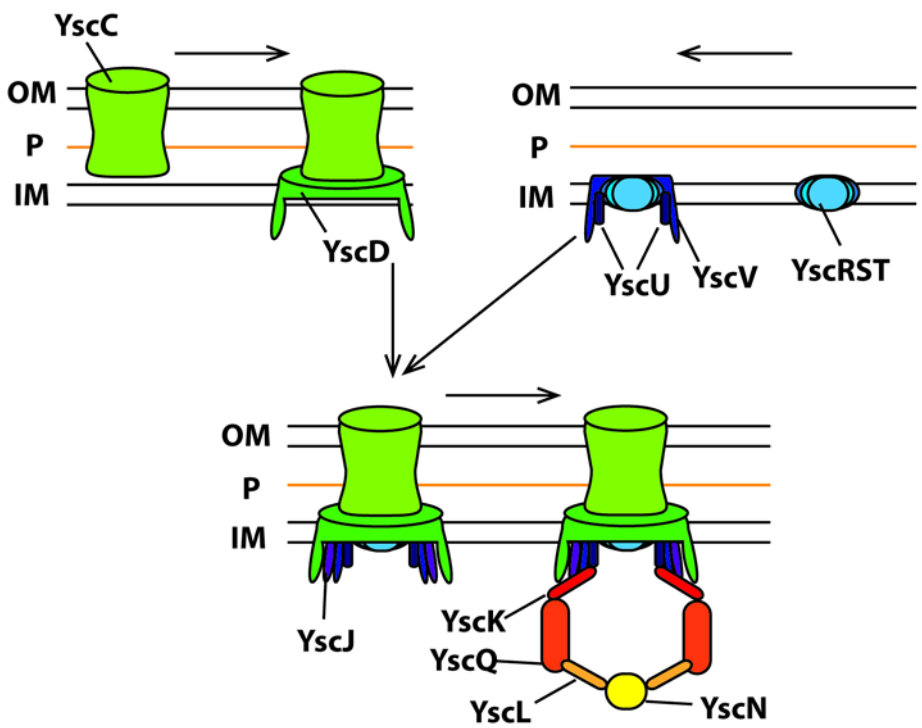

Figure 2. Assembly of the *Yersinia* T3SS basal body. Assembly of T3SS starts at the outer membrane (OM) with YscCD (top left panel) and at the inner membrane with YscUVRST (top right panel) independently. YscC also spans peptidoglycan layer (P) in the periplasm. These two complexes are then joined to each other by YscJ and a cytoplasmic complex also joins them (lower panel). Adapted from Diepold and Wagner, 2014 (86).

mediated mainly by protein-protein interactions (83) and is tightly regulated. Different studies from *Salmonella* and *Yersinia* have shown that the assembly starts by formation of inner and outer membrane rings (82, 169). According to these studies, OM secretins (YscC-family proteins) are localized in the OM and form a complex with YscD family proteins extending towards IM to form a base for the assembly (82, 169). In some systems, the correct localization of secretin requires a pilotin protein (82). Simultaneously, homologs of YscR, YscS and YscT form another complex in IM which is later joined by YscU and YscV family proteins (88, 318). These two independent complexes are then tethered to each other by YscJ-family proteins. Meanwhile, a third complex is formed by the cytoplasmic components and they join the basal body via their interaction with YscU and its homologs (43, 82, 85, 86, 256). The completion of the secretion organelle requires the secretion of first substrates, the needle and inner rod proteins, whose secretion and polymerization complete the construction of the basal body (87, 163).

The “ruler” protein, YscP, has been found to be important for the needle length determination. In the ‘ruler model’, YscP stretches with the growing needle and when the needle reaches its desired length, the secretion of further needle proteins is supressed and the secretion organelle opens up for new substrates (4, 97, 156, 169). In another model, termed as ‘timer model’, needle and inner rod proteins are recruited into the secretion channel together and the completion of inner rod assembly signals the completion of needle filament assembly (113, 197). Recent research showed that the ruler model fits *Salmonella* SPI-1 needle length determination better (326).

1.3.3. Cytoplasmic side of T3SS

Cytoplasmic components of T3SS carry out different functions including energizing the system, substrate specificity and substrate switch.

YscN family members are the ATPases of the T3SSs. YscN in *Yersinia*, InvC in *Salmonella* SPI-1, Spa47 in *Shigella* and EscN in *E. coli* are the most studied

members of this family (51). Their ATPase activity is crucial in developing a functional system and they should be associated with IM to exert their function (7, 49, 98, 331). It has been shown that the T3SS ATPases are related to F_0F_1-ATPases (142, 341). They are believed to act as homohexameric rings (7, 249). *In silico* analysis of possible EscN hexamers showed that the homohexameric structures created 6 ATP-binding sites (341). Also, Spa47 is a much better ATPase as an oligomer compared to its monomeric form (48, 49). One important common feature of the T3SSs and the F_1 ATPases is a conserved Arg residue. In EscN, mutation of the Arg residue disrupts the ATPase activity of the hexamer (341). The function of T3SS ATPases is negatively regulated by the stator protein, YscL in *Yersinia* and EscL in *E. coli* (36, 262).

Another member of the cytoplasmic complex is the so called 'C-ring protein' YscQ protein family. In *Yersinia* and *Salmonella* SPI-2, YscQ family members have a second inner translation start site in addition to the previously shown site both of which can be used to express YscQ or its shorter C-terminal variant YscQ-C (52, 202, 338). A possible internal translation initiation site was shown to exist in some other T3SS but not in all (52). Interaction of these two variants with each other is required for a functional T3SA (52, 84, 202, 338). Recent high resolution data showed that in *Shigella* the YscQ homolog forms 6 pod structures at the cytoplasmic side. These pods are bound to ATPase proteins via stator proteins which act as linkers (138, 195). This took the previous findings one step further where 'the C-ring proteins' were shown to bind IM complex proteins, ATPase, stator and YscK-family members of cytoplasmic complex (149, 151, 215). In *Yersinia*, YscQ, YscL and possibly YscK and YscN were shown to form a dynamic structure in cytosol. Under inducing conditions YscQ and YscL became less motile (84, 258).

Although not found in all T3SSs, other 2 small cytoplasmic proteins of the *Yersinia* T3SS are YscX and YscY. Basal body can be assembled without these two proteins but secretion channel cannot be opened (87). Initially it was thought that YscY is the chaperone of YscX which can be secreted by T3SS (74). However, later Diepold et. al. suggested that via their association with export

apparatus proteins YscV, YscX-YscY complex might have a role in secretion of the first substrates YscF, YscI and YscP (87).

1.3.4. Inner membrane

The export apparatus is composed of the YscR, YscS, YscT, YscU and YscV families of proteins all of which carry transmembrane domains (226). In addition to their transmembrane domains, the homologs of YscU and YscV also carry cytoplasmic domains (10, 112, 226, 243). Not much is known about YscR, YscS and YscT families except that they are required for the assembly of a functional system (88, 318). In *Shigella* it has been shown that the export apparatus is formed bound to a nonamer of YscV homolog, MxiA, which is located between IM rings and ATPase complex (2). As the central protein of export apparatus, YscV family members also oligomerize into a ring (88, 185, 332).

YscU family is the autoprotease of the export apparatus. Its main function is substrate specificity switch and it will be discussed in section 1.3.7.1. An NPTH motif is conserved in YscU family members and the cleavage occurs at the proline site when T3SS is induced (77, 175, 340). The resulting free C-terminal YscU (YscU-C) is a substrate for the system and is important for the increased secretion after induction (110, 175, 190).

1.3.4.1. Inner membrane ring

YscD and YscJ family proteins constitute the IM rings of T3SSs (280, 295). YscJ family members are lipoproteins. (51). They interact with YscD family proteins in 1:1 ratio and YscD family proteins cover both YscJ family and export apparatus proteins from outside (226, 247).

1.3.4.2. Connecting the two membranes

Of the two IM rings, YscD family members extends longer into the periplasm and interacts with the OM secretin YscC family members (82, 228, 266, 281). In the periplasm, an important mission to create a space within the continuous peptidoglycan layer is carried out by secretin protein. Both YscC and YscD in *Yersinia* and their homologs in *Shigella* have been shown to be elastic proteins and stretch to maintain a stable interaction between the IM and OM rings (170).

1.3.5. Secretin

Secretins are formed in OM by homo-multimerization of YscC family proteins into a ring structure and form a pore from which the needle filament protrudes from the bacterial surface. N-terminal of secretin protein extends into the periplasm, crosses the peptidoglycan layer and interacts with the IM rings to complete the channel for secretion of substrates (88, 166). In some systems they are localized into OM by their specific pilotin proteins (50, 68). Correct localization of secretin is important since it determines where the basal body would be constructed (82).

1.3.6. Needle protein

The needle filaments are the part of T3SA extending from the secretin and where substrates need to pass through to be exported (90, 253). Inner rod proteins form the connection between the needle filament and membrane rings (113). Needles are composed of YscF family proteins which are assembled helically (63, 192). YscF family proteins are not only the first substrates but also required for the secretion of other substrates. Growth of the filament is the result of the addition of single YscF family proteins to the distal end (248). After the completion, a pentamer complex, formed mainly of LcrV protein family (termed as 'tip proteins'), caps the needle filament (101, 217).

One common feature of the T3SSs from different species is that they require target cell contact for full induction and function (76, 207, 343). In addition to its role in secretion, the needle filament is also believed to transmit the contact signal to cytoplasmic complexes of the T3SA to induce secretion. Support for this comes from the work in *Yersinia* where some point mutants in the *yscF* gene resulted in a secretion competent but non-translocating T3SS (71). Both YscF and the *Shigella* needle protein MxiH could be locked in a constitutive 'ON' mode as results of different point mutations (159, 305). There is also data supporting that in addition to needle proteins, the inner rod protein MxiI in *Shigella* and tip proteins IpaD in *Shigella* and PcrV in *Pseudomonas* are involved in signal transmission (57, 177, 242, 310). This strengthens the idea that the needle proteins are involved in promoting the contact signal to T3SA.

1.3.7. Secretion and translocation in T3SSs

The final aim of the T3SSs is to direct effectors into target cells. This process includes two important steps. The first one is secretion which is the export of substrates from bacteria. Secreted substrates do not necessarily end up in target cells. Second one is translocation. It is the final step where anti-host effector proteins are directed into target cells by the help of translocator proteins.

1.3.7.1. Secretion of substrates through basal body

Once the T3SS basal body is assembled, a very narrow channel is formed for the secretion of later substrates. These substrates can only be secreted in their unfolded state. Any protein that is partially folded prior to its entry into the secretion channel blocks the channel and prevents the secretion of further substrates (90, 253).

An important question is how bacteria sense the completion of T3SA to initiate secretion of early substrates and induce the system. As discussed above, once the needle filament reaches its correct length, this is sensed by the basal body and a specificity switch from early to later substrates can occur (4, 113, 156). In

addition to their function in determining the length of needle filament, the ruler proteins (YscP family) are involved in this switch as well. (62, 169). It has also been shown that the autoproteolytic YscU family proteins, which can interact with ruler proteins, undergo a cleavage when the needle filament reaches to its desired length (302). Once cleaved, the exposed surface properties of YscU family proteins are proposed to be important in detection of further substrates by T3SA (77, 214, 293). However, we still do not know the exact mechanism behind this switch. Some further clues stem from the affinities of both YscP and YscU homologs to cytoplasmic components of T3SA. For example, in *Yersinia* an interaction with YscP and ATPase complex component YscO is essential to obtain a functional system (218). In addition, the YscU homolog in *Shigella*, Spa40, undergoes an affinity change between different cytoplasmic components upon its self-cleavage which might be important in the specificity switch (43).

The switch from early substrates opens the gate for further substrates. What is required for their secretion and how are they secreted? One essential component of substrates for their secretion is a non-cleaved N-terminal secretion signal (209). However, in some substrates, other sequences localized elsewhere can completely or partially be required for their secretion (11, 58, 161, 190). Surprisingly, unlike most secretion signals, T3SS secretion signals have no distinct consensus sequence. There are even reports suggesting that the secretion signal resides in mRNA sequence rather than in the amino acid sequence (19, 20, 223). On the other hand, mutations without an impact on the amino acid sequence in the N-terminal end of the substrates were found not to disrupt secretion (158, 189, 268) Yet, the properties of the signal sequence is conserved, though not well understood, and using machine learning T3S substrates can be predicted at a relatively high level. Moreover, this method can be used for detection of substrates among different bacterial species including plant pathogens (25, 193, 272). In support with this, heterologous secretion of T3SS substrates is observed among different species (17, 109, 264, 267). The N-terminal end of the substrates have been found to be unordered and a binding between a substrate and its chaperone introduces a more stable structure to the N-terminal end (259).

Having a secretion signal is not enough for secretion. Substrates also carry a chaperone-binding site which is located close to the secretion signal (56, 323, 325). It is believed that the key function of the chaperones is to keep the substrates stable and in an unfolded condition and to guide them to the T3SA (174, 297). The detailed mechanism of the substrate-chaperone complex recognition by T3SA is not yet known. However, it is known that the ATPase protein can bind and dissociate the substrate-chaperone complexes in an ATP-dependent manner (6, 117). Also, Lara-Tejero et. al. showed that at least the initial recognition of chaperone substrate complex is via interactions with the chaperone (174). However, binding to ATPase protein itself requires a heterocomplex of the chaperone and the substrate in *E. coli* (55). A recent study showed that chaperone/substrate complexes form homohexameric structures compatible with the hexameric structure of ATPase. It was also suggested that this structure leaves the N-termini of effectors free for T3SA to recognize and introduce them into the secretion channel (257). Although these findings may constitute an explanation for substrate recognition, it is not yet biochemically coupled to the secretion process (226).

Another cellular energy source, the proton motive force (PMF), is required for T3S as well through a yet not well-known mechanism (329). It is believed that PMF is the driving force of substrate export whereas ATP hydrolysis takes a role in chaperone-substrate complex dissociation. In support of this, recent studies in *Pseudomonas* showed that, together with regulatory protein PcrD, YscO-family protein PscO is involved in PMF dependent energizing of T3SS by modulating proton flow (178).

1.3.7.2. Translocation

Once the substrates leave the secretion channel, the effector proteins are then targeted into the host cell by the translocator proteins. This process is called ‘translocation’ and appears to be uncoupled from secretion.

T3SSs have 3 proteins required for translocation. One of them is the hydrophilic 'tip protein' located at the distal end of the needle filament and the two others are hydrophobic 'pore forming' proteins that are destined to target cell membrane.

1.3.7.3. Tip proteins

The LcrV family of tip proteins is found in all animal pathogenic T3SSs. They are located at the tip of the needle filament as a multimer (Figure 3) (101, 217). So far several roles have been proposed for these proteins including sensing target cell contact, promoting insertion of the hydrophobic translocators into the host cell membrane and regulating the T3SS (51, 216). Of these proposed roles for tip proteins, signal transduction and regulation have been separated from each other in *Shigella* (260). The exact composition of the tip complex varies between species. In *Yersinia*, 5 LcrV proteins form a ring structure that resembles the rest of the basal body (46). The ring structure has also been shown in other bacteria (118). Different from *Yersinia*, in *Shigella* one molecule of hydrophobic translocator IpaB replaces one hydrophilic IpaD resulting in a hetero-complex tip with 4:1 IpaD:IpaB ratio (229). Another difference observed among T3SS families is the chaperoning of the tip proteins. T3SSs of *Yersinia* and *Pseudomonas* express a distinct

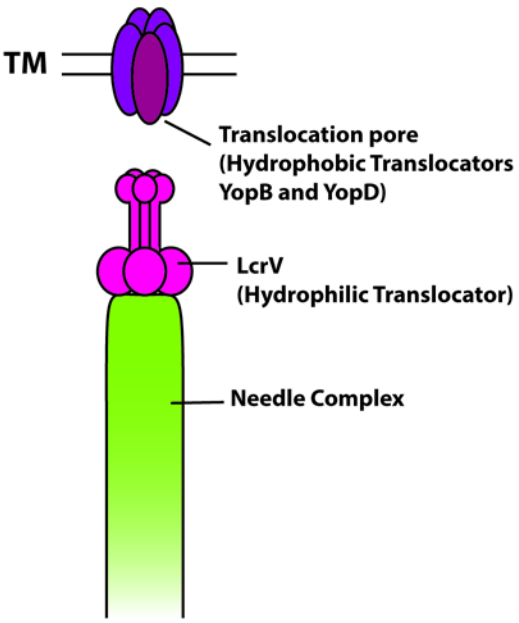

Figure 3. Translocators of *Yersinia* T3SS. There are two types of translocators in the T3SSs. Hydrophilic translocators (LcrV in *Yersinia spp.*) localize at the needle tip as a pentamer. Hydrophobic translocators (YopB and YopD in *Yersinia spp.*) form a pore inside the target cell membrane (TM). All three translocators are required for translocation.

chaperone for tip proteins (78, 200), whereas in another group including *Salmonella* and *Shigella*, tip proteins carry a self-chaperoning domain at the N-terminal (153). In *Yersinia*, the LcrV chaperone LcrG has also shown to be involved in regulation of T3SS (200). In *E. coli*, however, the tip protein complex is replaced by a helical filament (69, 164).

Recently, it was shown that the N-terminal of LcrV of *Yersinia* is important for efficient/early translocation of Yops (99). However, it is not yet known if this is *Yersinia* specific or a more general property of tip proteins.

Since tip proteins are important for multiple steps of the T3SS and exposed at the bacterial surface, they have also gained interest as potential targets of anti-virulence bacterial therapies (89, 176).

1.3.7.4. Pore formation

The hydrophobic YopB- and YopD- family proteins are believed to form a pore in the target cell membrane (Figure 3) (51). The proposed translocation pore is formed by a hetero-complex of these proteins and interactions between them are necessary for translocation (37, 141, 194, 221, 261, 299). These two proteins can form the ring structure only when they are inserted into the membrane (279). It was also reported in *Pseudomonas* that the pores tend to be formed in specific regions called lipid rafts which are also shown to be important for T3SS in other species (12, 173, 279, 311). The placement of hydrophobic translocator proteins into the target cell membrane is believed to require the tip proteins (61, 127, 196, 242, 310). Importantly, pore formation does not require only bacterial factors. Several host cell factors have also been shown to be important for formation of the translocator complex within target cell membranes (39, 269, 285). Furthermore, research on the direct effects of translocons on virulence showed that they can also act as pore forming toxins and induce an immune response from the host (30, 91, 172). In addition to these, the translocator complex of *Pseudomonas* can remain intact within the target cell membrane even after the bacterium detaches from the host cell (91). Similar to the tip

proteins LcrV and PcrV, PopD, the YopD homologue in *Pseudomonas*, is involved in signal transduction to bacteria after target cell contact (24).

1.3.7.5. Proposed mechanisms

Although the exact mechanism of translocation has not been clarified or directly demonstrated. So far two different mechanisms have been proposed: the injection model and the AB-toxin like two-step model.

1.3.7.5.1. Injection model

The widely accepted injection model proposes that the T3SA acts as a needle and inserts itself into the target cell membrane forming a continuous channel between the bacterial and target cell cytosols (Figure 4A). Thus, effector protein secretion and translocation are combined into a single step where the effectors are targeted directly into the host cell (113). Support for this model comes from the ability of *Yersinia* tip protein LcrV to bind translocator proteins YopB and YopD (67, 273). Here it is important to note that YopB and YopD are suggested to form a translocation pore in the host cell membrane and LcrV is localized at the needle tip. Therefore, interactions between these proteins would hint a continuous channel between two cytosols where proteins are transferred. In addition, no effector Yops have been found in the extracellular milieu during the infection indicating that they are translocated into target cells without being released to the external milieu (105, 180). Conversely, it was shown that a *Yersinia* effector, YopE, could be detected outside bacteria or target cells during a HeLA cell infection (225). Still, the injection model had been the only proposed mechanism of translocation for almost 2 decades until it was challenged with two-step model.

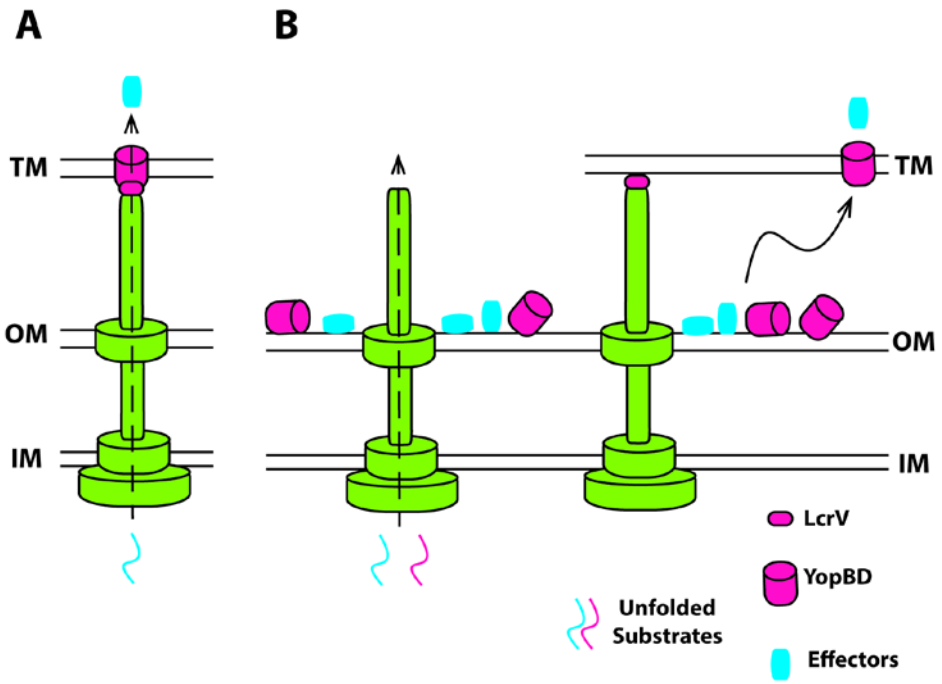

Figure 4. Proposed models of translocation by T3SS. (A) According to the injection model the T3SA forms a continuous channel between bacterial and target cell cytosols through bacterial inner (IM) and outer (OM) membranes as well as target cell membrane (TM). When the system is induced, substrates enter into the secretion channel in their unfolded state and fold directly after leaving it. Translocator proteins form a pore in the TM and facilitate the translocation of effectors into the target cell. (B) In the two-step model, under non-inducing conditions at 37°C, substrates are secreted in their unfolded state and localized on the bacterial surface. Upon induction a signal is transmitted resulting in the release of surface localized substrates. Translocators then direct effectors into the target cell.

1.3.7.5.2. Two-step model and localization of substrates on bacterial surface

In a previous study the effector protein YopE was found to localize outside the host cells during infection was still resistant to proteases; however, the exact location was not determined (225). Many years later not only YopE but also another effector YopH and the translocator YopD were shown to be at the bacterial surface under non-inducing conditions (8). Importantly, the surface localized YopH was also shown to be translocated into HeLa cells (8). This was

interesting since in the injection model no effector proteins are expected to be outside bacteria or host cells. In addition, the proteins found outside would not be expected to be translocated into target cells. Based on these findings, an AB-toxin like two-step mechanism was proposed (Figure 4B) (96). In this model, both translocator and effector proteins are secreted before target cell contact and initially localize on bacterial surface. This completes first step. Once host cell contact is established, a signal is transmitted (possibly through the needle filament) and the surface proteins are released. Then the translocators somehow facilitate the translocation of effector proteins into target cells. Even before these observations were made in *Yersinia,* T3SS substrates of *Shigella* had also been found to be at the bacterial surface before target cell contact and these proteins were then released after host cell contact and even translocated (206, 208, 322). Another observation supporting the two-step mechanism is that, for YopE and YopH in *Yersinia,* different sequences are functioning as secretion and translocation signals (240, 274, 294). This indicates that T3SS can discriminate between secretion and translocation and they might work as two different processes instead of one. Recently, it was shown that a T5SS substrate in *E. coli* localized at the bacterial surface could be translocated into a eukaryotic cell by a T3SS dependent mechanism (300). In another very recent study, it was shown that OMVs isolated from *Salmonella* carry SPI-1 T3SS effectors on their surface and that the effectors also could be translocated into host cells even in the absence of bacteria. These effectors were functional once they are targeted into the host cell, but it remains to be shown if the intracellular targeting of effectors required the translocator proteins (162). However, although in different bacteria translocation of surface proteins by T3SS has been observed, no direct evidence for the complete mechanism has yet been shown.

Importantly, neither mechanism can negate the other and it is possible that these two mechanisms can work independently or somehow be coordinated. The two-step mechanism could be optimal for rapid/early delivery of effectors from the bacterial cell surface directly after host cell contact. However, at later stages it might be slow since it would depend on initial secretion to the bacterial

surface and then there might be a switch to injection mechanism with direct targeting of the effectors.

1.4. The genus Yersinia

The *Yersinia* genus is an *Enterobacteriaceae* family member. There are at least 15 species under the genus but only 3 of them have been shown to be pathogenic to humans: *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* (140). All 3 species carry a plasmid encoding the T3SS and have similar pathogenicity dynamics although the infection routes can differ.

1.4.1. *Yersinia pestis*, the causative agent of the plague

Plague is a zoonotic disease mainly maintained in rodents and fleas that can be transmitted to humans. There are 3 pandemics that are believed to have occurred in the recent history. Out of these, the second pandemic (also known as the Black Death) has been estimated to have caused 25 million deaths in Europe. The plague agent, *Yersinia pestis* is evolutionarily the youngest of the pathogenic *Yersinia* species. It is highly pathogenic and believed to have limited ability to survive outside its hosts in the environment. The transmission to animal/human hosts occurs by flea bites. Bacteria then reach lymph nodes causing bubonic plague. Bacteria can also reach to bloodstream and thus different organs resulting in septicemic plague or to lungs to establish pneumonic plague. Pneumonic plague can rapidly spread from human to human via infected droplets. Most *Y. pestis* strains carry 3 plasmids, which support adaptation to different hosts (238, 291).

1.4.2. Enteropathogenic *Yersinia*

The other two pathogenic *Yersinia* species are both enteropathogens causing gastrointestinal self-limiting infections in humans. However, in some cases they can also cause systemic infections (291).

1.4.2.1. *Yersinia pseudotuberculosis*

Y. pseudotuberculosis is believed to have emerged within last 200 million years (94). Its name comes from granulomatous abscesses which look tuberculosis-like in livers and spleens of the hosts. It is the cause of more adult related Yersiniosis of the two enteropathogenic species (291). A *Y. pseudotuberculosis* strain, that has gained the ability to express the superantigen mitogen A, is the causative agent of the Far East scarlet-like fever outbreaks in Russia and Japan (16).

1.4.2.2. *Yersinia enterocolitica*

Y. enterocolitica is believed to have evolved around the same time as *Y. pseudotuberculosis (94)*. However, unlike *Y. pseudotuberculosis*, yersiniosis caused by *Y. enterocolitica* infections is more abundant in infants and young children. Some strains depend on exogenous iron to establish an infection (291). Pathogenic strains of *Y. enterocolitica* show strong specificity pattern for different serotypes (94).

1.4.3. Emergence of *Yersinia pestis* from *Yersinia pseudotuberculosis*

Y. pestis is evolutionarily much younger than the two other human pathogenic species. It has been estimated to have emerged as recently as 1,500 to 20,000 years ago from the *Y. pseudotuberculosis* O:1b strain (3, 94). Their close relation is reflected in both identical 16S rDNA sequences and DNA-DNA hybridization (32, 306). Thus it is intriguing to argue how they ended up differing this much in their pathogenic life style. Today we know that the evolution of *Y. pestis* was a stepwise process including both gain and loss of genes (Figure 5). These include the gain of two plasmids one of which encodes for Pla protein which is important for systemic spread from infection site and the other carries genes important for colonization in and spread from flea together with preventing phagocytosis (93, 131, 132, 292). One of the genes *Y. pestis* lost during its

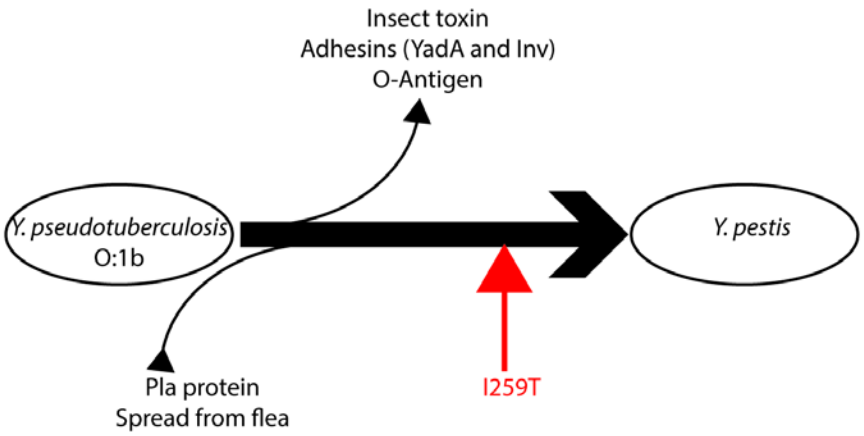

Figure 5. Evolution of *Yersinia pestis* from *Yersinia pseudotuberculosis* O:1b strain.

evolution is an insect toxin which would have prevented spread from flea when it is expressed (333). Other 2 genes lost during the evolution of *Y. pestis*, are the *yadA* and *inv* genes that encode for adhesin/invasin important for binding to and passing through intestinal barriers (287, 290). Another group of genes that were lost in *Y. pestis* genome are O-antigen synthesis genes. It is believed that the loss of these genes contributed to complement resistance of *Y. pestis* (245).

Although these changes in the DNA content of *Y. pestis* have been important in the emergence of one of the most virulent pathogens in the history of humanity, a small substitution in the *pla* gene is shown to be associated with 2 of the 3 plague pandemics of modern history (283, 317). The mutation that resulted in change of isoleucine at position 259 in Pla protein to threonine resulted in a more rapid systemic spread and cause of pneumonic plague (344).

1.4.4. Infection route

Since *Y. pestis* and *Y. pseudotuberculosis* are evolutionarily closely related and share the main virulence mechanism, T3SS, the latter has long been used to understand disease development of plague. However, the infection routes of the two pathogens differ. Below, the infection route of enteropathogenic *Yersinia* is discussed.

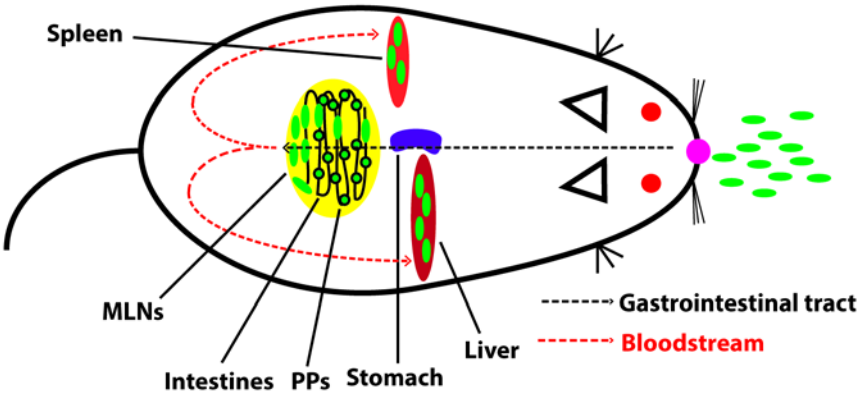

Figure 6. Infection route of *Yersinia pseudotuberculosis.* In oral infection route, *Y. pseudotuberculosis* first travels to intestines where it localizes in Peyer's Patches (PPs). From there, the bacteria continue to Mesenteric Lymph Nodes (MLNs). Further spread from MLNs to spleen and liver indicates the systemic spread of the bacteria.

Most research of *Yersinia* infections is done using mice as a model host organism (Figure 6). Enteropathogenic *Yersinia* infections are mostly spread through oral/fecal route i.e. contaminated food and water sources (42, 123, 179, 276). During oral infection, *Yersinia* reaches intestinal epithelium which forms a barrier for many pathogens, but not for *Yersinia*. Here, they interact with M-cells through the adhesin invasin (see section 1.4.5) on bacterial surface and β1-integrin on the host cell surface. Thereby they promote their uptake and transfer to lymphoid follicles called Peyer's Patches (PPs) (60, 145, 146, 183). From there *Yersinia* targets mesenteric lymph nodes (MLNs). Enteropathogenic *Yersinia*'s early internalization by naïve murine macrophages and ability to proliferate in them suggests that the dissemination to MLNs is mediated by migrating phagocytes (251, 312). Still, it is clear that in order to spread from MLNs to spleen and liver to cause systemic infection, the ability to block phagocytosis is essential. This ability is mediated by the T3SS effectors where YopH is clearly a player (191, 307). The ability to spread from MLNs leads to rapid spread of the infection thereby causing sepsis.

1.4.5. Adhesins

Adhesins are sticky proteins expressed on the surface of bacterial cells. Their function is to adhere to host target cells. *Y. pseudotuberculosis* has 3 major adhesins: Invasin, *Yersinia* adhesin A (YadA) and attachment and invasion locus (Ail).

Invasin is a chromosomally encoded protein whose optimal expression occurs at 26°C (211, 235). However, low level of expression is also seen under acidic conditions at 37°C (235). Therefore, it is not surprising that invasin is believed to be present on the bacterial surface and have important roles early during infection (236, 237). As discussed earlier, invasin is mainly required for crossing the intestinal barrier through its interaction with β1-integrins (60, 145, 146, 183). However, further systemic spread of infection does not require invasin (95, 236). Still, importantly, invasin is one of the adhesins that can promote cell contact and activate the T3SS effector translocation into host cells (45, 205).

Ail is also a chromosomally encoded and its expression is induced at 37°C (210). Surprisingly, in *Y. pseudotuberculosis* Ail appears not to have any major roles neither in adhesion nor in invasion (308).

Among these three adhesins, YadA is the only one not encoded on chromosome but instead on the 70 kb virulence plasmid (41). Its expression is induced at 37°C through the action of the same transcriptional regulator, LcrF, that regulates the T3SS genes (289). Primarily, it mediates serum resistance via inactivating complement system (277). YadA also mediates binding to different cell types via its interaction with extracellular matrix components (100). Although *Y. pseudotuberculosis* strains devoid of YadA do not lose their pathogenicity, it has been shown that YadA and invasin are involved in selective binding and Yop translocation into neutrophils. (125, 137, 230). However, YadA is clearly important for establishment of infections caused by *Y. enterocolitica* (298).

1.4.6. Regulation of T3SS in *Yersinia*

Since T3SSs are encoded by a large number of genes, their expression is highly energy consuming and in addition early secretion could result in early recognition of bacteria by the host immune system, expression of T3SSs are tightly regulated. In *Yersinia*, regulation is believed to occur at two main levels: the shift to the host body temperature and target cell contact. In addition, translocation of the T3SS effectors is also subjected to regulation.

1.4.6.1. LcrF and temperature sensing

During the infection of a mammalian host, the first major change *Yersinia* encounters is the switch to the host body temperature of 37°C. This results in a basic level expression of the T3SS genes mediated by the thermoregulator protein LcrF (64, 75, 324, 336). LcrF belongs to the AraC family transcriptional activators (64, 134). Upon the temperature shift, suppression by YmoA on transcription from the *lcrF* gene is relieved as a result of degradation of YmoA by proteases (65, 150). However, transcription is not the only level where LcrF production is regulated. LcrF is not synthesized, even when its transcription is forced at lower temperatures, as a result of 2 stem-loops which masks the Shine-Delgarno sequence of the *lcrF* mRNA. At 37°C, the stem loop structures melt making the Shine-Delgarno sequence accessible to tRNAs and ribosomes (40). Thus, LcrF synthesis is thermoregulated both at transcriptional and post-transcriptional levels. Once produced, LcrF functions as a master regulator for the expression of the T3SS encoding genes (64, 75, 324).

1.4.6.2. Target cell contact and Ca^{+2} depletion

Complete induction of T3SS requires a contact between the bacterium and target cell. Secretion after the contact is polar, i.e. occurs only close to contact site (265). Although it is not exactly known how this contact is sensed and how the signal is directed to the bacterium, it has been proposed that the signal passes through the tip complex and the needle filament (199, 260, 310).

Multiple proteins are involved in contact dependent regulation of T3SS and they will be discussed in more detail in following sections.

Unique to the T3SS of *Yersinia*, cell contact at least partly can be mimicked *in vitro* by depleting Ca^{+2} from the growth media at 37°C and has been a useful tool in studies of the *Yersinia* T3SS. During Ca^{+2} depletion *in vitro*, activation of the T3SS is also followed by growth cessation which is not assumed to occur during *in vivo* infections (47). Thus, although low Ca^{+2} induces expression and activates T3SS *in vitro*, it may not fully reflect the conditions the pathogen is exposed to during *in vivo* infection of animal/human hosts.

1.4.6.3. Virulence plasmid copy number

In *Yersinia*, Ysc-Yop T3SS encoding genes are located on the virulence plasmid. Recent research has shown that the temperature dependent regulation of T3SS also includes upregulation of the copy number of this plasmid. Plasmid copy number increases upon temperature shift to 37 °C and even more in Ca^{+2} depleted media. The upregulation of plasmid copy-number was shown to occur also during *in vivo* infection in the mouse infection model. When the plasmid was integrated into *Yersinia* chromosome, this increase of gene dose was suppressed and this also correlated with lowered virulence in mice compared to the wt strain (320). This further highlights that *Yersinia* has multiple ways to rapidly induce and activate the T3SS directly upon host cell contact. The rapid upregulation of T3SS gene dose makes the rapid cell contact activation even more powerful.

1.4.6.4. LcrQ and target cell binding

One of the proteins with a role in contact and Ca^{+2} dependent regulation is LcrQ (255). The exact mechanism of how LcrQ is involved is not known. One idea is that LcrQ forms a complex with YopD and LcrH (see Section 1.4.6.6) (53, 54, 334). A second possible mechanism suggested by Li et. al. claims that LcrQ:LcrF ratio inside the bacteria is important in T3SS gene transcription. If the ratio gets higher, the transcription is lowered and vice versa (184). In support of both

models, it is known that under secretion non-inducing conditions, intra-bacterial LcrQ level is high repressing the T3SS gene expression. Only after bacteria encounters secretion inducing environment, LcrQ is secreted by the T3SA which relieves the repression (241, 296).

1.4.6.5. Gatekeeper protein YopN

YopN is another protein with an established role in the induction of T3SS after target cell contact or Ca^{+2} depletion. It will be discussed more detailed in Section 1.4.8. In short, T3SS is supressed when YopN forms a complex with TyeA and this complex has been suggested to be targeted to T3SA by chaperones YscB and SycN (15, 57, 104). Under inducing conditions, YopN dissociates from all 3 proteins and, similar to LcrQ, becomes a T3SS substrate itself. This is accompanied by a dramatic increase of both expression and secretion of T3SS components (104).

1.4.6.6. Post-transcriptional regulation – YopD

Multiple substrates of the *Yersinia* T3SS have also been shown to be involved in the regulation of the system. YopD is one such protein involved in the negative regulation of the system. A complex of YopD with LcrH blocks the Shine-Delgarno sequence of the *yop* genes and destines them to degradation (18, 339). As mentioned above, LcrQ is suggested to be a member of this cascade as well. The YopD-LcrH complex is thought to be stabilized by LcrQ. However, upon activation, both YopD and LcrQ are recognized by T3SA as substrates thereby opening up the Shine-Delgarno sequences of the *yop* mRNAs to tRNA and ribosomes (53).

1.4.6.7. Regulation of translocation – YopK and YopE

The aim of T3SS is to translocate anti-host defense effector proteins into target cells. The amount of effectors translocated appears to be extremely important since not only too low but also high levels of translocated effectors could be detrimental for the pathogen. YopK is a negative regulator of translocation. In

its absence, effectors are over-translocated and this results in lowered *in vivo* virulence (136, 137, 304). It is possible that hyper translocation of Yop effectors results in an earlier and stronger recognition and immune response from the host. YopK connects translocon complex to actin cytoskeleton of the host via its interaction with β1-integrins of the host suggesting that the YopE-dependent disruption of actin cytoskeleton is signaled to YopK by eukaryotic signaling protein RACK1 and thereby YopK can regulate translocation (5, 303). It was also proposed that YopK regulates the pore size of translocon (136).

A second protein that regulates translocation is the effector protein YopE (see Section 1.4.7.1) (5). YopE is a GTPase activating protein (GAP) and a point mutant in the GAP domain not only disrupted its effector function but also resulted in increased translocation of all effectors (5). This also fits in a YopK-YopE model to regulate effector translocation levels.

1.4.7. Effectors and their function within the target cells

Pathogenic *Yersinia* species are mainly extracellular bacteria and the functions and mode of action of the translocated effector proteins also reflect this.

1.4.7.1. Cytotoxicity (YopE)

YopE is one of the two major effectors of the *Yersinia* T3SS that targets actin cytoskeleton of the host cells (263). This results in cytotoxic effect and rounding of adherent cells, e.g. HeLa cells, *in vitro* (263, 265). Since it is easy to monitor this change using light microscopy, it has been used as an indicator of T3SS and YopE activity for a long time. An arginine finger motif, with an Arg residue located at position 144, of the GAP domain of YopE has been shown to be critical for activity (35, 316). Eukaryotic RhoA and Rac1 proteins were proven to be targeted by YopE (316). In addition, *in vitro* studies suggested Cdc42 as a YopE target, as well (35). Effectors can differ in their importance against different host immune cells. It is known that YopE is important for the bacterial defense against uptake by dendritic cells (102).

YopE has also shown to be efficient in eliciting cytotoxicity, as even when low levels are translocated compared to wt, infected HeLa cells are still fully rounded within an hour of infection indicating complete disruption of the actin cytoskeleton. Therefore, it must be kept in mind that cytotoxicity or cell rounding should not be used as a sole indicator of YopE translocation.

In a recent paper it was shown that the YopE GAP activity could also be detected as a danger signal in macrophages and induces the killing of *Yersinia*. This may be one reason why the level of translocated YopE needs to be controlled. Interestingly, this effect could be counteracted by YopT (321).

When mice are infected with a Δ*yopE* mutant, bacteria can reach as far as to MLNs where they are cleared by the host and therefore the mutant is unable to cause systemic infection in mice (191).

1.4.7.2. Phagocytosis inhibition (YopH)

YopH is another major effector and its early translocation after target cell contact has proven to be very important for *in vivo* virulence (21, 22, 99). It is a protein tyrosine phosphatase (PTPase) and localizes at focal adhesion complexes within the host cell (34). Very soon after invasin mediates bacterial binding to β1-integrins, the host proteins such as FAK, $p130^{Cas}$ and Fyb are phosphorylated. YopH is quickly translocated into target cells and dephosphorylates these proteins, thereby blocking the cascade required for phagocytosis of bacteria (34, 124, 239).

The interaction between invasin and integrins also causes an increase in intracellular Ca^{+2} concentrations of target cells. YopH is responsible for blocking this Ca^{+2} influx, as well (22).

In *in vivo* virulence in the mouse infection model, YopH is very important as strains lacking a functional YopH protein do not reach beyond the PPs where they are cleared by the host (191, 307). A Δ*yopH* mutant was found to attract

more neutrophils to PPs compared to wt and this is probably the reason why in a co-infection of wt and Δ*yopH* there was increased clearance of wt strain (70).

1.4.7.3. Additional Effectors

Along with the major effectors, the *Yersinia* T3SS also translocates additional effectors into host cells all of which promote the infection to different degrees.

YopJ (or YopP in *Y. enterocolitica*) blocks cytokine production and induces apoptosis by inactivating the kinases of MAPK- and NF-κB signaling pathways (122, 126, 212, 213, 219, 275, 342). In addition, YopJ targets Nod2 signaling and thereby alters the permeability of intestines to promote bacterial dissemination (204).

YopM is different from other effectors by being targeted into the nucleus (31, 288). Its proposed function is to inhibit pro-inflammatory responses from the target cell (33, 201, 203). YopM can also induce apoptosis by activating Caspase-3 (335) and necrosis by targeting NLRP3 (327). In addition, YopM not only promotes infection but also prevents immune recognition of T3SS effector translocation by restricting the pyrin inflammasome (59).

YopT is not found in all *Y. pseudotuberculosis* strains. Similar to YopE, YopT also targets Rho family GTPases to disrupt cytoskeleton inside the host cells (143). The cleavage of GTPases by YopT leads to their mislocalization (284, 345). Therefore, it is not surprising that YopE can complement the absence of YopT in the strains that lack the *yopT* gene (313). Furthermore, as discussed earlier, YopT is responsible for counteracting the YopE induced danger signal inside the host cells by limiting YopE translocation. By competing for the same Rho GTPases as YopE, YopT has been suggested to counteract the danger signal (321).

YpkA (denoted YopO in *Y. enterocolitica*) also targets proteins involved in the regulatory cascade of actin polymerization, thereby preventing the formation of microfilaments. YpkA carries out these roles by its two enzymatic domains. The

interaction with globular actins of the host cell leads to activation of the serine/threonine kinase domain. This results in the phosphorylation of proteins involved in the actin polymerization (115, 181, 220). In addition, the guanine nucleotide dissociation-like inhibitor domain targets RhoA and Rac1 of the host. As a result, these proteins cannot be activated and take their role in actin polymerization (250).

1.4.7.4. Chaperones

The chaperones of the T3SS substrates carry multiple functions; they keep their respective substrates in an unfolded state prior to their entry to T3SS channel and target the substrates to the T3SA for secretion. These roles of the chaperones have already been discussed in previous sections. In addition, it has also been shown that some chaperones such as LcrG, chaperone of the tip protein LcrV in *Yersinia*, have important roles in T3SS regulation (200). Therefore, it can easily be concluded that chaperones have multiple significant roles for the function of T3SS.

In general, T3SS chaperones are divided into several classes. Class I chaperones specifically bind to effector proteins. They are further divided into class Ia which only binds to a single effector and class Ib which can bind to several effectors (233). Both classes have similar folded structures despite differing in sequence. Class I chaperones are acidic and form homodimers (152, 233). There is no significant difference between class Ia and Ib chaperones. What gives class Ib their binding promiscuity is that the sequence they target is found in multiple effectors (66). It is important to state that *Yersinia* T3SS does not have class Ib chaperones.

Class II chaperones are specific for translocators (233). They carry a triad of tetratricopeptide repeats (232). These repeats form helix-turn-helix conformation and constitute the protein binding site (9).

Class III chaperones are the third class and do not share any significant similarities with each other. In *Yersinia*, the YscF chaperone YscE, the LcrV chaperone LcrG and the YscX chaperone YscY belong to this group.

1.4.8. YopN

YopN is a 32 kDa protein that is encoded within an operon with 5 additional genes within the *lcrE* locus of the virulence plasmid (314). The coding sequence of the *yopN* gene overlaps with the downstream gene, *tyeA* (14). Previous research on the *Yersinia* virulence plasmid showed that the Δ*yopN* strain expressed and secreted Yops constitutively even in the presence of Ca^{+2} at 37°C. This phenotype was termed 'calcium blind (CB)', since a Δ*yopN* mutant is unable to sense the Ca^{+2} concentration to block expression and secretion (106, 314, 337).

YopN was also identified as one of the substrates of the T3SS (106). In fact, it was subsequently shown that YopN was not only secreted but also translocated into host cells by a YopB dependent mechanism, similar to T3SS virulence effectors (72, 116).

1.4.8.1. Structure of YopN

As depicted in its linear structure (Figure 7 upper panel), YopN carries a secretion signal (SS) and a chaperone-binding domain (CBD) within first 76 amino acids (aa) of its N-terminal (73, 148, 282). These are properties common to most T3SS substrates. In the C-terminal part, aa 212-222 and 243-293 are required for TyeA binding (144, 282), thus this region is termed the TyeA binding domain (TBD). In addition, at DNA level 3'-*yopN* and 5'-*tyeA* coding sequences overlap with 20 base pairs (bp). This means that sequences required for TyeA expression (i.e. 5'-UTR) localize within the 5' end of the *yopN* gene.

Obtaining the crystal structure of YopN was difficult since both ends have protein-binding domains and are unstable when not bound to their interacting partners. Schubot et. al. overcame this problem by crystallizing the protein

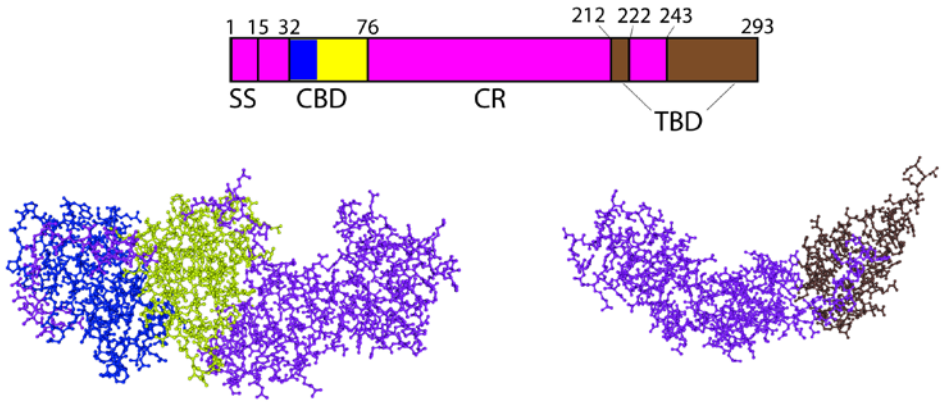

Figure 7. YopN protein with different functional domains depicted. Upper panel shows the linear structure of YopN. Lower left panel shows the 3D model of YopN (pink) in complex with its chaperones SycN (purple) and YscB (yellow). Lower right panel shows the 3D model of YopN-TyeA complex (TyeA is colored brown). SS, secretion signal; CBD, chaperone binding domain; CR, central region; TBD, TyeA-binding domain. Adapted from Schubot et. al. 2005 (282).

together with the binding partners; either to the chaperones (YscB and SycN) or to TyeA (Figure 7 lower left and right panels respectively) (282). The two structures were shown to be superimposable and could be merged into one model that constitutes a modelled structure for the YopN-TyeA-YopB-SycN complex. Altogether, these results showed that the chaperones and TyeA bind to two distal ends of the elongated YopN structure. Importantly, interaction with neither chaperones nor TyeA required YopN residues between aa 76 and 212 (from here this part will be referred to as the central region, CR).

1.4.8.2. YopN homologs in other T3SSs

YopN has homologs in the T3SSs of many animal pathogens. The most studied ones include InvE in *Salmonella* SPI-1, MxiC in *Shigella*, SepL in *E. coli*, PopN in *Pseudomonas* and CopN in *Chlamydia*. One significant difference among YopN homologs is that in Ysc/Yop family of T3SSs, including *Yersinia* (YopN-TyeA) and *Pseudomonas* (PopN-Pcr1), the YopN and TyeA homologs are expressed as two distinct proteins whereas in the other families they are expressed as a single protein with the TyeA homolog extending the C-terminal

of the protein (231). In *Yersinia*, a single frameshift mutation is all that is required for generation of a YopN-TyeA chimeric protein. The chimeric protein could regulate the T3SS similar to the wt *in vitro* and was secreted under inducing conditions. However, the mutant was slightly less competitive compared to the wt strain in mixed cell infection in the oral mouse infection model, therefore somewhat attenuated compared to wt (14).

In some T3SSs, there is evidence for a hierarchy of secretion of different substrates. According to this hierarchy, the YscF-, YscI- and YscP-protein family members are secreted first and have therefore been coined as early substrates. The middle substrates are the translocators that are secreted next and finally the effectors or late substrates are secreted. YopN homologs of some of the other systems have been shown to be involved in the secretion specificity switch from middle to late substrates. In *Salmonella*, a sorting platform composed of homologs of YscQ, YscK and YscL has been suggested to mediate the secretion switch both from early to middle and from middle to late substrates. Before secretion, substrates form high molecular complexes with the sorting platform. However, in a Δ*invE* mutant, translocators were not loaded onto the sorting platform and therefore could not be secreted. This implicates a role for InvE in the switch (174). In *Shigella*, MxiC is important for the timely secretion of effector proteins (44, 286). An interaction between MxiC and inner rod protein MxiI is critical for the switch from middle to late substrates. In line with this, disruption of this interaction resulted in constitutive secretion (57). Unlike InvE, a Δ*mxiC* mutant strain can secrete translocators but the secretion is delayed compared to the wt strain (57). SepL in *E. coli* acts together with another switch regulator SepD to control the substrate switch. Deletions of either of these two genes results in very similar phenotypes as the Δ*invE* deletion in *Salmonella*, i.e. repressed secretion of translocators and hyper-secretion of effectors (81). Recently, Portaliou et. el. added the YscV homolog EscV to SepD-SepL complex and suggested a more detailed mechanism for the switch. In this mechanism, EscV recruits SepL and SepD to the T3SA. This causes a conformational change in SepD which results in recognition of and interaction with the translocators via their cognate chaperones. The secretion of

the recruited translocators then occurs by the help of SepD. After translocator secretion is completed, SepL and SepD dissociate from each other allowing effectors to be recognized and secreted (246). The inability of translocator secretion from the Δ*invE* and Δ*sepL* mutants, as expected, prevents the translocation during cell infection (168, 227).

Another difference among YopN homologs is their own secretion. YopN has long been known to be secreted by the T3SS and more recently also shown to be translocated into infected host cells (72, 106, 116). However, neither InvE nor SepL are secreted and are therefore not expected to be translocated (167, 168). Importantly, CopN of the intracellular pathogen *Chlamydia* is the only YopN-homolog that has been shown to function as an effector inside the host cells. When expressed in yeast, it caused cell cycle arrest, which might be a result of prevention of microtubule assembly. In addition, CopN can also sequester tubulin (23). However, expression of YopN in yeast did not result in any detectable impact or phenotype (23, 139).

1.4.8.3. YopN binding partners and their role in suppression of secretion

All of YopN's intra-bacterial binding partners have been shown to have a role in blocking secretion under non-inducing conditions and the binding domains in all these proteins are well established (15, 73, 103, 104, 144, 148, 282). Initially, it was thought that YopN blocks secretion physically from the surface of the bacteria (106). However, later studies showed that YopN regulates secretion from the inside of the bacteria (Figure 8) (144). Under non-inducing conditions, TyeA specifically binds YopN at two residues (aa 212-222 and aa 248-293) where the tryptophan residue at position 287 being very critical for binding (15, 103, 144, 282). Substitution of the tryptophan residue diminishes the YopN-TyeA interaction and results in deregulation of the T3SS (15). This shows that the TyeA-YopN interaction is critical for regulation of expression and secretion of the *Yersina* T3SS. The YopN-TyeA interaction does not however require the interactions with the two YopN chaperones (282).

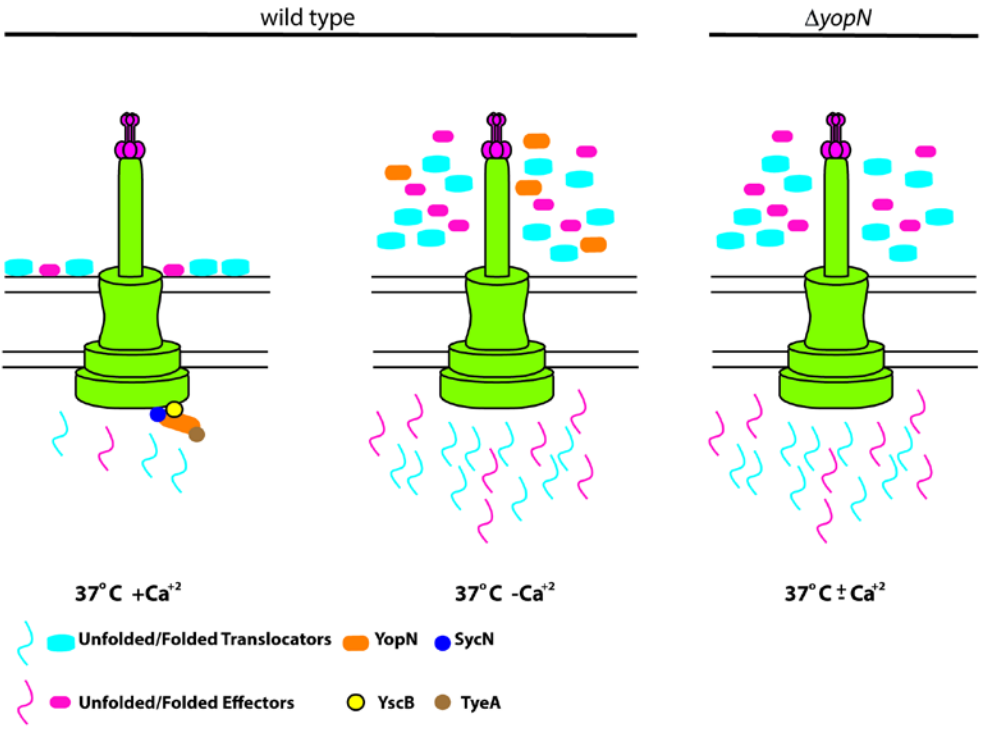

Figure 8. Regulation of T3SS by YopN and the effect of the Δ*yopN* mutation. Left panel illustrates non-inducing conditions at 37°C. Under this condition, YopN is bound to SycN, YscB and TyeA limiting the secretion to low levels. Exported proteins are localized at the bacterial surface. Middle panel illustrates the inducing conditions where YopN dissociates from its binding partners and becomes a substrate of T3SS itself. YopN's secretion is followed by a dramatic increase in secretion and expression of the T3SS substrates. Right panel illustrates Δ*yopN* mutant at 37°C regardless of Ca^{+2} concentrations. In this mutant T3SS cannot be blocked and is constitutively active at 37°C.

This complex is believed to be targeted to the T3SA by two chaperones, SycN and YscB (57). The target sequences of these two chaperones are adjacent to each other (155). Surprisingly, aa substitutions in the SycN binding part of CBD did not release the block of effector Yop secretion under non-inducing conditions. In addition, most of the aa substitutions within the SycN or YscB binding sites did not prevent the binding of the chaperones to YopN (155). This supports the idea that SycN and YscB should first form a heterodimer before binding to YopN (73) and also indicates that binding of one chaperone can bring the other in close proximity to YopN. Importantly, none of these substitutions blocked YopN secretion under non-inducing conditions suggesting that either

the CBD has a specific role in YopN secretion regulation or that the YopN-SycN-YscB complex cannot be formed correctly to block secretion (155).

1.4.8.4. Secretion of YopN and desuppression of the T3SS

The idea that T3SS substrate secretion follows YopN secretion comes from *Shigella* where MxiC secretion is followed by secretion of other substrates (44, 199). The exact mechanism behind the up-regulation of secretion is not known but it is believed that YopN dissociates from all three binding partners since none of them are secreted and loss of any of the proteins results in early secretion of YopN (73, 144). This might somehow trigger the switch of YopN from being a secretion suppressor to a T3SS substrate (Figure 8) (104).

1.4.8.5. Translocation of YopN

Any additional functions of YopN and its homologs have not been addressed for some time. One reason for this might be that neither InvE nor SepL are secreted and therefore unlikely to be translocated. To my knowledge, translocation of MxiC has not been addressed yet, although it is known to be secreted. The only YopN homolog shown to have an additional role is CopN which exerts an effector-like function inside eukaryotic cells (23, 139).

Translocation of YopN was first shown by Day et. al. where ELK-tagged YopN (YopN-ELK) was shown to be translocated into HeLa cells. Furthermore, YopN-ELK translocation was influenced by intra-bacterial regulators SycN, YscB, TyeA and LcrG. In addition, a Δ*yopB* mutant which is defective in building a functional translocon was found not to translocate YopN-ELK proving that the translocation was T3SS specific. However, this study was done in a mutant where all other effector *yop* encoding genes were deleted. Therefore, any competition for translocation between effectors was eliminated (72).

In a subsequent study it was also shown that GSK-tagged YopN (YopN-GSK) was also translocated in a YopB-dependent manner. Importantly, in a Δ*yopE* background, both YopN-ELK and YopN-GSK were translocated. In this

background competition between effectors still exists despite being at a lower level as a result of the absence of one of the effectors (116).

However, neither of these studies directly compared YopN translocation between non-enhanced and enhanced translocation derivatives of the T3SS which means that it is not clear if YopN translocation is regulated in a similar way as for the other effectors.

1.4.8.6. YopN impact on in vivo virulence

Activation of the T3SS *in vitro* comes together with growth cessation which can be readily observed when *Yersinia* is grown at 37°C without Ca^{+2}. Under this condition, *Yersinia* stops growing whereas it can continue to grow at 37°C with Ca^{+2} or at 26°C regardless of the Ca^{+2} concentration (171). The exact reason of this growth cessation is not known but it is believed that the energy required to support T3SS is maintained by shutting down metabolic pathways resulting in growth cessation. This can partly be supported by the Δ*yopN* mutant. When bacteria lack YopN protein, as a result of CB phenotype, T3SS secretes Yops continuously at 37° (Figure 8) (104, 106).

The CB phenotype of the *yopN* mutant is problematic for evaluation of the role of YopN during *in vivo* virulence. Firstly, when *Yersinia* enters to target organism's body, it needs to be able to replicate to establish infection. Being unable to replicate, a Δ*yopN* mutant can never reach to sufficient numbers within the host. Secondly, the Δ*yopN* mutant starts to secrete T3S substrates as soon as the environmental temperature is 37°C which is the host temperature. This could be expected to result in a strong early host immune response. Altogether, this makes it difficult to assess a role of YopN in *in vivo* virulence by using the full length Δ*yopN* mutant.

This, in turn, makes it hard to work with YopN and any direct role in virulence although the fact that YopN is translocated is very interesting. Thus, any study that targets YopN needs to discriminate between its regulatory and non-

regulatory functions, which were one of the initial challenges of this project that will be outlined in the coming pages. How did it end? Let's see!

2. Main objectives of the thesis

YopN has been established to be important for the function of the T3SS. However, due to its function in regulation and that the mutants characterized so far are deregulated for expression and activation of the T3SS there are essentially no studies on an additional function of YopN in the *Yersinia* T3SS. In this study my aim was to investigate potential additional roles of YopN. More specifically the aims of this study were:

- To investigate the kinetics of T3SS activation and any function of YopN in this process.

- To characterize the central region of YopN and any role of this region in the function of the T3SS.

- To dissect any direct role of YopN in virulence from its role in the T3SS regulation.

3. Results and Discussion

3.1. Kinetics of the T3SS induction upon Ca^{+2} depletion

T3SSs are tightly regulated at multiple levels by multiple factors (See section 1.4.6). In *Yersinia*, the regulation of the T3SS has been quite extensively studied. However, the kinetics of expression and secretion of T3SS substrates upon the activation of the system has not been directly addressed. Therefore, we decided to address this and to study the kinetics after induction of the T3SS of *Yersinia*.

When we expressed YopN-HA *in trans* and the system was induced by depleting the Ca^{+2} from the culture medium after an hour of growth in the presence of Ca^{+2}, we observed that the secretion of YopN could be detected already 5 minutes after the activation (Paper 1, Figure 2A). In addition, secreted YopN levels increased over time up to 30 min after depletion. YopN was first identified as a negative regulator of the *Yersinia* T3SS and upon switching into T3SS inducing conditions YopN was found to be secreted, thereby relieving the suppression of both Yop expression and secretion (104, 106, 337). The mechanism behind the suppression/de-suppression process is not well known. A widely accepted hypothesis is that YopN blocks secretion of the T3SS substrates including another negative regulator LcrQ (241, 255, 296). LcrQ suppresses Yop expression but has no impact on the regulation of secretion (255). Upon the secretion of YopN, LcrQ is secreted as well presumably relieving the block on Yop expression. This mechanism can also explain the deregulated expression observed in the Δ*yopN* mutant seen under non-inducing conditions.

As YopN secretion is known to be followed by induction of Yop expression and activation of secretion (104), we also analyzed this in the wt strain YPII/pIB102 (Paper 1, Figure 2B and C). YopH, the effector protein that has been shown to be required during early infection, was expressed and secreted at detectable levels around 5 minutes after activation (Ca^{+2} depletion) and both expression and secretion of YopH dramatically increased around 15 minutes after activation.

Expression and secretion of the translocator YopD and the effector YopE both reached detectable levels 15 minutes after induction, whereas the translocators YopB and LcrV were detected 30 minutes after induction.

Considering the requirement of early translocation of YopH during infection (99, 328), presumably all translocators are expressed and secreted at the earlier time points despite being below the limits of our detection levels. It is also known that the levels of YopB required for functional translocation are very low (99). In addition, previous studies showed that the detectable levels of the *yopE* transcript were not reached until 45 minutes after the temperature shift in inducing media (107). This is longer than the 15 minutes where we detected YopE expression. Importantly, in an actual oral route of infection the bacteria directly encounter temperature shift. However, the induction of the T3SS would be expected to occur only after host cell contact. These conditions are likely to be better mimicked in our experiment and we believe 15 minutes is a better representation of the required time to acquire detectable YopE levels.

In the '2-step model' for translocation by the T3SS, Yops first localize at the bacterial surface from where they are released upon target cell contact and the translocators somehow direct the effectors into the host cells (96). We wanted to analyze how quickly YopH was released from bacterial surface after a shift into inducing conditions. The experiment was designed in the same way with activation by Ca^{+2} depletion and a final sample taken 1 h after induction. Bacteria were fixed onto the cover slides, immunostained using anti-HA antibodies and analyzed by fluorescence microscopy. Surprisingly, the YopH levels on the bacterial surface increased over time with a major increase around 15 minutes. The exposure time required to detect YopH-HA on the surface was 10 times shorter in the 15 minute-sample compared to 0- and 5-minute samples. In addition, the Δ*yscF* mutant, that is unable to secrete Yops, did not show any surface staining even after 1 h induction (Paper 1, Figure 3A). An explanation to these findings might be the absence of target cells in the experimental setup. We probably can rule out that the increasing levels of secreted YopH-HA somehow

results in the aggregation of proteins on the bacterial surface since an over-secreting Δ*yopN* mutant did not show any surface staining (Section 3.2.2).

However, if the signals transmitted to induce the T3SS and to release surface protein are different and if the latter cannot be mimicked *in vitro* by Ca^{+2} depletion, the bacteria would not get the signals to release the proteins and proteins might accumulate on the surface. Our experimental setup where we activate the T3SS by depleting Ca^{+2} from the medium to mimic host cell contact gave valuable information about the kinetics of induction of expression and activation of secretion. Therefore it would be of great interest to include host cells and compare the activation of the system via binding to host cells and Ca^{+2} depletion and include studies of localization of translocators and effectors at the bacterial surface during activation and initiation of functional translocation.

3.2. Multiple functions of YopN

Various substrates of the T3SS in *Yersinia* carry regulatory functions in addition to their identified function. For example, YopE is not only an effector protein but also involved in translocation regulation (5). Another example is the translocator protein YopD which also is involved in transcriptional regulation of T3SS-related genes (18, 339). In addition, it is intriguing to note that YopD has also been found to localize inside infected human cells (108). YopN is another substrate with an established role in regulation of expression and secretion activation of the T3SS. In addition, YopN has been shown to be translocated into target cells by the T3SS (72, 116) which raised the question if it also has an additional function in the *Yersinia* T3SS. Furthermore, these studies indicated that YopN's translocation is not only YopB dependent, but also intra-bacterial T3SS regulators can influence its translocation (72). Here it is important to note that (i) YopB is a translocator protein and its localization within the target cell membrane is required for translocation; and (ii) intra-bacterial regulators are not secreted and cannot directly influence translocation. Thus, these studies did not answer one important question: Is YopN translocation regulated like the other Yop effectors?

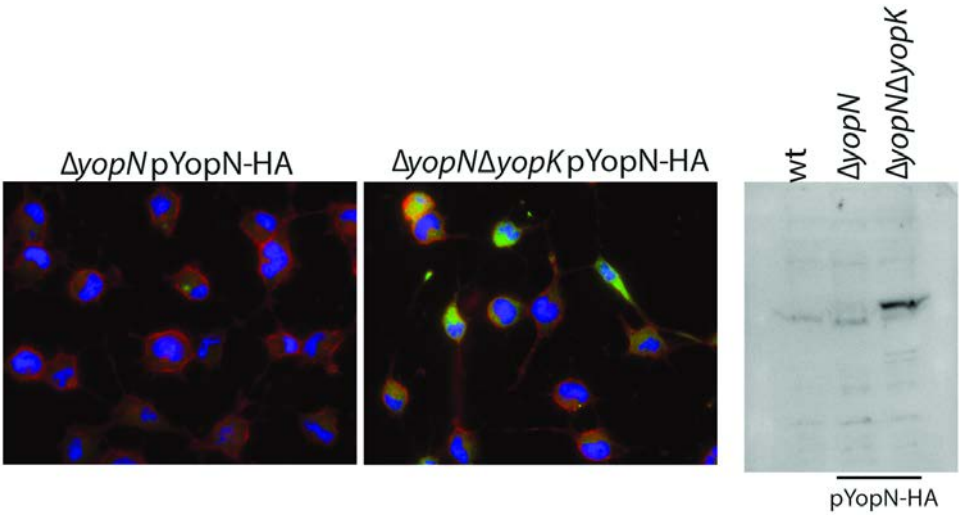

Figure 9. Regulation of YopN translocation by YopK. HeLa cells were infected for 2 h with indicated strains. Left panel shows immunostaining of infected cell membranes by Texas Red-conjugated WGA (red), cell nuclei by DAPI (blue) and translocated YopN-HA with anti-HA antibody (Sigma Aldrich, Now MERCK) followed by Alexa-488-conjugated secondary antibody (green). In the right panel, a second set of cells were fractionated and cytosolic proteins of the HeLa cells were precipitated. The samples were analyzed by western-blot using anti-HA antibody. Reprinted with permission from Sofie Ekestubbe ISBN 978-91-7601-639-8.

In *Yersinia*, YopK is a negative regulator of translocation and translocation of all known effectors is enhanced in a Δ*yopK* background (136). To verify if YopK has a similar role in YopN translocation, we infected HeLa cells with a strain expressing YopN-HA from an expression plasmid in either wt or a Δ*yopK* background. As can be seen in Figure 9, deletion of the *yopK* gene resulted in enhanced translocation of YopN (Ekestubbe, unpublished data). This verified that *Yersinia* YopN translocation is regulated in a similar way as the other Yop effectors. This gives support to the idea that YopN may have an additional function inside the host cell.

3.2.1. Lack of secretion hierarchy in *Yersinia spp.*

Although the exact molecular mechanism is not known, YopN and its homologs in other T3SSs of other pathogens are all involved in T3SS regulation albeit acting at different levels. InvE of *Salmonella* SPI-1 and SepL of *E. coli* are required for the secretion of the translocators (168, 227). In *Shigella*, MxiC is

not required for translocator secretion but its absence results in delayed secretion of translocators (57). In all these organisms, effector secretion is induced or up-regulated when genes encoding the YopN homologs are mutated or deleted (44, 81, 168). Based on these findings, the YopN family of proteins has been suggested to be part of a sorting platform that discriminates between translocators and effectors and thereby establishes a secretion hierarchy (174). According to the secretion hierarchy model, substrate secretion occurs at 3 levels; early substrates required for secretion of other substrates are secreted first and they are followed by secretion of the translocators and finally the effectors.

As expected a non-polar Δ*yopN* mutant, verified to have no effect on the expression of the overlapping downstream *tyeA* gene, expressed and secreted high levels of T3SS substrates under both inducing and non-inducing conditions (Paper 1, Figure 2). To specifically address the translocator secretion, we analyzed YopB and YopD secretion by western blot. Unlike in *Salmonella* and *E. coli*, in *Yersinia*, deletion of the *yopN* gene did not result in supressed secretion of the hydrophobic translocators. YopB and YopD secretion levels were high and similar not only under inducing conditions but also under non-inducing conditions (Paper 2, Figure 5). These results are in line with previous findings showing that the hydrophilic translocator LcrV was also secreted in a Δ*yopN* mutant (73, 148). To ensure that the loss of YopN had no impact on kinetics of translocator secretion similar to the *Shigella* homologue MxiC, we also monitored YopB/YopD secretion within the first 30 minutes after induction. As early as 15 minutes after induction, both YopB and YopD were secreted at higher levels compared to the wt strain (Paper 2, Figure 6). These data are in accordance with the earlier studies where it was shown that a Δ*yopN* mutant was able to translocate effector proteins into infected host cells *in vitro*, a process that strictly requires secreted translocator proteins (104). Taken together, these results show that in the *Yersinia* T3SS, YopN does not discriminate between translocator and effector proteins and the hierarchy identified in other T3SSs via a proposed sorting platform does not appear to exist in *Yersinia*.

3.2.2. YopH surface localization under non-inducing conditions requires YopN

The effectors YopE, YopH and the translocator YopD have all been shown to localize to the bacterial surface under non-inducing conditions (8, 225). In addition, surface localized YopH has been shown to be translocated into target cells in a T3SS dependent manner (8). Δ*yopN* mutant strains are 'calcium blind', i.e. they cannot sense the Ca^{+2} concentration in the environment and the T3SS is constitutively active at 37°C regardless of Ca^{+2} concentration or target cell binding. In other words, at this temperature the Δ*yopN* mutant acts as it is constantly under inducing conditions for Yop expression and secretion (106, 337). Therefore, we decided to analyze how well a Δ*yopN* mutant represents inducing conditions when grown in non-inducing conditions (Paper 1, Figure 1). We grew the strains expressing YopH-HA at 37°C under non-inducing conditions for 2 h. The samples were fixed on cover slides, immunostained by anti-HA antibodies and analyzed by fluorescence microscopy. As expected, YopH was detected at the bacterial surface of the wt strain under non-inducing conditions. On the other hand, the Δ*yopN* mutant showed essentially no surface localization of YopH and most of the bacteria were similar to secretion negative control, the Δ*yscF* mutant. Importantly, we verified that YopH-HA was expressed at similar levels in all 3 different strain backgrounds. These results suggest another role of YopN, namely to be required for the surface localization of Yops. One possible mechanism is that YopN directly interacts with secreted proteins and guide them to the surface. Another possible explanation is that YopN is involved in the signal transduction cascade that results in the release of Yops from the bacterial surface and when YopN is missing, Yops no longer can bind to the surface. Key to understanding the potential function of YopN in the surface localization of the Yops prior to activation will be to study the localization of YopN during these conditions. Is YopN also present on the bacterial surface in low amounts under non-inducing conditions as it was previously suggested (106)?

3.2.3. The central region of YopN is dispensable for T3SS regulation

Although regulation of T3SS in some way is common to all YopN homologs that are identified, the secretion of YopN homologs themselves vary. For example InvE and SepL are not secreted (167, 168); whereas MxiC and YopN are secreted by the T3SA (44, 106). Importantly, in *Yersinia* and *Shigella*, the secretion of YopN and MxiC are also connected to activation of the T3SS. In both species, secretion of substrates follows the secretion of YopN and MxiC (44, 104, 199). In this way induction and regulation of secretion is tightly regulated in both species.

In *Yersinia*, regulation of YopN secretion depends on TyeA which binds amino acids 212-222 and 248-293 and the chaperones SycN and YscB that bind within the C-terminal 76 amino acids (144, 282). This leaves a large central region (CR) between these binding sites that is essentially uncharacterized and without any known function. Thus, we hypothesized that, if YopN has any function other than that in regulation, it might involve the CR (Papers 2 and 3). We first wanted to rule out that the CR of YopN had any function in the regulation of T3SS. Therefore, we constructed 3 different deletions within the CR of YopN: $yopN_{\Delta 76\text{-}181}$, $yopN_{\Delta 131\text{-}167}$ and $yopN_{\Delta 170\text{-}207}$ and expressed them from the arabinose inducible promoter of the pBAD24 plasmid in a Δ*yopN* background (Paper 2, Figure 1). These regions were selected according to published 3D-structure of YopN (282) and predicted to have limited impact on the overall YopN structure. We also included wt YopN expressed from the same plasmid as a control.

We next analyzed the impact of these deletions on the regulation of the T3SS. Out of the three mutants, $YopN_{\Delta 170\text{-}207}$ was the most deregulated. Expression and secretion levels were similar to wt YopN under inducing conditions. However, there was also some Yop secretion under non-inducing conditions. Therefore, we concluded that $YopN_{\Delta 170\text{-}207}$ could not fully suppress Yop secretion under non-inducing conditions (Paper 2, Figures 2-4). It is not very surprising that this mutant was partly deregulated since the deleted region included a

small part of TBD. Thus, the deletion might influence the complex formation between TyeA and YopN that in turn resulted in the deregulated phenotype.

The second mutant, $YopN_{\Delta 131\text{-}167,}$ was not as deregulated as $YopN_{\Delta 170\text{-}207}$, but still not as tightly regulated as the wt. Both expression and secretion of $YopN_{\Delta 131\text{-}167}$ were similar to wt YopN (Paper 2, Figure 3). However, $YopN_{\Delta 131\text{-}167}$ could not completely block Yop secretion under non-inducing conditions (Paper 2, Figures 2 and 4).

The third mutant, $YopN_{\Delta 76\text{-}181}$, was also expressed at comparable levels to wt whereas the secreted $YopN_{\Delta 76\text{-}181}$ levels were lower compared to wt and the other mutants (Paper 2, Figure 3). Importantly, $YopN_{\Delta 76\text{-}181}$ could still efficiently block Yop secretion under non-inducing conditions and the Yop secretion levels were similar to wt under inducing conditions (Paper 2, Figures 2 and 4). We do not know if the lower level of extracellular $YopN_{\Delta 76\text{-}181}$ was the result of a problem with its secretion or if it was less stable outside bacteria. Thinking that YopN blocks secretion intra-bacterially (144), non-secreted $YopN_{\Delta 76\text{-}181}$ would be expected to suppress the secretion of other substrates under inducing conditions. However, in $YopN_{\Delta 76\text{-}181}$ expressing strain Yop substrates are secreted as wt hinting that $YopN_{\Delta 76\text{-}181}$ is secreted but less stable outside the bacteria.

Here, it is important to note that all YopN variants functioned in the general induction of T3SS. Although the secretion block under non-inducing conditions was impaired at different levels, all the strains expressing the different YopN variants secreted significantly more Yops under inducing conditions (Paper 2, Figure 2). This means that all the YopN variants could sense induction signals and regulate the T3SS accordingly. These findings are very important as we now can establish for the first time that the CR of YopN is dispensable for regulation and activation of the T3SS. One consequence of this finding is that it opens up for new studies addressing any additional role involving the CR.

3.2.4. YopN is required for efficient early translocation of YopE and YopH

YopE is one of the two major effectors translocated by the *Yersinia* T3SS. Its activity within the target cell leads to disruption of the actin cytoskeleton and thereby causes rounding of the infected cells, which can easily be tracked directly by light microscopy (263, 265). When we infected HeLa cells with *Yersinia* expressing wt YopN or the deletion variants at multiplicity of infection (MOI) of 2:1 (bacteria per cell), all mutants caused delayed rounding of the HeLa cells. Out of the three mutants, the strain expressing $YopN_{\Delta 76\text{-}181}$ was most delayed and complete cell rounding was not evident even after 4 hours of infection whereas wt and the Δ*yopN* mutant strain caused rounding of all infected cells already after 1 hour of infection (Paper 2, data not shown).

In order to determine if this effect was general and if the translocation of another major effector, YopH, was also affected, we introduced the YopN variants into a strain expressing $YopH_{6\text{-}99}$-Bla (YopH-Bla) in a Δ*yopN* background and infected HeLa cells with these strains with a MOI of 20:1 for 30 minutes. Using the beta lactamase reporter assay we measured blue (translocated) and green (non-translocated) signals and calculated the translocation levels as blue:green ratio. As expected, the Δ*yopN* mutant translocated even more YopH than the wt strain. This is likely the result of pre-prepared pool of translocators and effectors already expressed prior to host cell contact. Therefore, at the initiation of the infection, the already expressed proteins before host cell contact could be directly translocated into HeLa cells. The strain expressing $YopN_{\Delta 170\text{-}207}$ translocated similar levels of YopH-Bla as Δ*yopN*. It is important to state that this strain was leaky in Yop expression and secretion under non-inducing conditions. Thus, we believe that, similar to the Δ*yopN* mutant, the high level of YopH-translocation in this mutant is caused by the leaky phenotype. The less leaky $YopN_{\Delta 131\text{-}167}$ expressing strain translocated similar amount of YopH with wt and wt YopN complementation strains. Importantly, the strain expressing $YopN_{\Delta 76\text{-}181}$, which was regulated as tightly as the wt in Yop expression and secretion, translocated significantly lower levels of

YopH-Bla compared to the wt and the wt YopN complementation. This suggested that YopN actually has a role in promoting efficient effector translocation somehow mediated by the CR (Paper 2, Figure 7). Importantly, we could hereby differentiate this role from YopN's role in regulation. Both in *Salmonella* and *E. coli*, YopN homologs InvE and SepL respectively have been shown to be involved in translocation (168, 227). However, in these cases the effect is indirect since the Δ*invE* and Δ*sepL* mutants are unable to secrete the translocators. Importantly, our findings support that YopN could have a more direct role in translocation.

One key point to consider regarding the role of YopN in effector translocation was that the secreted level of $YopN_{\Delta 76\text{-}181}$ was lower compared to the wt YopN. This in turn could at least in part be the reason for the less efficient translocation. In the translocation assays, expression of the different YopN variants was induced by adding 0.2 % arabinose. This high level of arabinose was critical for $YopN_{\Delta 76\text{-}181}$ to regulate the T3SS since lower levels of arabinose resulted in deregulation of the system (Bamyaci, unpublished data). Therefore, we induced expression of wt YopN using lower arabinose levels and found that addition of 0.03 % arabinose resulted in similar exported levels of wt YopN to fully induced $YopN_{\Delta 76\text{-}181}$ (0.2 % arabinose) (Paper 2, Figure 8A). The YopN levels expressed using this low arabinose concentration were still sufficient to restore T3SS regulation of a Δ*yopN* mutant (Paper 2, data not shown). In addition, when wt YopN expression was induced by 0.03 % arabinose during HeLa cell infection, it translocated not only YopH-Bla but also $YopE_{6\text{-}86}$-Bla (YopE-Bla) at similar levels to when its expression was induced by 0.2 % arabinose and significantly more than $YopN_{\Delta 76\text{-}181}$ where expression was induced by 0.2 % arabinose (Paper 2, Figure 8B and C). This confirms that the less efficient translocation seen for the $YopN_{\Delta 76\text{-}181}$ expressing strain was not a result of lower levels of exported $YopN_{\Delta 76\text{-}181}$. Another possible reason for the less efficient translocation is that the deletion of aa 76-181 disrupted the overall structure of YopN and that this resulted in a physical block of translocation. To address this issue, we expressed wt YopN and $YopN_{\Delta 76\text{-}181}$ from pBAD24 plasmid in a wt YopN ($YopN^+$) background. We hypothesized that if $YopN_{\Delta 76\text{-}181}$ acts by

physically blocking translocation, then the phenotype of the mutant protein should be dominant when expressed concomitant with the wt YopN. On the other hand, if the less efficient translocation in the $YopN_{\Delta 76\text{-}181}$ expressing strain is linked directly to loss of the CR, than wt YopN should restore the mutant protein's effect to wt phenotype. When we infected HeLa cells, expression of $YopN_{\Delta 76\text{-}181}$ had no impact on translocation levels in $YopN^{+}$ background. This argues against that the impact on translocation is due to a physical block of the translocation channel (Paper 2, Figure 8D) and suggests a direct role for CR of YopN in promoting efficient early translocation of YopH and YopE.

While it was convenient to perform the initial studies expressing the YopN variants *in trans* in a Δ*yopN* mutant background, our plan was to introduce the most interesting mutations *in cis* on the virulence plasmid. However, to our disappointment, when we introduced $yopN_{\Delta 76\text{-}181}$ mutation *in cis* on the virulence plasmid of *Y. pseudotuberculosis*, Yop expression was deregulated at 37°C (Paper 3, data not shown). The most likely explanation is that the levels of $YopN_{\Delta 76\text{-}181,}$ when expressed from the native promoter, were lower compared to when expressed from pBAD24 plasmid and too low to facilitate regulation of the Yop expression and secretion. Therefore, we needed to find a way to design YopN mutants with retained regulatory function when expressed from the native promoter *in cis*. When we made an *in silico* analysis of the CR of YopN, we could identify three putative coiled coil domains (CCDs) located between amino acids 65-100. In addition, the published crystal structure of YopN also identified an α-helix that covered two of the three putative CCDs. Combining these, we decided to disrupt the CCD between aa 80-86. We used two different methods to achieve this. In the first approach we mutated the charged amino acids Asp-82 and Glu-84 into Ala and the critical hydrophobic residues Val-80 and Val 83 into Gly ($YopN_{GAGA}$), Arg ($YopN_{RARA}$) or Ser ($YopN_{SASA}$). In the second method, the Val-83 residue was substituted by Pro ($YopN_{V83P}$). All these mutations were predicted to disrupt the CCD (Paper 3, Figure 1). All the mutant variants were expressed and secreted at similar levels as wt YopN with fully retained regulatory function. (Paper 3, Figure 2A-D). In addition, similar to

$YopN_{\Delta 76\text{-}181}$, the strains expressing these proteins from the pBAD plasmid were all impaired for YopH translocation into HeLa cells (Paper 3, Figure 2E).

Of the substitution variants, $yopN_{GAGA}$ and $yopN_{RARA}$ were chosen to be introduced *in cis* on the virulence plasmid of *Y. pseudotuberculosis*. The resulting strains were similar to wt in their ability to block the T3SS under non-inducing conditions as well as removing the block after switching into inducing conditions (Paper 3, Figure 4). In order to study the impact of $YopN_{GAGA}$ on effector translocation, *yopH-bla* and *yopE-bla* gene fusions were introduced into the strain expressing $YopN_{GAGA}$ *in cis* from the virulence plasmid. Both YopH and YopE were translocated at significantly lower levels by $yopN_{GAGA}$ compared to the wt strain (Paper 3, Figure 5). Similar to the studies of $YopN_{\Delta 76\text{-}181}$ (Paper 2), the impact on YopE translocation was bigger than on YopH translocation.

3.2.5. YopN central region is required for YopN translocation

For more than a decade, it has been known that YopN is translocated into host cells by the T3SS (72, 116). Therefore, we tested if the YopN variants were also translocated. We constructed Bla fusions of full length YopN, $YopN_{\Delta 76\text{-}181}$ and the substitution variants (YopN-Bla). When we infected HeLa cells and measured the YopN translocation using the Beta-lactamase assay, wt YopN-Bla was found to be translocated. Surprisingly, translocation levels of the mutants were very low and comparable to the levels of a translocation negative Δ*yopB* mutant expressing wt YopN-Bla (Paper 3, Figure 3). This means that the CR is important not only for the translocation efficiency of effectors but also YopN's own translocation. One interpretation of these findings is that YopN, similar to YopK and YopE, exerts its function in translocation from inside the host cell and that mutations that result in the loss of intracellular targeting of YopN therefore also has an impact on translocation of all Yops. However, at this point we do not know if these two events are connected.

3.2.6. YopN-YopD interaction

My work suggests multiple functions for YopN including being required for surface localization of YopH under non-inducing conditions and the role of the YopN-CR with the putative coiled-coil domain in efficient Yop translocation as well as YopN's own translocation. These findings suggest that YopN might interact with other Yops such as the translocators. Therefore we attempted to co-purify secreted Yops with YopN-HA from the supernatant of a culture grown under inducing conditions. First, we confirmed a specific binding of YopN-HA to magnetic beads coated with anti-HA antibodies that we used for immuno-precipitation (Paper 1. Figure 4A). When we analyzed the Yops in the same samples by Western blot, we found that YopD was co-purified with YopN (Paper 1, Figure 4B). In line with this, when we repeated the assay using a Δ*yopD* mutant, we did not detect any binding further confirming that YopN interacts with YopD (Paper 1, Figure 4C).

3.2.7. Possible targets of YopN inside the host cell

Although YopN is known to be translocated into the target cells, no function for YopN inside the target cell has been assigned or suggested. To my knowledge, the only published effort to attain an effector function to YopN was done by Huang et. al. (139). In that study, they expressed YopN and its homolog in *Chlamydia*, CopN, in yeast cells. While CopN expression gave a phenotype and resulted in cell cycle arrest possibly via altering cytoskeleton, YopN expression could not be linked to any phenotype or toxic activity (139).

Our results suggested that YopN has a direct role in translocation and it is inevitable to think that YopN's role within the host cell might be related to this role. We also showed that the YopN variants were not translocated. However, we do not have any indication that the inability to translocate YopN mutants is directly related to the YopN variants' lack of efficiency in Yop effector translocation. Therefore, we initiated a yeast-two-hybrid (Y2H) screen to see if we could identify any possible targets for YopN within the host cell that could lead us to either an effector function or a role in translocation regulation. To

cover the entire genome of a potential target cell for *Y. pseudotuberculosis*, we used ULTImate Y2H screen by Hybrigenics Services, France. In short, we sent amplified *yopN* gene together with its sequence to Hybrigenics Services that generated fusion of the *yopN* gene to the *lexA* gene in the pB29 plasmid to be used as bait in the screening. As prey we chose a cDNA library obtained from activated human leukocytes cloned into the pB6 plasmid. Hybrigenics Services stated that they their tests did not show any toxic activity of YopN towards the yeast cells. The company performed the initial Y2H screening and delivered the obtained potential hits. From the hits provided, we chose two proteins, Heterogenous nuclear ribonucleoprotein U-like 2 (hnRNPUL2) and SH3 domain binding protein 1 (SH3BP1, Exo70) to be tested further. In order to characterize the binding domain of YopN to these proteins, in addition to full length *yopN*, we fused $yopN_{76\text{-}181}$, $yopN_{\Delta 76\text{-}181}$, $yopN_{1\text{-}181}$, $yopN_{76\text{-}293}$, $yopN_{RARA}$, $yopN_{SASA}$ *and* $yopN_{VP}$ to *lexA* gene in pB29 plasmid (Figure 10A) and performed the β-Galactosidase assay of the Yeast β-Galactosidase Assay Kit (Thermo Scientific) according to the manufacturers' protocol to test their binding to hnRNPUL2 and SH3BP1. As seen in Figure 10B, the strongest binding of both putative target proteins was obtained from full length YopN. Significant binding was also seen for $YopN_{1\text{-}181}$; however, these interactions were weaker than the interactions with full length YopN.

Interestingly, none of the other YopN variants, including $YopN_{\Delta 76\text{-}181}$ and $YopN_{76\text{-}181}$, could bind to the putative target proteins. Similar results were obtained when the yeast expressing the prey and a bait were grown on selective media (data not shown). hnRNPUL1 is a nuclear targeted protein and was first found to be involved in RNA metabolism and export (27, 111). Later research also identified its importance in double stranded DNA break signaling and repair (244).

Exo70 (or SH3BP1) is an SH3 domain binding protein and is also a component of exocyst which is important for spatial and temporal control of exocytosis. One established role of Exo70 is tethering the secretory vesicles to plasma membrane via its ability to bind to inner leaflet of plasma membrane (128, 188).

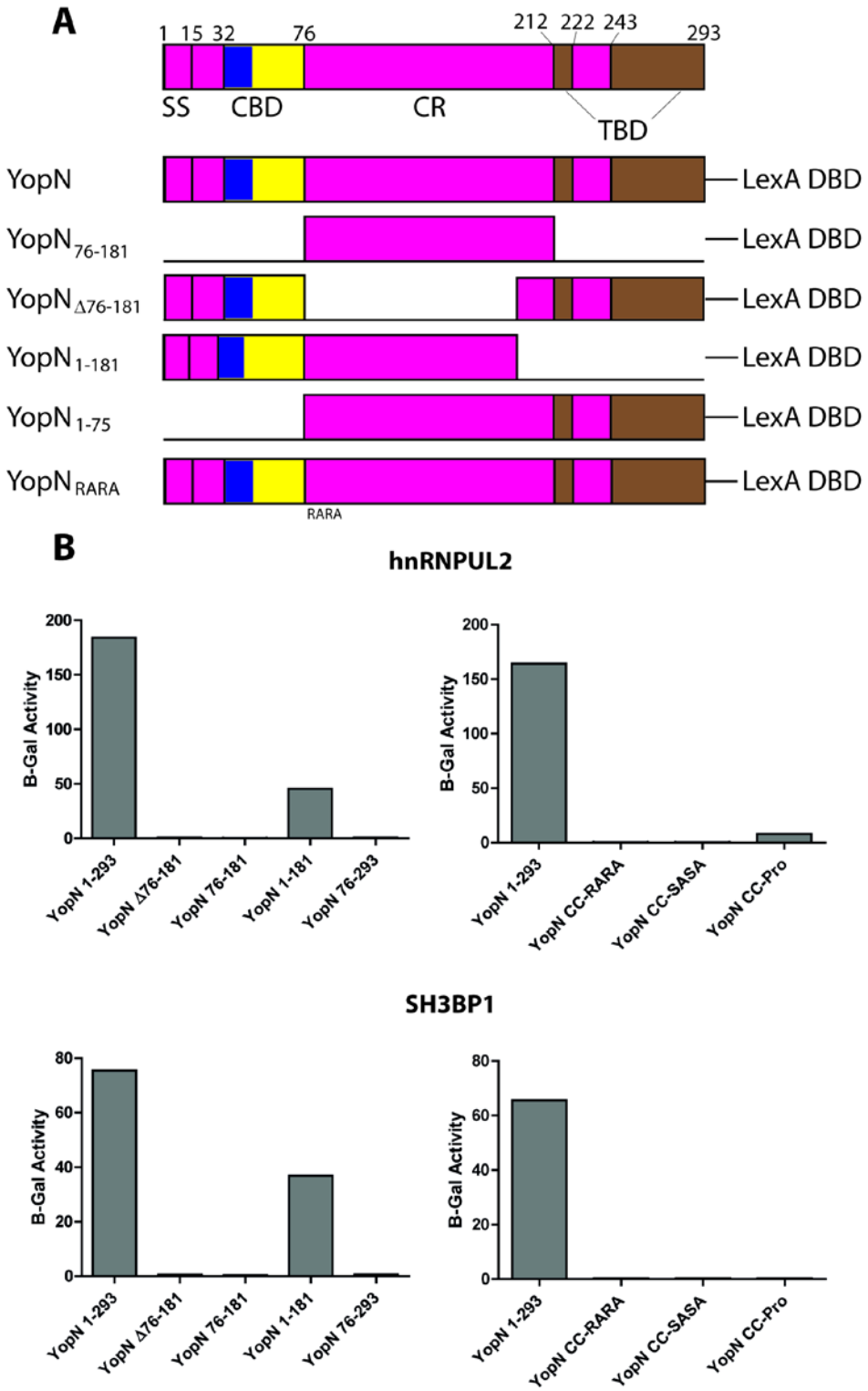
A
1 15 32 76 212 222 243 293
SS CBD CR TBD
YopN
YopN 76-181
YopN Δ76-181
YopN 1-181
YopN 1-75
YopN RARA
LexA DBD
RARA
B
hnRNPUL2
B-Gal Activity
YopN 1-293
YopN Δ76-181
YopN 76-181
YopN 1-181
YopN 76-293
YopN CC-RARA
YopN CC-SASA
YopN CC-Pro
SH3BP1

Figure 10. Characterization of YopN interaction with host proteins. (A) YopN variants used to characterize the YopN interaction with host proteins. (B) Depending on the results we received from Hybrigenics Services, we characterized the interaction of YopN with two of the putative targets, hnRNPUL2 and SH3BP1. We have repeated the yeast-2-hybrid assay by using different YopN variants as baits. Shown are one representative experiment for each assay.

In addition, Exo70 has a role in cell migration by inducing actin branching which, in turn, pushes the plasma membrane (187).

Although its function makes hnRNPUL2 less likely to be a biological target for YopN, the roles of Exo70 project a possible target for YopN. However, since the same domain of YopN ($YopN_{1-181}$) binds to both proteins, this might suggest a promiscuous structure of this domain that results in the detected bindings instead of a specific interaction. More experiments needs to be performed to test the specificity of these interactions to get support for any implications of the interactions for infection.

3.2.8. Lack of efficient translocation results in increased phagocytosis of *Yersinia pseudotuberculosis* by macrophages

Pathogenic *Yersinia* spp. are mainly extracellular during infection and their virulence strategy involving the T3SS, aim to prevent bacterial uptake. Thus, to test if the inefficient translocation of YopE and YopH had any impact on phagocytosis inhibition, we infected J774 macrophages with the strains expressing YopN CR deletion variants to determine their uptake within the first 30 minutes of infection. As expected $YopN_{\Delta131-167}$ and $YopN_{\Delta170-207}$ expressing strains were somewhat resistant to phagocytosis although not as resistant as the wt strain. However, $YopN_{\Delta76-181}$ expressing strain was phagocytosed to almost the same extent as a Δ*lcrV* mutant which recently was shown to be avirulent in a mouse infection (Paper 2, Figure 9) (99). This showed that the inefficient translocation of effectors within the first 30 minutes of infection also resulted in

significant loss of phagocytosis resistance. This finding is very significant as phagocytosis inhibition is really crucial for the ability of *Yersinia* to establish *in vivo* infection.

3.2.9. The putative coiled-coil domain within the YopN-CR is necessary to establish a systemic infection *in vivo*

Since $YopN_{GAGA}$ was stably expressed *in cis* and showed wt regulation of the T3SS but was unable to support efficient translocation, we decided to evaluate the impact of the mutation in the systemic mouse infection model.

YopE and YopH are major effector proteins of the *Yersinia* T3SS and a mutant that lacks either protein cannot establish a systemic infection in mouse (191, 307). Therefore, we hypothesized that the $yopN_{GAGA}$ strain, which was unable to translocate YopE and YopH efficiently within the first 30 minutes of a HeLa cell infection, would also be unable to establish a systemic infection. We therefore introduced the $yopN_{GAGA}$ gene into the Xen4 strain that expresses luciferase activity from the virulence plasmid and that can be readily tracked in real time during infection using the *In vivo* Imaging System (IVIS).

In the oral infection route, *Y. pseudotuberculosis* first reaches to intestine and PPs (60, 145, 146, 183). From here the bacteria disseminate to MLNs and later to spleen and liver to establish systemic infection (251, 312). We infected groups of mice with either wt or $yopN_{GAGA}$ Xen4 strains via oral route by adding bacteria into sterilized drinking water. On average, each mouse consumed 3.8 x 10^8 wt Xen4 or 4.4 x 10^8 Xen4 $yopN_{GAGA}$. At day 3 post infection (p.i.), intestines, MLNs, livers and spleens of 2 sacrificed mice, one infected with Xen4 wt and the other with Xen4 $yopN_{GAGA}$, were examined by IVIS. Both strains were able to establish infection by colonizing in intestines and PPs. Unlike Xen4 $yopN_{GAGA}$, wt Xen4 had already disseminated to MLNs at high numbers and to liver and spleen at low numbers. This showed that wt strain started to spread systemically as early as 3 days p.i. At day 5 p.i., the levels of both strains in the intestines of the sacrificed mice were lower. The wt Xen4 strain established systemic infection with increasing numbers in both spleen and liver. Despite increasing

in numbers in MLNs, the mutant strain showed lower numbers in spleen and liver compared to wt Xen4. At this day, the mobility of all mice infected with wt Xen4 was very low and their weight loss reached a critical level. Together, these were symptoms of a severe infection and all mice infected with wt Xen4 were therefore sacrificed. We continued to monitor mice infected with the Xen4 $yopN_{GAGA}$ strain. At day 8 p.i., the numbers of Xen4 $yopN_{GAGA}$ increased slightly in MLNs. On the other hand, its numbers in liver and spleen were lower compared to day 5 p.i. This means that the mutant strain was not able to remain systemic and the mouse started to clear the infection. At day 16 p.i., the mouse infected with Xen4 $yopN_{GAGA}$ had cleared the infection and only showed background level signals (Paper 3, Figure 6). Taken together, these data show that the $yopN_{GAGA}$ mutant that could neither efficiently translocate the major effectors nor promote its own translocation also failed to establish a systemic infection and was finally cleared by the host.

All in all, my work shows that YopN CR has a direct role in establishing a systemic infection in the host by promoting efficient translocation of YopE and YopH into target cells. We also attempted to test translocation of YopJ and YpkA in the same *yopN* substitution mutant backgrounds but the translocated levels of these two effector proteins were below the level of detection of the system we used even in a wt strain. Very importantly, the direct function of YopN translocation and virulence is independent of the role of YopN in T3SS regulation.

4. Main findings

- YopN is secreted rapidly after switching into T3SS inducing conditions.
- Yops are expressed and secreted rapidly after Ca^{+2} depletion and YopH appears to be secreted first.
- In contrast to most other T3SSs, in *Yersinia* there is no YopN dependent secretion hierarchy facilitating translocator secretion prior to effector secretion.
- YopN is required for the localization of YopH at the bacterial surface under non-inducing conditions.
- The central region of YopN spanning amino acids 76-181, is dispensable for the regulatory function of YopN in T3SS.
- A *yopN* mutant lacking amino acids 76-181 is impaired for translocation of YopE and YopH virulence effectors.
- Disruption of a putative coiled-coil domain localized at the N-terminal part of the central region is also dispensable for T3SS regulation and is also impaired for YopE and YopH translocation.
- The putative coiled-coil domain located within the central region of YopN is required for the T3SS dependent translocation of YopN into the target cells.
- The interaction between YopN and YopD might be involved in the role of YopN in translocation and/or Yop localization at the bacterial surface under non-inducing conditions.
- The central region of YopN is required for blocking uptake by macrophages.
- The impaired translocation of effectors within the first 30 minutes of infection is detrimental for the ability of *Yersinia* to establish *in vivo* infection.

5. Future Perspectives

The questions about YopN resolved in this thesis are just the tip of the iceberg to understand the role of this understudied protein of the *Yersinia* T3SS. Although we have verified and suggested multiple roles for YopN from regulation of the T3SS to *in vivo* infection, we do not know much about the molecular mechanisms. Thus, future experiments on YopN should focus on YopN function at a molecular level.

One very significant and novel finding is that we have established a role for YopN in effector translocation. It remains to be shown if YopN supports effector translocation from inside the host cell or acts extracellularly. The YopN substitution mutants' inability to be translocated could support the idea that YopN impacts translocation from inside the host cell. One way to better understand this could be to clone YopN variants into eukaryotic expression vectors and introduce these clones into different cell lines. Infection of these cells with strains expressing different YopN variants could then help us to understand how YopN acts during translocation. It is important to note that YopK and YopE are two proteins translocated by the *Yersinia* T3SS and both are involved in translocation regulation. Therefore, these cell lines might also be useful to reveal if YopN interacts with any of these proteins inside the infected host cell.

Even though we could not verify the significance of the initial findings, our data suggests that YopN has two potential targets inside the host cells. Cell lines expressing the different YopN variants could be helpful to confirm these interactions inside eukaryotic cells. The impact of expressing YopN variants could possibly be supported by biochemical assays to detect any impact or function of these target proteins and how YopN impacts these functions.

Another significant finding in this thesis is the interaction between YopN and YopD. A follow up of these findings should certainly include the characterization of this interaction. A possible binding domain of YopN would be expected to be

the CR. However, our initial studies suggest that the CR is not involved in this interaction. Another strong candidate for the interaction is the YopN C-terminal region. Different from most of their homologs in other pathogens, YopN and TyeA are expressed as two distinct proteins instead of a single fusion protein and the YopN-TyeA interaction is clearly important for the regulatory role of YopN. So far, no explanation has been suggested for the evolutionary advantages and/or disadvantages of this difference. If YopN-YopD interaction occurs via YopN C-terminal, YopN-TyeA interaction inside the bacteria could prevent or affect this binding. However, since the YopN-YopD interaction would be significant for the virulence of *Yersinia*, the separation of YopN and TyeA into two proteins could be advantageous for the bacteria. In addition, further optimization of co-immuno-precipitation experiments would be useful to see if YopN can interact with any other protein in the supernatant.

The T3SSs of *Yersinia* and *Pseudomonas* belong to the same sub-family. In line with this the homologs of YopN and TyeA, PopN and Pcr1 respectively, are expressed as two independent ORFs in *Pseudomonas*. Interestingly, PopN also encodes a putative coiled-coil domain located at the corresponding part of the protein with the one we mutated in our assays. It would be very interesting to see if PopN has similar additional roles as YopN has on the pathogenicity of *Pseudomonas*.

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