Controlling substrate export by the Ysc-Yop type III secretion system of *Yersinia*

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In memory of my father

To my mother

With love and eternal appreciation

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Abstract

Several pathogenic Gram-negative bacteria invest in sophisticated type III secretion systems (T3SS) to incapacitate their eukaryotic hosts. T3SSs can secrete protein cargo outside the bacterial cell and also target many of them into the eukaryotic cell interior. Internalized proteins promote bacterial colonization, survival and transmission, and can often cause severe disease. An example is the Ysc-Yop T3SS apparatus assembled by pathogenic *Yersinia* spp. A correctly assembled Ysc-Yop T3SS spans the *Yersinia* envelope and also protrudes from the bacterial surface. Upon host cell contact, this system is competent to secrete hydrophobic translocators that form a translocon pore in the host cell membrane to complete the delivery channel bridging both bacterial and host cells. Newly synthesized effector Yops may pass through this channel to gain entry into the host cell cytosol.

As type III secretion (T3S) substrates function sequentially during infection, it is hypothesized that substrate export is temporally controlled to ensure that those required first are prioritized for secretion. On this basis three functional groups are classified as early (*i.e.* structural components), middle (*i.e.* translocators) and late (*i.e.* effectors). Factors considered to orchestrate the T3S of substrates are many, including the intrinsic substrate secretion signal sequences, customized chaperones, and recognition/sorting platforms at the base of the assembled T3SS. Investigating the interplay between these elements is critical for a better understanding of the molecular mechanisms governing export control during *Yersinia* T3S.

To examine the composition of the N-terminal T3S signals of the YscX early substrate and the YopD middle substrate, these segments were altered by mutagenesis and the modified substrates analyzed for their T3S. Translational fusions between these signals and a signalless β-Lactamase were used to determine their optimal length required for efficient T3S. This revealed that YscX and YopD export is most efficiently supported by their first 15 N-terminal residues. At least for YopD, this is a peptide signal and not base upon information in the mRNA sequence. Moreover, features within and upstream of this segment contribute to their translational control. In parallel, bacteria were engineered to produce substrate chimeras where the N-terminal segments were exchanged between substrates of different classes in an effort to examine the temporal dynamics of T3S. In several cases, *Yersinia* producing chimeric substrates were defective in T3S activity, which could be a consequence of disturbing a pre-existing hierarchal secretion mechanism.

YopN and TyeA regulatory molecules can be naturally produced as a 42 kDa YopN-TyeA hybrid, via a +1 frame shift event somewhere at the 5'-end of *yopN*. To study this event, *Yersinia* were engineered to artificially produce this hybrid, and these maintained *in vitro* T3S control of both middle and late substrates. However, modestly diminished directed targeting of effectors into eukaryotic cells correlated to virulence attenuation *in vivo.* Upon further investigation, a YopN C-terminal segment encompassing residues 278 to 287 was probably responsible, as this region is critical for YopN to control T3S, via enabling a specific interaction with TyeA.

Investigated herein were molecular mechanisms to orchestrate substrate export by the T3SS of *Yersinia.* While N-terminal secretion signals may contribute to specific substrate order, the YopN and TyeA regulatory molecules do not appear to distinguish between the different substrate classes.

Abbreviations

5’-UTR – 5’-untranslated region

ATP – Adenosine -5’-triphosphate

AT – Autotransporter pathway

Bla – Beta-Lactamase

CBD – Chaperone binding domain

CD – Calcium dependent growth

CFU – Colony forming units

CI – Calcium independent growth

CU – Chaperone usher pathway

Cya – Adenylate cyclase

DM – Deletion mutagenesis

DNA – Deoxyribonucleic acid

EPEC – Enteropathogenic *Escherichia coli*

EHEC – Enterohaemorrhagic *Escherichia coli*

GST – Glutathione S-transferase

GTP – Guanosine-5'-triphosphate

HR – Hyper sensitive response

H – NS Heat-stable nucleoid-structuring protein

IM – Inner membrane

IL – Interleukin

IPTG – Isopropyl β-D-1-thiogalactopyranoside

LCR – Low Calcium response

LEE– locus of Enterocyte Effacement Pathogenicity Island

MLN – Mesenteric lymph nodes

mRNA – Messenger RNA

NMR – Nuclear magnetic resonance

OM – Outer membrane

PAIs – Pathogenicity Islands

RBS – Ribosome binding site

SD – Shine Dalgarno site

Sec – general secretion pathway

SM – Site-directed mutagenesis

SRP – Signal recognition particle

T1SS – Type one secretion system

T2SS – Type two secretion system

T3SS – Type three secretion system

T4SS – Type four secretion system

T5SS – Type five secretion system

T6SS – Type six secretion system

Tat – Twin arginine secretion system

TPS – Two partner secretion

TRF – Translational reporter fusions

TS – Temperature sensitive growth

WHO – world health organization

Yop – *Yersinia* outer protein

Ysc – *Yersinia* secretion

Papers included in this thesis:

This thesis is based on the following publications and manuscripts referred to by their roman numerical (I – IV).

I. Amer A. A., Åhlund M. K., Bröms J. E., Forsberg Å., and Francis M. S. 2011. Impact of the N-terminal secretor domain on YopD translocator function in *Yersinia pseudotuberculosis* type III secretion. J Bacteriol 193(23):6683-700.

II. Amer A. A., Gurung J. M., Francis M. S. 2013. *Yersinia pseudotuberculosis* type III secretion is reliant upon an authentic N-terminal YscX secretor domain. (Submitted manuscript)

III. Amer A. A., Costa, T. R., Farag S. I., Avican U., Forsberg Å., Francis M. S. 2013. Genetically engineered frame shifted YopN-TyeA chimeras influence type III secretion system function in *Yersinia pseudotuberculosis.* (Submitted manuscript)

IV. Amer A. A., Costa, T. R., Gurung J. M., Avican U., Forsberg Å., Francis M. S. 2013.Functional consequences of site-directed mutagenesis in the C-terminus of YopN, a *Yersinia pseudotuberculosis* regulator of Yop secretion. (Manuscript)

Papers not included in this thesis:

V. Costa T. R., Amer A. A., Fällman M., Fahlgren A., Francis M. S. 2012. Coiled-coils in the YopD translocator family: a predicted structure unique to the YopD N-terminus contributes to full virulence of *Yersinia pseudotuberculosis*. Infect Genet Evol 12(8): 1729-42.

VI. Costa T. R., Amer A. A., Farag S. I., Wolf-Watz H., Fällman M., Fahlgren A., Edgren T., and Francis M. S. 2012. Type III secretion translocon assemblies that attenuate *Yersinia* virulence. Cell Microbiol. doi: 10.1111/cmi.12100. (Epub ahead of print)

1 Introduction

1.1 Bacterial infections and disaease

Survival of a species requires that a reasonable identity be maintained. Over time, mechanisms to maintain “self” have evolved. To keep persisting, most of microorganisms including bacteria can develop a relationship with a host (87). This relationship is either beneficial for both partners or only for the invading microorganism. In this later type, the microorganism often utilizes an infection strategy; by which it can compromise the eukaryotic host immune system to cause disease. However, it should be clarified that disease is not the purpose of an infection; it is simply the complication that comes with host colonization. Nevertheless, infectious diseases account for a major part of the global health problem, with most of the burden falling in developing countries (116). According to World Health Organization (WHO) reports, diarrheal diseases for example kill 1.8 million people every year, the majority of these are children. By far, most of this burden is caused by contaminated food and water, and poor hygiene. Furthermore, diseases that use to be restricted geographically, such as cholera, are now striking in regions once thought safe. While some diseases have been almost completely subdued, others such as tuberculosis and plague that have always been among our greatest enemies are fighting back with renewed ferocity. Modern research tools and an ability to understand the molecular infection mechanisms employed by these disease-causing bacteria will hopefully overcome the rapidly emerging treatment obstacles, such as antibiotic resistance, and lead to totally new approaches for combating these threats.

1.2 Pathogenic Gram-negative bacteria

Late in the 19th century Robert Koch stated his postulates that tried to define the relation between the disease and its causative microbe. Although these postulates did not meet all the criteria of a huge population of microorganisms, they did shed light on the concept of pathogenesis. Hence, the term pathogen was given to any microbe that causes damage or disease to the host (50). Higher eukaryotes such as humans, animals, and plants are always subjected to bacterial infections, which often lead to severe and even lethal diseases. Major infectious agents are Gram-negative bacteria, which employ at least six different protein secretion systems to deliver protein toxins (virulence factors) to the host cell cytosol. There they can subvert metabolic and signaling pathways that often lead to host cell death (see section 1.4) (47).

1.2.1 Virulence factors

By the end of 20th century, Koch’s postulates were expanded to the molecular level, reflecting the dawn of a molecular age in microbial pathogenesis research. The microbiologist Stanely Falkow proposed a set of fundamental experimental criteria that must be satisfied with respect to the study of microbial genes and their products in order to cause disease (99).

If so, this gene then is given the term virulence factor. Pathogenic bacteria differ from their harmless relatives in having genes coding for virulence factors that facilitate host invasion and infection (265). Genetic screens, in particular transposon mutagenesis, led to the identification of many virulence factors in a wide range of bacterial pathogens (288). These factors either are encoded by the host chromosome or are carried on mobile genetic elements. The latter include transposons, viral prophages, and plasmids (see section 1.7) (265). This in turn led to better understanding of the molecular paradigms in bacterial pathogenesis such as; regulatory two component systems, pilus-mediated adhesion, secretion systems, and pathognecity islands. Moreover, continuous trials are being made in order to investigate the role(s) of individual virulence factors in the manipulation of host DNA metabolism, cell cycle control, cytoskeletal dynamics, membrane structure and function, and programmed cell death (288).

1.3 Enteropathogenic bacteria

Enterobacteriaceae is a large Gram-negative bacterial family. Members of this family are rod-shaped, non-sporulating facultative anaerobes. Moreover, the majority use flagella for swimming-dependent motility. Some of these enteric bacteria, e.g. *Escherichia coli,* are part of the normal gut flora and incidentally cause disease while others, e.g. *Salmonella*, *Shigella*, and *Yersinia,* are regularly pathogenic to humans and animals. Therefore, the later species are usually called enteropathogenic. These enteropathogenic bacteria invade the intestinal tract causing severe and in some cases lethal diseases to their hosts. In particular, they use several protein secretion machineries to deliver multiple toxin substrates to the host cell cytosol causing infection. The following sections in this study will discuss in details the pathogenicity of *Yersinia*. In particular, the molecular mechanisms that control type three secretion system (T3SS), a major virulence strategy employed by *Yersinia* to cause infection in their eukaryotic hosts.

1.3.1 The genus *Yersiniae*

The *Yersinia* species are Gram-negative rod-shaped bacteria that belong to the family Enterobacteriacae. There are at least eleven known species of *Yersinia,* three of them are human pathogenic while the rest are not. The eight non-pathogenic strains have not been studied extensively and were not shown to cause diseases to human (278). On the other hand, the human pathogenic species are; *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* that invade the intestinal tract causing gastro intestinal disorders, and *Yersinia pestis* that is considered the main causative agent of plague (Black Death). These pathogenic species carry a common 70-kb virulence plasmid that encodes for the T3SS machinery, an injection device used by these bacteria to target their eukaryotic host (see section 1.7). All these species usually target lymphoid tissue where they resist phagocytosis by disruption of the host innate immune response and primarily proliferate extracellularly (173).

1.3.2 *Yersinia pestis*; an ancient plague agent

Plague is one of the oldest known epidemic diseases of humans. It emerged in three major pandemics. The first was the 'Justinian plague' that appeared during the Roman Empire era in the 6th century and killed about half of the population in this region. The disease followed the trade routes coming from Africa and central Asia. The second appeared in Europe in the 14th century where approximately one third of the worlds' population was lost. It was given the name 'Black Death' because of the blackening of the skin around the swellings. The third pandemic started in China during the middle of the 19th century and spread to the rest of the world (186, 238). At that time the Swiss-French bacteriologist, Alexandre Yersin, could isolate the plague bacillus and use an antiserum he raised against it to cure a plague patient. In 1970, the plague bacillus was later named as *Yersinia pestis* in his honour (233). Interestingly, each of these pandemics was caused by a different biovar of *Yersinia pestis*; Antiqua (still in Africa and central Asia), Medievalis (limited to central Asia), and Orientalis (still distributed worldwide) respectively (277). These biovars differ from each other in their *in vitro* capabilities of fermenting glycerol and reducing nitrates. In contrast, the three biovars show no differences in virulence or pathology in animals or humans (233). Plague is still detected in many parts of the world, particularly in Africa and Asia. Over the last 20 years there were 1000 to 5000 plague cases worldwide, with 100 to 200 deathes every year (as reported to the WHO) (277). Some of these plague-causing isolates have even been found with resistence to commonly used antibiotics (121, 297). Hence, plague is considered a real re-emerging threat, and there is an urgent need for better understanding of its etiology, diagnosis, and treatment.

1.3.3 *Yersinia pestis* pathogenesis

As discussed, *Y. pestis* is the causative agent of plague; a disease associated severe symptoms of fever, chills, headache, and weakness with a high mortality rate. *Yersinia pestis* primarily infects wild rodents and uses them as natural environmental reservoirs. *Xenopsylla cheopis,* an arthropod vector, transmits *Yersinia* between animals and from infected animals to human (244). Upon being ingested within the contaminated blood, *Y. pestis* replicate forming biofilm aggregates that consequently create a blockade of the flea gut. The starving flea then regurgitates the bacteria in contaminated blood while trying to bite another host (17). For successful pathogen transmission, *Y. pestis* uses plasminogen activators to degrade complement and adhere to the extracellular matrix laminin promoting fibrinolysis. The bacteria then disseminate, by surviving inside the macrophages, to the adjacent lymphatic vessels causing an inflammatory response with swollen lymph nodes 'buboes' – a disease state called 'bubonic plague'. Moreover, *Y. pestis* can continue their way reaching to the bloodstream giving rise to severe sepsis, the second form of the disease called 'septicaemic plague'. In rare cases *Y. pestis* reach the lungs causing inflammation associated with chest pains and cough with bloody sputum; a third form of the disease called 'pneumonic plague' (179, 238). In this later type, there is more risk of plague transmission by breathing in of contaminated aerosol droplets produced by coughing.

1.3.4 Pathogenic *Yersinia* surviving in the environment

Unlike *Y. pestis*, both *Y. pseudotuberculosis* and *Y. enterocolitica* are not vector-borne pathogens. They can survive free in the surrounding environment like soil, and water. Moreover, they can be transmitted to their eukaryotic hosts via ingestion of contaminated food and liquids and follow a similar enteric infection route (270). In the small intestine, like many enteric bacterial pathogens, these *Yersinia* attach to the M-cells (epithelial cells that overlay the lymphoid tissue in the intestinal follicles) (208) . This facilitates the pathogens' passage to the Peyer's patches (lager aggregates of lymphoid nodules). There, *Y. pseudotuberculosis and Y. enterocolitica* replicate extracellularly resisting phagocytosis, and this facilitates their spread to the mesenteric lymph nodes (MLN) (16). This causes gastroenteritis, and mesenteric lymphadenitis accompanied with symptoms of abdominal pain, diarrhea, vomiting, and fever. While systemic infections with these bacteria are rare, in rodents both *Y. pseudotuberculosis and Y. enterocolitica* can reach the liver and spleen causing systemic lethal infections that mimic human plague (Figure 1) (49, 188, 286).

1.3.5 *Yersinia pseudotuberculosis*; evolutionary ancestor of *Yersina pestis*

Recent findings state that both *Y. pestis* and *Y. pseudotuberculosis* share an extremely high degree of similarity in their genetic make up. Both strains showed almost identical 16S rRNA sequences and high degree of sequence similarity in DNA-DNA hybridization analysis (133, 285), suggesting that *Y. pestis* evolved from its ancestor *Y. pseudotuberculosis* 1,500-20,000 years ago, i.e. before the first pandemic era (1). This is a fascinating finding because the two species obviously have different ecology and epidemiology. How was *Y. pestis* converted from a soil enteropathogen to a vector-borne obligate pathogen that causes the lethal plague? Looking at the differences in the genetic content of the two pathogens, it was clear that *Y. pestis* genome had undergone some genetic rearrangements. Moreover, during evolution, in addition to the pYV virulence plasmid, *Y. pestis* had acquired other unique plasmids by horizontal gene transfer (309). The pPla plasmid (9.5-kb), encodes for the plasminogen activator, a surface protease required for efficient pathogen transmission from the flea to the human/rodent host (see section 1.3.3). The second plasmid is pMT1(100-kb), which encodes a phospholipase toxin Ymt, required for bacterial replication and colonization in the flea midgut (146), and the F1 capsule protein that inhibits bacterial phagocytosis by macrophages (88).

These differences in genetic content alone can not be the sole reasons for *Y. pestis* evolution. It is also important to consider the differential regulation of several pre-existing common genes between the two species (51, 255). Overall, these changes in genetic content and in regulation enable the pathogen to adapt to different environmental stresses, nutrient variability, and host availability during evolution.

1.4 Secretion systems in Gram-negative bacteria

Protein export through the cell envelope is a basic process in all bacteria. Several

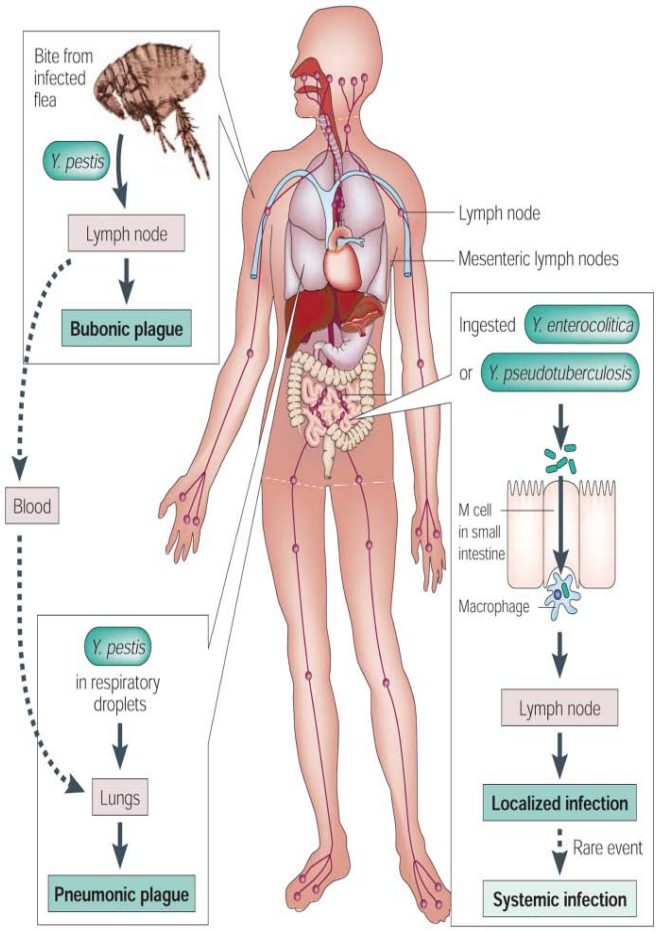

Figure 1: Illustration for different routes of infection by pathogenic *Yersinia* species. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Microbiology] (309), copyright (2003)
http://www.nature.com/nrmicro/index.html

protein secretion systems have evolved in most bacterial species. Many protein substrates have to be secreted in order to perform various functions such as; cell wall biogenesis, acquisition of nutrients, motility, intracellular communication and virulence. Moreover, adhesins are another group of secreted proteins that are found in both pathogenic and environmental bacteria, they facilitate attachement of the bacteria to their hosts. For a protein to be secreted it has to pass through the bacterial inner membrane, periplasm, and the outer membrane. In the coming sections, various protein secretion systems will be summarized, followed by a detailed discussion on type three secretion system (T3SS), one of the best understood virulence strategies in Gram-negative bacterial pathogens.

1.4.1 Two-step secretion through the bacterial envelope

On their way to the extracellular milieu, secreted proteins have to cross through the bacterial inner membrane passing by the periplasm. At least three secretion pathways are available to pass the inner membrane barrier. The Sec system is found in bacteria, eukarya and archaea. It transports unfolded proteins from the cytoplasm to the periplasm. Due to its role in transporting membrane biogenesis proteins, the Sec system is essential for bacterial survival (73). Proteins exported by this pathway are usually translated as pre-proteins with 20-30 N-terminal amino acids secretion signal. The newly synthesiszed secretion signal is recognized by SecA and the general chaperone SecB. SecA catalyzes ATP hydrolysis, and together with the proton motive force drives the substrate to be secreted to the periplasm through the integral inner membrane Sec-YEG pore complex. The SecDFYajC complex enhances membrane cycling of SecA. Upon secretion, periplasmic signal peptidases cleave off the signal peptide of the secreted pre-protein (97, 310) (Figure 2). A second possible pathway is the SRP (signal recognition particle) system. SRP is composed of a single protein 'Ffh' and a RNA molecule 'Ffs'. These components recognize transmembrane α-helices (a characteristic of membrane proteins) in newly synthesized polypeptides and target them to the Sec pathway in the inner membrane (IM) proteins in a co-translational manner (266), (Figure 2). The third gate to the periplasm is the Tat (Twin-argnine translocation) system. It is a simple system with three components; TatB and TatC that function to target proteins through the inner membrane by TatA. It secretes folded proteins that posses an N-terminal twin-arginine motif (contains two arginine residues) (266) (Figure 2).

Unlike the Sec system, the later two machineries recognize specific non-cleavable signal peptides.Type two (T2SS), and type five (T5SS) secretion systems, in addition to the chaperone usher (CU) pathway are possible secretion gates through the outer membrane if the secreted substrates in the periplasm are meant to be further targeted to the extracellular space (Two step process) (Figure 2) (125). The T2SS apparatus is evolutionary related to type four pili system. It consists of at least 12 proteins that form the machinery required for the export of folded perplasmic proteins to the extracellular space (258). Briefly, these proteins include the secretin that spans the outer membrane forming a pore, through which a pili-like organelle is formed, a cytoplasmic ATPase, an integral inner membrane complex, and a periplasmic peptidase that cleaves off and then N-methylates the prepilins (229). This system transports proteins with diverse functions such as proteases, celluloses, phospholipases, and toxins (258). The T5SS is composed of the autotransporter (AT) or two partner secretion (TPS) pathway. The AT pathway secretes proteins produced as pre-pro-proteins having an N-terminal Sec signal (cleaved off upon reaching the periplasm), a passenger domain (functional domain of the protein) which is secreted extracellulary by the help of a C-terminal translocation domain (makes a β-barrel hollow structure in the bacterial outer membrane) (125). The TPS pathway secretes proteins that differ from their AT counterparts by having the passenger and C-terminal domains translated as two separate proteins (125). Finally the Chaperone usher (CU) secretion system, mainly assembles pili, and fimbriae, but also can assemble amorphous capsule-like structures. Upon secretion of unfolded polypeptides to the periplasm via the Sec system they bind to a periplasmic

chaperone component of the CU system allowing them to refold properly. The chaperone then guides the folded cargo to the outer membrane where the usher protein acts as a platform for assembly and secretion to the cell surface (174).

1.4.2 Direct secretion to the outside; a one step process

Gram-negative bacteria can deliver their protein substrates from the cytoplasm to the extra-cellular milieu either in a two step process as described above or direct through a one step process in the form of other secretion channels that span the entire bacterial envelope. Several secretion systems e.g. type one (T1SS), type three (T3SS), type four (T4SS), and type six (T6SS) secretion systems do posses an injection device that spans both inner and outer bacterial membranes guiding their substrates to be secreted extracellulary without any periplasmic intermediate (Figure 2). T1SS secretes diverse substrates such as proteases, and lipases. The apparatus is made up of three proteins, an outer membrane protein (OMP), an inner membrane ATP binding cassette (ABC), and a periplasmic adaptor protein (MFP). Substrates for this system are characterized by Glycine rich repeats that bind Ca^{2+} ions. Moreover, they are recognized via non cleavable C-terminal secretion signals (80, 198). T3SS are a highly complex nanomolecular machines utilized by many Gram-negatvie bacteria that interact with eukaryotic cells in virulence or mutualism. These systems are evolved originally from the bacterial flagella (47). From section 1.6 this fascinating protein export machinery in *Y. pseudotuberculosis* will be discussed in more detail. T4SS is one of the most versatile secretion systems. In general, it consists of 12 proteins that build up the injection apparatus. There are three T4SSs depending on their functions. For instance, *Escherichia coli* and *Agrobacterium tumefaciens* deliver DNA substrates into fungal, plant or human cells. *Helicobacter pylori* and *Neisseria gonorrhoeae* use T4SSs to mediate DNA uptake from and release into the extracellular milieu, promoting genetic exchange. *Helicobacter pylori*, *Brucella suis* and *Legionella pneumophila*, inject virulence proteins into mammalian host cells via T4SS. Despite having different functions in different bacteria, all these subtypes are evolutionary related (117). Last but not least, T6SS is the most recently discovered secretion system. In 2006, the Mekalanos laboratory, described it as a novel secretion system while they were investigating virulence genes in *Vibrio cholerae* and *Pseudomonas aeruginosa* (215, 243). Substrates of this system do not posses an N-terminal secretion signal. Many bacteria contain more than one T6SS loci, which are independently regulated suggesting that they might have different functions. It is believed that it is required for virulence of several bacteria (22), quorum sensing (295), biofilm formation (294) or even in interbacterial community competition (151). The exact functions and mechanism of substrate export by T6SS is not yet clearly understood (25, 248). Figure.1 illustrates these various secretion systems through both inner and outer membranes in Gram-negative bacteria.

1.5 Secretion systems in *Yersinia*

Pathogenic *Yersinia* species cause several disease symptoms ranging from gastero-intestinal disorders (e.g. *Y. pseudotuberculosis*, and *Y. enterocolitica*) to the

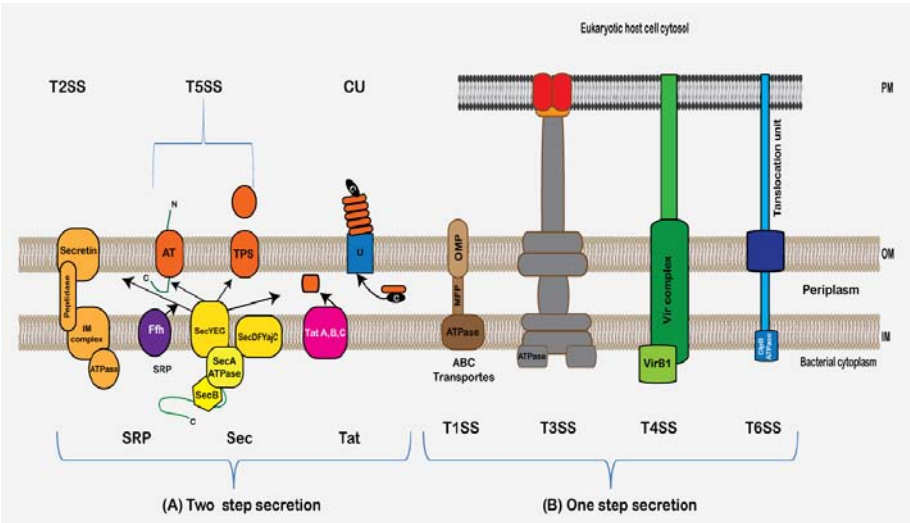

Figure 2: Illustration of different secretion systems employed by Gram-negative bacteria. (A) Two step secretion through inner then outer membrane (substrates are first secreted to the periplasm via Sec, or Tat systems, followed by export to the extracellular space via T2SS, T5SS, or the CU pathway) (B) One step secretion directly from bacterial cytoplasm to the extracellular milieu (substrates are directly secreted to the extracellular space via a conduit that spans both bacterial membranes via T1SS, T3SS, T4SS, or T6SS)

lethal epidemic plague (e.g. *Y. pestis*) (see sections 1.3.3 and 1.3.4). The *Yersinia* genome encodes for multiple secretion systems that, in addition to virulence, are employed to do several functions. For example, bioinformatic analysis of the *Y. pestis* genome showed that it possesses Sec, Tat, and SRP secretion systems required for membrane biogenesis and two step secretion processes for secretion to the extracellular milieu. Additionally, T1SS, T2SS, T3SS, and T6SS systems were mapped in its genome while a T4SS was lacking (315). Interestingly, the Tat secretion system was shown to be essential for virulence in *Y. pseudotuberculosis* (183). T2SS was found in all *Yersinia* species, but is mostly studied in *Y. enterocolitica*. The T2SS (Yts2) was shown to influence *Y. enterocolitica* dessimination and colonization in liver and spleen (308). The various autotransporter systems (T5SS) exist in *Yersinia* and encode various essential virulence related substrates. Ten autotransporter ORFs were identified in *Y. pestis* (KIM) genome (315). For instance, Invasin, a major adhesin that binds to β1-integrins on the host cell surface (156), and is an essential virulence determinant in both *Y. pestis* and *Y. pseudotuberculosis,* is a T5SS AT (156, 157, 222). Another autotransporter, YadA, is utilized by *Y. enterocolitica* and *Y. pseudotuberculosis* to promote adhesion to host cell surface, and cause autoagglutination suggesting a role in biofilm formation (306). In *Y. pestis*, two chaperone/usher secretion systems (CU) have been well characterized, the caf1 system and the pH 6 antigen system. They contribute in antiphagocytic capsules and adhesins assembly respectively (88, 153). While absent in all other sequenced *Yersiniae*, T4SS (subtype B) was detected in *Y. pseudotuberculosis* (strain IP31758) having been gained by lateral gene transfer. It is suggested that type T4SSB system mediates the intracellular survival of this *Yersinia* strain in epithelial cells (94). Whereas most bacterial genomes harbour only one or two T6SS gene clusters, the closely

related *Y. pseudotuberculosis* and *Y. pestis* contain four and five such clusters respectively (319). Their physiological roles in either organism have not been elucidated. T3SS, the major focus of this study (see section 1.6), was discovered in the 1980s. This system is found in all pathogenic *Yersinia* species, and is used to deliver their toxins cargo directly from the bacterial cytoplasm to the eukaryotic host cell cytosol.

1.6 Type three secretion systems (T3SS) in pathogenic bacteria

As briefly described in the previous sections, type three secretion systems (T3SS) are nano-complex protein secretion machineries employed by most Gram-negative pathogenic bacteria including the pathogenic *Yersinia* species to deliver their toxins (virulence factors) to the eukaryotic host cell cytosol leading to various disease symptoms which are lethal in some cases. Several bacterial pathogens such as *Shigella*, and *Salmonella*, utilize T3SSs to induce uptake to multiply intracellulary within host cells evading its immune response (120, 205), while human pathogenic *Yersinia* species use T3SSs to resist phagocytosis to promote extracellular replication (268). Plants are also targets for bacteria using this virulence strategy. For example, the plant pathogenic *Pseudomonas syringae* deliver a large set of Hrp toxins via T3S machinery to plant cells alerting the hypersensitive response (HR, a plant defense response) in resistant plants, and diseases in susceptible plants (143). Interestingly, some non-pathogenic bacteria are still able to use T3SSs in their symbiotic relationships with their hosts (plants and insects) (225).

In general, The T3S apparatus, termed the injectisome, is a complex needle-like structure that spans both bacterial inner and outer membranes, allowing direct delivary of T3S toxins to the extracellular space, or to the host cell cytosol upon host cell contact. However, production of T3S protein components, assembly of the apparatus, and controlled secretion of different T3S substrates, are complicated events that occur upon induction of the system, and are required to be performed in an efficient manner to result in a fully functional T3SS. Induction of T3SS occurs as a response to host cell contact (see section 1.8.4), and can be mimicked *in vitro* by using special culture media and growth temperatures. Extensive research studies are being done to better understand the events accompanied with induction of T3SS. In this study, we are trying to understand the molecular mechanisms underlying substrate export via this fascinating T3SS in the enteric pathogen *Y. pseudotuberculosis*.

1.6.1 Type three secretion system families

Despite their different lifestyles, T3SSs exist in various proteobacterial genera. Phylogenetic analyses have been used to uncover the evolutionary/functional relationships among these systems. Interestingly, T3SSs existed not only in proteobacteria but also in *Chlamydiae* including the environmental species that infect amoeba raising the possibility that they might harbour the ancestral T3SS (152, 225). Sequence comparisons revealed that T3SSs from these different species can be classified into seven families (61, 110, 143) (Table.1). This classification is based on the most well known T3SSs in genera from each of

the seven families. For example, the Ysc family is based on the extensively studied Ysc-Yop T3SS found in all pathogenic *Yersinia*, whereas the Inv/Mxi/Spa family is based on the T3SSs studied in intracellularly replicating *Salmonella* and *Shigella* species. Exploring the evolution of T3SSs and the functional and evolutionary relationships between the different bacteria harbouring one or more of these systems, may lead to better understanding of how these bacteria could adapt to different environmental conditions and/or diverse hosts using T3SSs.

1.6.2 Origin of T3SS

The existence of several T3SS families in many different bacteria brought up the question; what is the origin of T3SSs? The fact that these T3SSs share high similarties in many of their core components and basic regulatory mechanisms supported the assumption of them having a common ancestor. Interestingly, these systems also showed very high similarities to the non-translocating flagellar systems (31). This has given rise to the flagella-T3SS and non-flagella T3SS terminology. Knowing that flagella appeared in a wider variety of microorganisms, they were even used for motility before the divergence of archea and bacteria (219), reinforced the possibility that non-flagella T3SSs have evolved from the flagellar system (219, 257). On the contrary, noticing that the non-motile *Chlamydiae* species posses a fully functiontal T3SS suggested that these may even be the origin of the flagella T3SS (128). Some evidence even supported a third possibility of a simpler independant common ancestor system for both flagella and T3SSs (257). This was claimed since analysis showed that the 16S RNA and T3SSs phylogenetic trees are completely different (128).

Genes encoding for T3SSs are mobilized into pathogenic Gram-negative bacterial genomes via lateral gene transfer, either as chromosomal pathogenicity islands (PAIs) or on extrachromosomal large plasmids (virulence plasmids) (304). The PAIs are large gene clusters (up to 200 kb) located on the chromosome; they carry genes for virulence proteins and the regulation phase integrase genes, and an origin of replication for plasmids which facilitates their mobilization among bacterial species (134, 135). This is supported by the unique G/C content and associated codon usage in these PAIs when compared to the bacterial core chromosome (134). While most Gram-negative bacterial pathogens do posses flagella, some have multiple non-flagella T3SSs (Table.1). This is interesting because it might mean that those bacteria have acquired additional T3SSs to adapt to multiple environmental conditions e.g. to gain the ability to infect different hosts. Possessing multiple T3SSs requires more complicated regulatory control, meaning that each system will be optimally activated only in its specific niche (317). An extensively studied example is the enteropathogenic *Salmonella enterica* which possess two PAIs each expressing independent non-flagella T3SSs; SPI-1 for invasion into eukaryotic cells, and SPI-2 for intracellular survival in *Salmonella* containing vacuoles (132, 139). Moreover, pathogenic *Yersinia* species also contain multiple T3SSs. *Y. enterocolitica* utilizes a chromosomally encoded Ysa system for colonization in the gastrointestinal tract (109, 298). The other two pathogenic species, *Y. pestis* and *Y. pseudotuberculosis* encode a Ssa/Esc system which still has an unclear function (227, 242). As already noted, all these three pathogenic *Yersinia* contain a pYV virulence plasmid (see section 1.7) that encodes for a Ysc-Yop T3SS (63, 64), a main

infection machinery used by pathogenic *Yersinia* species to infect their eukaryotic hosts. Starting from the next section the Ysc-Yop T3SS will be discussed extensively.

Table 1: Representation of the Seven T3SSs families in pathogenic bacteria.

Family	Species	System	Function
Ysc	Pathogenic *Yersinia* spp.	Ysc	Block phagocytosis
	Pseudomonas aeruginosa	Psc	
	Aeromonas salmonicida	Asc	Induce cytokine expression
	Photorhabdus luminescens	Lsc	
	Vibrio parahaemolyticus	Vsc	Induce apoptosis
	Bordetella pertussis	Bsc	
	Desulfovibrio vulgaris	Dsc	
Inv/Mxi/Spa	*Salmonella* spp.	SPI-1	Trigger bacterial uptake in non-phagocytic cells
	Shigella flexneri	Inv-Mxi-Spa	
	Yersinia enterocolitica	Ysa	
	Sodalis glossinidius	Inv-Spa	
	E. coli (EIEC)	Eiv-Spa	
	Yersinia ruckeri	Inv-Mxi-Spa	
	Burkholderia pseudomallei	Bsa	
	Chromobacterium violaceum	Inv-Spa	
Ssa/Esc	*Yersinia pestis*	?	Invasion and intracellular survival
	Yersinia pseudotuberculosis	?	
	E. coli (EPEC)	Esc	
	E. coli (EHEC)	Esc	
	Salmonella enterica	SPI-2	
	Citrobacter rodentium	Ssa	
	Edwardsiella tardo	?	
	Chromobacterium violaceum	?	
Hrp1	*Pseudomonas syringe*	Hrp-1	Induce hypersensitive response (HR) in resistant plants and disease in non- resistant plants
	Erwinia spp.	Hrp-1	
	Vibrio parahaemolyticus	Hrp-1	
Hrp2	*Xanthomonas campestris*	Hrp-2	
	Burkholderia pseudomallei	?	
	Ralstonia solanacearum	Hrp-2	
Rhizobium	*Rhizobium* spp.	?	Plant symbiosis
	Mesorhizobium loti	?	
Chlamydiales	*Chlamydia trachomatis*	?	Intracellular survival and pathogenicity
	Chlamydia pneumoniae	?	

1.7 The Ysc-Yop T3SS in pathogenic *Yersinia* – the players!

Although it is believed that the Ysc-Yop T3SS originally evolved from the flagella T3SS, it is used by *Yersinia* for a totally different function other than motility. *Yersinia* species build up a syringe-like apparatus (injectisome) to inject a small but effective set of protein toxins and enzymes from the bacterial cytoplasm into the host cell interior through a pore created in the host cell membrane; a process called translocation. These translocated toxin substrates then subvert the host immune response and metabolic pathways for the benefit of *Yersinia* to grow and replicate extracellulary. Essentially, the Ysc-Yop T3SS is tightly regulated in

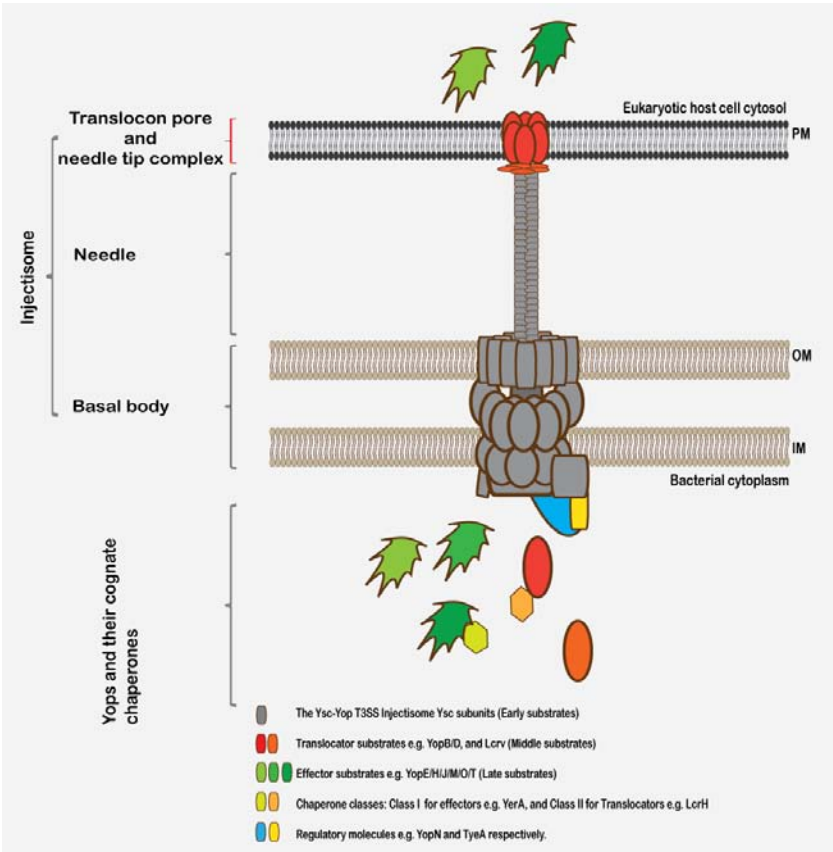

Figure 3: Schematic representation of the *Yersinia* Ysc-Yop T3SS. *Yersinia* utilizes a pre- assembled injectisome to establish contact with eukaryotic host cell. This triggers secretion of translocator proteins that form a translocon pore in the host cell membrane, a conduit for subsequent effector delivery to the host cell cytosol. Consequently, host physiology is disarmed establishing survival and spread for *Yersinia*.

response to host cell contact, divalent cations concentrations, and temperature, although other environmental cues are likely to contribute to regulatory fine-tuning. In general, components of this system are encoded on a ≈70 kb plasmid (the virulence plasmid). The name of the virulence plasmid depends really on the strain being studied (pCD1 in *Y. pestis*, pYV227 in *Y. enterocolitica* and pIB1 in *Y. pseudotuberculosis*) (64). The genetic makeup of these plasmids is essentially resolved; about 50 genes required for *Yersinia* virulence occupy three quarters of the plasmid. Genes encoding for the T3SS machinery are arranged in big blocks of poly-cistronic operons, flanked on both sides by mono-cistronic operons that encode for effector toxins and regulatory proteins (64). In general, the *Y. pseudotuberculosis* pIB1 virulence plasmid carries genes that encode for; (1) Ysc (*Yersinia* secretion) proteins, the building blocks of the injectisome, (2) Yops (*Yersinia* outer proteins) which include i) translocator proteins, ii) effector substrates, and iii) regulatory proteins that control secretion of this group of effectors and translocators upon host cell contact. Note also that translocator proteins control the synthesis and subsequent translocation of Yop effectors and also form

pores in the host cell membrane for this purpose. Effectors are translocated to the host cell interior to downregulate the host immune response for the benefit of *Yersinia* to survive. (3) Lcr (low calcium response) proteins that tightly control gene expression of T3SS components.

Additionally, the plasmid harbours other genes required for the its replication, stable maintenance and other non-functional genes (67). Loss of the virulence plasmid or disruption of its genetic content affects *Yersinia* survival and virulence (64, 124).

1.7.1 The injectisome; a toxin delivery apparatus

The injectisome is the secretion apparatus. More than 25 Gram-negative bacterial species are equipped with T3SS injectisomes. Unlike other T3SS components, the components and the structure of the injectisomes among these different bacteria are relatively conserved and share a high degree of similarity to the bacterial flagellum (61). Upon induction of the *Yersinia* Ysc-Yop T3SS several T3SS protein substrates (Yops) traverse this injectisome to get outside of the bacteria. Basically, about 25 proteins (Ysc components) build up this injectisome apparatus in *Yersinia* (85). Genes encoding for these Ysc proteins are arranged in adjacent poly-cistronic operons termed *virA*, *virB*, *virC*, and *virG* (7, 19, 105) (Table 2). Expression of Ysc genes from these operons is induced upon elevating temperature to 37 °C. Transcription of *virC* is controlled via the transcriptional activator LcrF (also termed VirF in *Y. enterocolitica*) while *virA* and *virB* are LcrF independent (180). Moreover, *virB* is needed for optimal transcription of Yop substrates (207).

Table 2: Arrangement of genes encode the Ysc proteins.

Operon	Gene(S)
virA	*yscX,Y,V*
virB	*yscN,O,P,Q,R,S,T,U*
virC	*yscA,B,C,D,E,F,G,H,I,J,K,L*
virG	*yscW*

The apparatus structure mainly consists of a basal body (two inner membrane rings, an inner rod, and an outer membrane ring) that assemble together to span the bacterial inner membrane (IM), periplasm, and outer membrane (OM). A hollow appendage (the needle) protrudes through the basal body and extends beyond the bacterial surface to serve as a conduit for secretion of Yops and a bridge to connect the bacteria with the host cell (85) (Figure 3). For complete translocation of Yops to the host cell interior a translocon pore must be formed in the host cell membrane that is helped to form by the distal needle tip assembly platform (201) (see section1.9.1). Additionally, a complex of five transmembrane Ysc proteins forms a cytosolic ring (C-ring) which is connected to the bottom of the basal body extending into the cytoplasm where it is thought to have a role in substrate recognition (86). The major constituents of the injectisome subunits are listed below (Table 3). It is important to note that not all exist simply as building blocks for the T3SS inectosome. Some other Ysc components serve regulatory, chaperoning, or as yet unknown functions and are located in the bacterial cytoplasm or are transiently bound to the export apparatus (Table 4).

Table 3: Summary of the major constituents of the Ysc-Yop T3SS apparatus.

Subunit	Components	Features of assembly
The translocon pore	YopB, and YopD	A hetero-polymer of about 500-700 kDa which is inserted into the host cell membrane forming a pore with an average inner diameter of 2.3 nm (212).
The needle	YscP, and YscF	About 150 units of YscF polymerize at the bacterial surface forming a 60 nm hollow needle structure (148). Needle length is controlled by the molecular ruler YscP (3) (see section 1.10.2).
The OM ring	YscC, and YscW	Both are secreted via Sec system to the periplasm (44, 175). YscW pilots YscC promoting its multimerization (12-15 units) in the bacterial OM (44, 45).
The Inner rod junction	YscI	Multimers of YscI build the inner rod which connects both IM and OM rings together (196). It acts as a periplasmic docking and extension platform for the needle (195).
The IM rings (Two MS rings)	YscJ, and YscD	Both are lipoproteins. YscJ is secreted via Sec system and forms the bottom IM ring (267). YscD forms the upper IM ring in the periplasmic side and interact with YscC connecting both IM and OM rings together (256).
C-ring (Export apparatus)	YscR, YscS, YscT, YscU, YscV, YscX, and YscY	The first five make a complex that penetrates the inner membrane and binds to the IM ring (8, 105, 237). A YscV, X, and Y (the YscX chaperone (77, 86)) complex help in substrate secretion specificity (86)
Cytoplasmic components	YscQ, YscN, YscK, and YscL	Might help in substrate export (181) (see section1.10.5.3) YscN is the system energizer (305) (see section 1.10.5.1)

Table 4: List of additional Ysc proteins required for other functions in T3SS.

Ysc component	Characteristics
YscA	Unknown function
YscB	Together with SycN form a heterodimeric chaperone for YopN (103).
YscE	YscF chaperone (279).
YscG	YscF chaperone (279).
YscH (YopR)	Controls YscF secretion and polymerization (28).
YscO	Chaperone substrate recycling (95).

1.7.2 The secreted substrates

Upon successful assembly of the T3SS apparatus, *Yersinia* respond to target cell contact or depletion of Ca^{2+} levels in the culture media (*in vitro*) at 37 °C by production and instant delivery of different Yop substrates to the outside of the bacterial cytoplasm, a process called secretion. Different Yops having various functions are secreted. The translocator proteins are primarily secreted to help subsequent translocation of effector substrates into

eukaryotic cells. In *Yersinia* those translocators, LcrV, YopB, and YopD, are all encoded on the *lcrGVHyopBD* operon (20). With the help of LcrV located at the needle tip, both secreted YopB and YopD are further oligomerized in the host cell membrane forming a pore; serving as a gate for passage of effector Yops to the host cell interior (translocation) (see section 1.9.1). Moreover, YopD plays a role in negative regulation of effector Yops synthesis (see section 1.8.4).

The effector substrates interfere with the host cell signaling pathways trying to create a more hospitable environment for *Yersinia* to survive and replicate. Pathogenic *Yersinia* species posses six plasmid encoded T3S Yop effectors (YopE, YopH, YpkA/YopO, YopM, YopJ, and YopT). Upon delivery of these effectors into the host cell, they target cellular components with their enzymatic activities downregulating the host innate immune response (199).

Last but not least, several proteins are secreted by the Ysc-Yop T3SS of *Yersinia* for regulatory functions. For example, host cell sensing or depleted Ca^{2+} levels in culture media induces LcrQ secretion, this in turn promotes derepression of the Yop synthesis block (see section 1.8.4). Another *Yersinia* secreted substrate, YopK, is involved in maintaining proper translocon pore size. *Yersinia* lacking this protein form larger pores and hyper translocates Yop effectors while over expression of YopK reduces Yops translocation (150). A third essential regulator, YopN, forms a 42kDa heterodimer with TyeA protein creating a cytosolic blockade for Yops secretion in non inducing conditions (see section 1.10.5.4.1) (103). After being secreted, YopN helps in establishing directional translocation of Yop effectors into the host cell interior (55, 108). Finally, each of these secreted substrates has at least one cognate chaperone that confers its presecretory stability. A detailed description of these different classes of secreted substrates is shown in table 5.

Table 5: Translocator and effector Yops function and their cognate chaperones in *Yersinia.*

Class	Substrate name	Functions	Cognate chaperone
Translocators	YopB	• With YopD forms a pore in the host cell membrane and both are required for effector translocation (113, 137). • Stimulates host pro-inflamatory responces (e.g. production of IL-8), an immune response counteracted by multiple *Yersinia* effectors (301).	LcrH (SycD)
	YopD	• With YopB forms a pore in the host cell membrane and both are required for effector translocation (113, 137). • In complex with LcrH and LcrQ; regulates of Yops synthesis (11, 112). • Has been found translocated into HeLa cell cytosol (113). Moreover, it interacts with YopE *in vitro* (142). Hence, it might chaperone YopE to the host cell interior or have an effector function itself.	LcrH (SycD)
		• Regulation of *Yersinia* Ca^{2+} dependent growth (239). • The protective antigen against plague (46). Helps in immune evasion (suppress pro-inflamatory cytokine production) (104).	

	LcrV	• Localized at the needle tip, and interacts with both YopB and YopD acting as a platform for translocon pore assembly and further as a physical junction between the needle and the formed pore (216, 259). • Initiatiates polarized Yop effector translocation upon sensing target cell contact (235).	LcrG
Effectors	YopE	• Inactivates Rho GTPases (key regulators of actin polymerization) altering the host cell cytoskeleton and consequently inhibiting phagocytosis (27, 249, 307). • Feedback regulation of translocon pore formation (137, 299).	YerA (SycE)
	YopH	• A tyrosine phosphatase targets proteins in focal complexes leading to their disruption and a consequent inhibition of phagocytosis (26, 234). • Inactivates both B and T lymphocytes inducing apoptosis in the latter (43, 314). • Blocks Ca^{2+} signalling upon infecting neutrophils, an anti-inflamatory response (14).	SycH
	YopJ	• Downregulates the host pro-inflamatory responses by blocking the transcriptional activator 'NF-κB' involved in these processes (260). • Induce apoptosis of infected dendritic cells and macrophages (32, 211)	None
	YopM	• A Leucine rich repeat protein (LRR) that targets Ser/Thr kinases involved in cell proliferation, and apoptosis (202, 204) • Binds and antaognize the activity of Caspase-1 inhibiting inflammasome assembly (182). • Localizes to the nucleus with unknown function (269).	None
	YpkA (YopO)	• A Ser/Thr kinase that is targeted to the interface of the plasma membrane of the host cell, and binds Rho and Rac1 GTPases inhibiting phagocytosis (89, 168)	SycO
	YopT	• A Cysteine protease that cleaves and further inactivates Rho, Rac, Cdc42 GTPases inhibiting phagocytosis (210, 300).	SycT
Regulators	LcrQ	• In complex with YopD and LcrH; regulates of Yops synthesis (11, 112).	SycH
	YopK (YopQ)	• Regulation of translocon pore size (2-3 nm)(150). • Regulate rate of Yops translocation from inside the host cell (82).	None
	YopN	• In a complex with TyeA, prevent Yops secretion prior to induction of the Ysc-Yop T3SS (103). • Secreted YopN promotes polarized Yops translocation to the host cell interior (55, 108)	SycN and YscB

1.7.3 The unique T3SS chaperones

Like most of Gram-negative bacterial pathogens, *Yersinia* possesses a variety of T3S chaperones. These are small acidic cytosolic molecules that bind one or more cognate Yop substrates conferring their stability in the bacterial cytoplasm prior to secretion (66). This may happen by preventing unfavourable aggregations or interactions with other proteins, and by

protection from proteolysis (276, 293). Some T3S chaperones also are unique in having other regulatory functions in controlling T3SS (see section 1.8.4). Additionally, a role in piloting their specific substrates to the T3S machinery may help to establish ordered secretion via the recognition of specific substrate-chaperone tertiary structures (see section 1.10.4.3). T3S chaperones differ from the universal molecular chaperones since they function independent of ATP hydrolysis and are not involved in protein folding (293).

According to the chaperone structure and the type of cognate Yop substrate(s), T3S chaperones are grouped into three classes (228). Class I chaperones, which are further classified into 'Class Ia' and 'Class Ib'. Class Ia T3S chaperones bind to one effector substrate, have a common α/β fold and almost always exist as homodimers with the exception of the YopN heterodimer chaperone SycN/YscB (187, 263). They bind to internal regions near to the N-terminus of their substrates, where the effector chaperone binding domain is partially wrapped around the chaperone surface (23, 187). Class Ib T3S chaperones differ from Class Ia in their ability to bind to more than one effector substrate (68). Several members from both classes have crystal structures resolved. Interestingly, both classes have related structures even though they have unrelated amino acid sequences (289).

Class II chaperones are specific to the two pore-forming translocators (see section 1.7.2). They form homodimers and consist of three tetratricopeptide repeat (TPR) structures (92). Each TPR consists of two anti-parallel α-helices which mediate protein-protein interactions (72, 226). The exact nature of interaction between chaperones from this class and their substrates is still poorly understood. Nevertheless, these homodimers are shaped to have concave and convex surfaces for substrate binding in a conformation that prevents premature interaction between the two substrates (226). As member of this class, the LcrH chaperone in *Y. pseudotuberculosis* (SycD in *Y. enterocolitica*) binds to both YopB and YopD translocators (92).

Class III chaperones represent a heterogeneous group of chaperones (96). For example, the YscE/YscG heterodimer functions to prevent premature polymerization of monomeric YscF units in the bacterial cytosol (279). YscY, the chaperone for YscX, has no definite crystal structure, yet it is grouped in this class despite the prediction it might possess TPR motifs, a characterstic of Class II chaperones (40, 77). In conclusion, diverse groups of chaperones act in concert with their corresponding T3S substrates to guarantee efficient T3S.

1.8 Induction and inhibition of the Ysc-Yop T3SS

The Ysc-Yop T3SS is turned on in response to specific environmental signals that alarm to the presence of the eukaryotic host. These signals include a temperature change from less than 30 °C (optimal for *Yersinia* growth) to 37 °C (interior temperature of the host) and the depletion in divalent cations concenteration (lower concentrations inside the host). However, it is not yet clearly understood how *Yersinia* sense and transmit these signals into the cytoplasm to ensure the correct temporal and spatial operation of the Ysc-Yop T3SS.

1.8.1 The low Calcium response phenomenon

A common signal used by several pathogenic bacteria to detect and combat their hosts, is sensing the divalent cation concentration. For example *Salmonella* SPI-2 (needed for intracellular survival) T3SS gene expression is induced upon sensing low intracellular Mg^{2+} concentration. Consequently, the PhoP/Q two component system downregulates SPI-1 (needed only for invasion) T3SS gene expression in response to these depleted levels of Mg^{2+} (131). Already in the 1950s it was shown that *Yersinia* species required Ca^{2+} for normal *in vitro* growth at 37 °C, while growth arrest occurs upon Ca^{2+} depletion (a Ca^{2+} dependant 'CD' growth phenotype) (178). Initially, it was thought that this might be due to a metabolic requirement for Ca^{2+} (145, 178), but later on it was shown that regardless of this growth arrest a massive expression and secretion of T3SS Yop proteins is induced, a phenomenon known as 'the low calcium response (LCR)' (106). The growth arrest then might be a consequence of the cost of production and secretion of Yops. Although the exact mechanism behind the LCR is not fully uncovered, it still represents a useful means for functional analysis of T3SS regulation and its regulatory components (126).

In general, mutagenesis of the *Yersinia* virulence plasmid T3S genes result in three distinct growth phenotypes related to the LCR. First, mutations that do not affect the LCR of *Yersinia* i.e. strains that still maintain the CD growth phenotype with a requirement for Ca^{2+} ions at 37 °C (34, 35). Other mutants keep growing at 37 °C irrespective of Ca^{2+} concentration, a phenotype refered as Ca^{2+} independant 'CI' growth (316). For example, strains that are defective in T3S apparatus assembly are unable to secrete the Yop synthesis negative regulatory proteins (see section1.8.4), which in turn hinder Yops synthesis and makes *Yersinia* tend to have this continuous growth phenotype. Furthermore, *Yersinia* lacking their T3SS positive regulators (e.g. LcrF) cannot produce Yops, and these also show a CI growth phenptype (64). The third group of mutants are those unable to grow at 37 °C regardless of the presence or absence of Ca^{2+}, a temperature sensitive 'TS' growth phenotype (240). Instead, they continuously produce Yops even in non inducing conditions despite this inability to grow. Usually, this is a phenotype of *Yersinia* lacking their T3SS negative regulators (e.g. LcrQ, YopD, or LcrH) or having them non-functional. It is still controversial if this *in vitro* LCR situation applies similarly to growth *in vivo.* After all, it seems counterintuitive for *Yersinia* to produce Yop virulence factor only when they stop growing. Although Ca^{2+} levels inside the eukaryotic host cell are much lower than outside, *in vitro* production and secretion of Yops is probably massive if compared to the *in vivo* scenario. Hence, *Yersinia* probably responds to multiple signals upon host cell contact in order to orchesterate a fine-tuned Yops production and secretion to ensure only enough Yops are made that is needed to overcome the host immune system.

1.8.2 Sensing host temperature: LcrF transcriptional regulator

The optimal growth temperature for the *Yersinia* species is usually below 30 °C. Upon eukaryotic host cell contact *Yersinia*, like most other pathogenic bacteria, senses a temperature change from its normal growth temperature to 37 °C, the host body temperature.

This is believed to be a key environmental signal for activation of the Ysc-Yop T3SS in all pathogenic *Yersinia* (321). Activation of the system induces the rapid production of a collection of T3SS proteins required for infection. Most studies on the mechanisms of the T3SS thermoregulation have been performed in *Y. pestis* and *Y. enterocolitica*.

At low temperatures (below 30 °C), T3S genes cannot be transcribed due to the binding of YmoA (nucleoid associated protein) to their promoter regions (253, 254) (Figure 4). YmoA is a chromosomally encoded histone-like protein that binds to the DNA stabilizing its curved architecture (65). This curved structure makes T3S promoters less available for the transcription machinery. Moreover, the gene encoding for the master T3SS positive transcriptional regulator LcrF (VirF in *Y. enterocolitica*), is not yet transcribed (Figure 4). In *Y. pestis*, even those *lcrF* mRNA transcripts that are generated at low temperatures cannot be translated because of the presence of two stem-loops at the *lcrF* mRNA Shine-Dalgarno region (a post-transcriptional inhibition) (Figure 4) (147), a feature which has been also investigated in *Shigella* species (98, 241). Elevating temperature to 37 °C (host cell contact) relaxes DNA curvatures which in turn release YmoA from the T3S gene promoters. YmoA is then degraded by the ClpP and Lon proteases (161). The stem-loops at the *lcrF* mRNA ribosomal binding sites are melted allowing its translation (Figure 4). LcrF, a member of the AraC transcriptional activator family (59), has a helix-turn-helix C-terminus DNA binding domain which facilitates its binding to the exposed promoters of the T3SS genes (several *lcr, ysc*, and *yop* operons on the virulence plasmid) allowing their transcription and subsequent translation (Figure 4) (180). It was previously shown that YmoA from *Y. enterocolitica* interacts with the general transcriptional regulator, H-NS (220). Interestingly, in *Shigella* species, H-NS was shown to down regulate T3S gene expression via binding to the promoter region of the transcriptional activator *virF* (98). Thus, YmoA might act as a cofactor by promoting H-NS binding to the promoter regions of T3S genes in *Yersinia,* and this might explain the difficulty of detecting Ysc-Yop promoters bound by YmoA.

1.8.3 Cell contact sensing via the injectisome

In order to start using their T3SS weaponry, the Gram-negaive pathogenic bacteria have to ensure close proximity and proper localization on the target host. This is supposed to occur via a signal that is sensed by these pathogens upon direct host cell contact. Exactly how *Yersinia* sense contact with host cell surface, what the nature of the signal actually is, and how they transmit these signals to initiate Yops production and secretion is still unknown What is established is an extremely controlled secretion mechanism in response to these signals that enables a functional T3SS in *Yersinia* (see section 1.10).

In *Shigella flexneri,* functional and structural analysis revealed that the interaction between IpaB (a YopB homologue) and IpaD (a LcrV homologue) promotes proper IpaB insertion into the host cell membrane (296), an event that transmits a signal through the needle (MxiH; equivalent to YscF) to the bacterial cytoplasm (197). This signal transmission might occur through a conformational change in the needle structure, and the signal probably

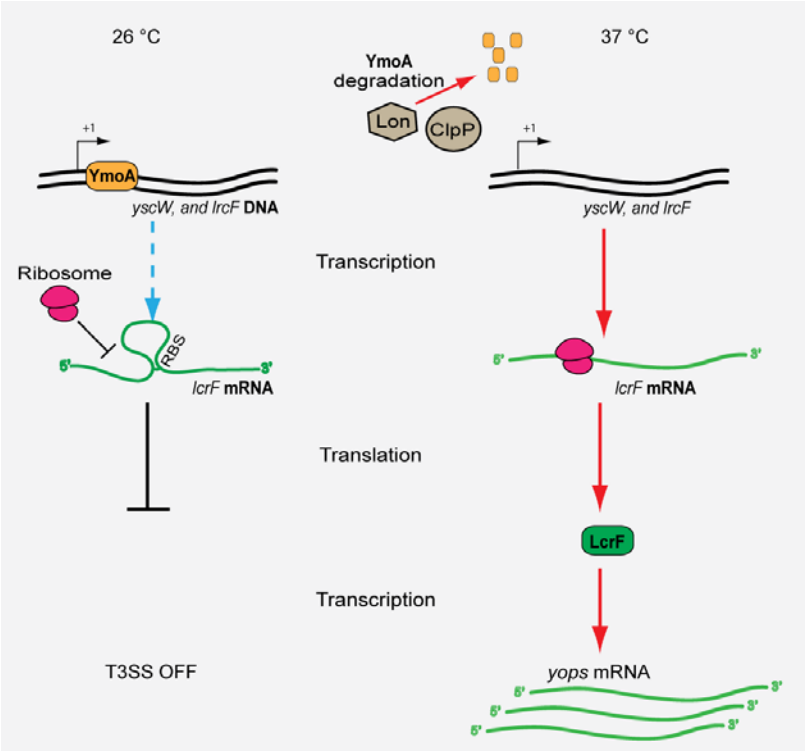

Figure 4: Thermoregulation of LcrF synthesis, the Ysc-Yop T3SS transcriptional activator. At 26 °C, YmoA binds DNA promoter region upstream of *yscW-lcrF* operon preventing its transcription. Additionally, any *lcrF* mRNA that is made forms a stem-loop structure at its ribosome binding site (RBS), thus preventing its translation. Upon temperature shift to 37 °C, YmoA is released from the DNA and further degraded by the ClpP and Lon proteases inducing transcription of the *yscW-lcrF operon.* Stem loops at the RBS region of *lcrF* mRNA are relieved allowing *lcrF* translation. LcrF in turn induces transcription of *Yops* genes.

reaches a regulatory protein MxiC (a YopN-TyeA homologue) in the *Shigella* cytoplasm (119, 197). Consequently, MxiC is released, allowing IpaC (a YopD homologue) secretion to complete the translocon pore formed by IpaB/IpaC (197). IpaC release, then allows translation and secretion of *Shigella* effectors.

Inspite of the high similarity between *Shigella* and *Yersinia* T3SSs, a similar signal transduction mechanism is not yet proven for the later pathogen. However, some experimental indications support a similar mechanism in *Yersinia*. For instance, alanine scanning mutagenesis of the needle component (YscF) in *Y. pestis* gave rise to a strain that constitutively secretes Yops regardless of the presence or absence of Ca^{2+} , although no longer able to translocate Yops to the host cell interior (283). Moreover, a translocation deficient mutant, altered in its YscF sequence, secreted Yops similar to parent *Y. pseudotuberculosis* (74). Fascinatingly, these mutants were perfectly able to assemble intact YscF needles and form translocon pores in the host cell plasma membrane. These findings reinforce the probability of a communication between the YscF needle and the rest of T3S

machinery to ensure a perfectly localized *Yersinia* onto the host being ready for Yops injection. Similar to *Shigella*, the communication in *Yersinia* could be through needle transmission of the signal to the bacterial cytosol promoting the dissociation of the YopN-TyeA complex, the T3SS cytosolic plug , to allow full release of the partially secreted YopN. This opens the way for secretion and further translocation of other Yop substrates (103, 138).

1.8.4 Post-transcriptional control; feedback inhibition

Prior to target cell contact (mimicked *in vitro* by growth in the presence of Ca^{2+} in laboratory culture media), expression of Yop substrates is shut off in *Yersinia*. This is believed to occur at a post-transcriptional level (11). YopD in complex with its cognate chaperone LcrH, both bind to AU rich regions in the 5'-UTR (untranslated region) of *yop* mRNA transcripts hindering protein translation, most probably because the Shine-Dalgarno sequences on these mRNA are not properly exposed to the ribosomes (11, 112). Consequently, acumulated untranslated mRNAs are further degraded by bacterial ribonucleases (312). LcrQ (YscM1 and YscM2 in *Y. enterocolitica*), an additional negative regulator, is thought to have a role in establishing this negative regulatory loop (250, 275). As long as LcrQ is in the *Yersinia* cytoplasm, Yops translation is repressed (236). Presumably, it binds to the YopD-LcrH complex to from a hetero-trimer that blocks Yops translation (112). In support of this, null *yopD* and/or null *lcrH Yersinia* are not able to downregulate Yop synthesis in these non-inducing conditions even if LcrQ is made to accumulate in the cytoplasm of these strains (112, 303). As soon as *Yersinia* sense the contact with its host cell (Ca^{2+} depletion *in vitro*), SycH (the YopH chaperone) binds and facilitates secretion of LcrQ (311). This in turn leads to its dissociation from the hetero-trimer complex and YopD-LcrH release from the *yop* 5'-UTR allowing ribosomes to bind to the available Shine-Dalgarno sequences initiating Yops translation. LcrH further pilots YopD to be secreted via an active T3SS (48) (Figure 5). It is thought that this negative regulatory loop prevents toxic accumulation of Yops inside *Yersinia,* in other words Yops are made only when they are needed for injection into the host cell. It also indicates that YopD, a component of the translocon pore, is secreted before Yop effectors are produced and secreted i.e. much like the IpaC example described for *Shigella* (see section 1.8.3).

1.9 Yops translocation; understanding the concept

The host immune response to bacterial infections is very rapid. *Yersinia,* like other pathogenic bacteria, need to develop very quick and efficient strategies to counteract these defence mechanisms. The Ysc-Yop T3SS is an effective strategy used by *Yersinia* to fight against the host immune system. Its success is based on the accurate delivery of Yop effectors into the cytosol of the eukaryotic host cell which is a process defined as translocation. Once translocated, Yop effectors perform collaborative functions to overcome the host immune defence (Table 5).

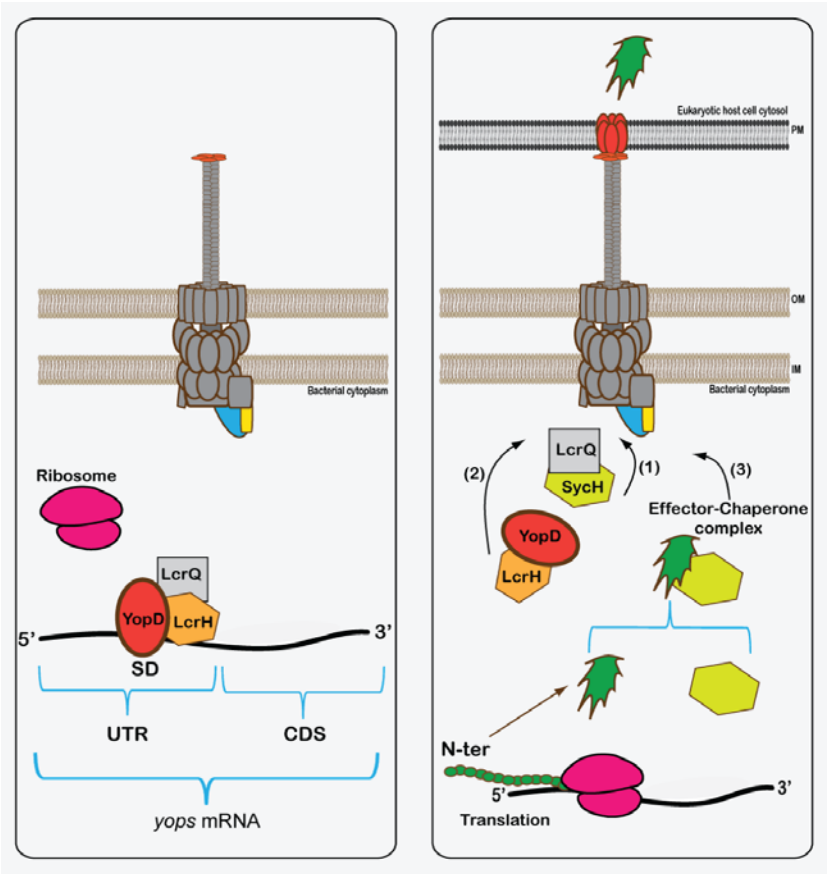

Figure 5: Ysc-Yop T3SS post-transcriptional regulation in *Yersinia*. (A) Prior to host cell contact (+Ca^{2+} *in vitro*): YopD-LcrH-LcrQ hetero-trimer complex binds to 5'-UTR of *yop* mRNA preventing ribosome binding to SD sequence to initiate protein translation. (B) Upon host cell contact (-Ca^{2+} *in vitro*): The YopD-LcrH-LcrQ complex is dissociated. SycH guides LcrQ for secretion, then LcrH pilots YopD for secretion, *yop* mRNA translation starts, and then Yop effector secretion is initiated.

As discussed in previous sections, multiple stages are required in order to reach the goal of effector translocation. This includes assembling as injectisome, sensing the host cell and signal transduction, opening the secretion channel, and synthesizing and secreting Yops Only then can a pore be formed in the host cell membrane for Yop delivery into the cell. In the coming few sections, the dynamics of Yops translocation by *Yersinia* will be discussed, including further descriptions of essential requirements for this process.

1.9.1 The translocon pore; a gateway to the target cell interior

Yersinia and other T3S utilizing bacteria essentially need to puncture the eukaryotic host cell membrane creating a pore through which they can translocate their effector toxins into the target cell interior. This happens using certain proteins (translocators) which interact to

oligomerize in the host cell membrane creating this gateway for effector passage. Both YopB and YopD are integral membrane proteins; they possess hybrophobic domains that facilitate their insertion into the host cell membrane forming the translocon pore (70, 136). Translocon assembly can be studied *in vitro* using sheep erythrocytes as a eukaryotic cell infection model. Upon infection, YopB and YopD translocon complex levels in the membrane can be detected indirectly by measuring the rate of cell hemolysis (137). Recently, a heteropolymer of about 500 to 700kDa representing the YopB-YopD translocon complex was purifired from erythrocyte membranes upon infection with *Y. enterocolitica* (213). The stoichiometry of YopB-YopD translocators in this complex was estimated to be about 2.4 YopD molecules per each YopB molecule (281). Consistent with this, both translocators were found to form pores in liposomes, and in other biological and artificial membranes (149, 218, 280). Using osmoprotection and dye-exclusion experiments, the inner diameter of the translocon pore was estimated to be 2-3 nm; this is consistent with the size of the inner diameter of the T3SS needle appendage (58, 137, 149). *Yersinia* lacking *yopB* and/or *yopD* is translocation deficient although effector Yops secretion remains intact (113, 137). While analysis by scanning transmission microscopy showed that the hydrophilic protein LcrV is located at the YscF needle tip, it is not believed to be a component of the translocon pore (216). Instead, LcrV was shown to be a platform for translocon assembly directly helping YopB and YopD insert into the target cell plasma membrane (194). In addition, YopK (YopQ in *Y. enterocolitica*), is a regulatory protein in *Yersinia* that has no known homologues in other bacteria, is thought to have a role in controlling the translocon pore size. *Yersinia* lacking this protein form pores with bigger diameter, and hypertranslocates Yop effectors (150).

Homologues for the three T3SS *Yersinia* translocators are present in other bacterial pathogens and these are also able to form translocon pores with approximatly similar sizes. For example, PopB and PopD translocators from *Pseudomonas aeruginosa* are very similar to the *Yersinia* YopB and YopD respectively, and these oligomerize forming a ring-like structure that disrupts artificial liposomes (42, 261). Moreover, PcrV from *P. aeruginosa* was shown localized at the YscF needle tip, and to functionally complement the absence of LcrV in *Yersinia* (42, 216). This suggest that the effector delivery mechanism through a translocon pore is universal in T3SS (200). However, unique components such as the *Yersinia* YopK protein may be required to customise each T3SS to suit each individual bacterial lifestyle.

1.9.2 Polarized translocation

As soon as *Yersinia* and other T3SS utilizing bacteria sense contact with the target cell membrane, they start secreting their toxins through the injectisome for purposeful delivery into the host cell cytosol. Upon getting out of the bacterial cytosol, the effector toxins have to be guided properly to the translocon pore for efficient translocation, otherwise they will be scattered into the extracellular milieu. For example, the YopE cytotoxin from pathogenic *Yersinia* (Table 5) was shown to be expressed, secreted, and further translocated into the HeLa cell cytosol in direct response to pathogen host contact. Moreover, almost no YopE was detected in the surrounding culture media. This indicated that YopE translocation was polarized or directional, occuring solely at the attachment site of T3S injectisome to the host

cell membrane (107). Moreover, detaching the needle appendage from the host cell surface by altering the length of either the surface adhesin YadA or the YscF needle reduced the efficiency of injecting YopP (induce apoptosis) into J774A.1 macrophages, a measure of compromised translocation in *Yersinia* (214).

Multiple key T3S regulatory components are required to maintain this polarized translocation phenomenon. Together with its chaperone LcrG, the multifunctional needle tip protein LcrV is thought to contribute in establishing this directional translocation of Yops by modulating the targeting repression caused by cytosolic LcrG (79). Moreover, secreted LcrV is localized at the needle tip and further interacts with YopB and YopD translocon components suggesting its role as a physical junction between the needle and the fully assembled translocon complex to confer directional translocation of Yops. Studies also indicate a role of the extracellularly secreted YopN, a key regulatory molecule for T3SS in *Yersinia*, in controlling directional Yops secretion and translocation. It has even been proposed that through interplay between the surface localized YopN, and TyeA and the translocon component YopD that directional translocation of YopE and YopH effectors inside the host occurs (108).

1.9.3 One step or two – an alternative translocation model

A central dogma in T3SS research is that translocation occurs in one step via direct injection of substrates from the bacterial cytoplasm into the host cell interior without any intermediates. Several lines of circumstantial evidence support this theory, although direct translocation through the needle-translocon pore has never been visualized microscopically. Studies on tracking the secretion and translocation of YopE-Cya (Adenylate cyclase) hybrids indicated that the 15 first residues of YopE were enough for its secretion while at least 50 residues are required for its translocation into the host interior (273, 274). Curiously, equal levels of Cya hybrids were detected outside and inside the host cell suggesting the presence of an intermediate step for secreted Yops before being targeted inside the host (33).

Recently, several studies could clearly detect the docking of T3S effectors at the bacterial surface prior to being deliverd inside the host. For instance, the *Shigella* substrates, IpaB, IpaC and IpaD were detected on the bacterial surface prior to host cell contact (292). Interestingly, latex beads coated with IpaB and IpaC are internalized into epithelial cells, inducing consequences that mimic aspects of *Shigella* T3S infection (206). Consistent with this, using electron microscopy, Hans Wolf-Watz and collegues proved that YopE, YopD, and YopH are localized on the surface of *Y. pseudotuberculosis* prior to target cell contact (5). Moreover, *Yersinia* coated with recombinant purified YopH could deliver the toxin in a T3SS dependent manner into neutrophils inhibiting the cytosolic calcium signalling inside the host (5). Knowing that no clear experimental evidence could visulalize the direct injection of T3S effectors through the needle to inside the host; this two step translocation model limits the role of the needle to host cell sensing and substrate secretion. In the two step model, Yops translocation from the bacterial surface into the host cytosol is proposed to occur in a similar mechanism to AB toxins delivery. It has been previously proven that translocator-effector

complexes are formed in *Y. pestis* (158). Hence, the translocator might act as the host cell membrane binding domain 'B moiety' while the effector mimics the enzymatic domain 'A moiety' of AB toxins (5, 90). Collectively, these fascinating findings directed thoughts towards two possible translocation mechanisms for T3S substrates.

1.10 Putting all the pieces together: Orchesterating the Ysc-Yop T3SS

Significant research efforts are being invested to understand the molecular mechanisms that maintain an optimally functioning T3SS in Gram-negative bacteria. Essentially, coordination between different stages of T3SS is a prerequisite to establish a system that can combat the immune response of the eukaryotic host. For a successful Ysc-Yop T3SS in *Yersinia* a sequence of events are presumed to occur. First a full and correct assembly of the apparatus, followed by induction of T3S triggered upon host cell contact (181). This promotes synthesis of Yop substrates, but only after a substrate secretion specificity switch from the secretion of Ysc needle components to the secretion of Yop translocator components (181). Translocator Yops are supposed to be secreted first to form a pore in the host cell membrane; this facilitates secretion of Yop effector toxins out of the bacterial cytoplasm, followed by their translocation to the host cell cytosol through the pre-formed translocon pore inserted in the host cell plasma membrane. Once internalized, the effectors start their job in manipulating the host cell physiology (181). In the above sections, these fundamental stages required for setting up a functional Ysc-Yop T3SS in pathogenic *Yersinia* were discussed. Furthermore, focus was on the regulatory mechanisms that maintain harmony between these different T3SS stages. Different classes of T3S proteins are produced for different purposes in each stage of T3SS, some of them have to be secreted to do their functions (Tables 3, 4, and 5). Recent findings are starting to indicate functional coordination not only between the different steps of T3SS but also among the different protein classes within the same stage. The remainder of this chapter will highlight these studies that are leading to a better understanding of the multiple mechanisms that orchestrate injectisome assembly, production and further export of different protein classes via T3SS.

1.10.1 Coordinated assembly of the injectisome

The structure of the T3S injectisome shares high similarity among many Gram-negative bacterial species. In *Yersinia*, it is believed that this syringe-like structure, originally evolved from the flagellum, consists of about 25 protein building blocks. The genetic make up and protein composition of the *Yersinia* injectisome have already been described in section 1.7.1. The Ysc-Yop T3S injectisome assembly starts when temperature reaches 37 °C and this process is a critical step to proceed to the next T3SS stages (84). Several studies revealed that the injectisome units tend to assemble in an ordered manner. In principle, the multi-ring basal body structure that spans the bacterial envelope and the export apparatus located at the cytoplasmic face of the inner membrane are assembled first. Several of the basal body components like YscC, YscW, and YscJ in *Yersinia* are secreted to the periplasm via the Sec system to build up the basal body ring structures. This unit is then secretion competent for

promoting the secretion of the components required for assembly of the periplasmic YscI inner rod (which connects assembled components in the inner and outer membrane and is the scaffold on which the YscF needle monomers are polymerized), and the extracellular YscF needle (sometimes given the name early substrates) (78, 287).

Recently, epistasis experiments using fluorescent fusion proteins of various T3S Ysc components revealed an outside-in assembly order of the basal body rings, i.e. the outer ring composed of YscC is assembled first, followed by the two inner rings made by YscD then YscJ, and finally the ATPase and the C-ring are put in place (84). The ATPase activity of the YscN ATPase ring is not required for the assembly process. Interestingly, YscV, a component from the (YscR, S, T, U, V) export apparatus in *Yersinia*, required only YscR, S, T for its oligomerization but not any other Ysc component of the injectisome. Hence, aspects of the export apparatus can assemble independently of the integral membrane rings structures. Similar examples were also seen in *Salmonella*, where the formation of the needle complex base requires a successfully pre-assembled export apparatus, but this export apparatus can assemble independently (287). Furthermore, the *Salmonella* export apparatus components are themselves assembled in a hierarcheal way, i.e. SpaP/YscR, SpaQ/YscS, and SpaR/YscT are assembled first, followed by InvA/YscV, and SpaS/YscU (287).

A next step after building a complete basal body structure is to assemble an appendage that extends on the bacterial surface called 'the needle'. In *Yersinia*, this requires the T3S proteins YscX and YscO for secretion of the early substrates YscI, YscP, and YscF (86). YscI the component that polymerizes to build a periplasmic inner rod is required for secretion of YscP, which means that the inner rod should be formed before the YscF needle polymerization starts (86). Helical polymerization of 100 to 150 monomers of the 9 kDa YscF protein build up a 60 nm needle like hollow structure (148). The length of the needle is controlled by YscP that functions like a 'molecular ruler' (167). Upon completion of YscF needle polymerization, a substrate specificity switch occurs by the help of YscP and YscU (see section 1.10.2). YscX and YscO are secreted followed by secretion of Yop substrates. The focus in the following sections will turn from assembly control to secretion control of Yop substrates.

1.10.2 From Ysc to Yop secretion; a specificity switch.

The successful completion of T3S apparatus assembly is a sign for the bacterial pathogen to move from a building step to a secretion step i.e. to shift from secretion of building blocks to secretion of substrates used for invasion of the host. Hence, a switch control is required. In *Salmonella* flagellar T3SS, FliK/YscP was shown to control the length of the hook during assembly. Together with FlhB/YscU, both interact to activate a secretion specificity switch from rod and hook substrates to filament substrates (209). This is activated upon cleavage of the C-terminus of the cytoplasmic domain of FlhB (see section 1.10.5.2), which might then recognize and promote secretion of filament substrates. A similar control occurs in *Yersinia* by the two components YscU and the YscP 'molecular ruler'. Indeed, YscP controls the extent of YscF polymerization in order to end up with the optimal needle length. This occurs when YscP enters the secretion channel directly after basal body assembly,

followed by YscF molecules that continue polymerization until the needle reaches its optimal required length. At this moment, YscP C-terminus switches substrate secretion to from Ysc to Yop proteins via cross-talk with YscU (93), then YscP itself is fully secreted (167). However, this mechanism has been challenged by work with *Shigella;* structural analysis of the needle showed the diffuculty for both MxiH/YscF and Spa32/YscP to coexist in the same secretion channel (119). Instead, it was proposed that the *Shigella* proteins travel alternately with continuous check for needle length by Spa32, and upon reaching the optimal length, Spa32 interacts with the C-terminus of Spa40/YscU eliciting the secretion switch signal (37). Whatever the mechanism, the substrate specificity secretion switch ultimately establishes a first level of T3S secretion hierarchy, involving a switch from needle components termed 'early substrates' to secrete translocator and effector substrates. This concept of T3S hierarcheal substrate secretion is assumed to extend beyond this level to even propose a temporal secretion control among translocators (middle) and effectors (late), a topic that will be discussed soon in details.

1.10.3 Differential T3S gene expression; for what reasons?

Bacteria tend to sense their eukaryotic target cells via their fully assembled T3S apparatus (see section 1.8.3). Most probably the signal is triggered by feeling the lowered levels of divalent cations inside the host than those outside. As soon as the signal is transmitted to the bacterial cytosol, the T3S translational repression is relieved and various substrates are produced and immediately guided by their chaperones towards secretion via the injectosome (see section 1.8.4). Different T3S substrate classes having different functions at different stages of infection are secreted (Table 5). While the most obvious way to control substrate secretion is to use substrate recognition mechanisms by the T3SS machinery (see section 10), recent evidence proposes that this temporal control can occur at the level of gene expression. In *Salmonella*, differential expression levels of various protein classes from SPI-1 T3SS were detected *in vivo* at different times post-infection and from different organs (127). For example PrgJ/YscI, the inner rod component and SipD/LcrV the needle tip complex were expressed continuously in all tested tissues showing the requirement for full needle assembly, and well guided effector translocation during the whole course of infection (127). On the other hand, SipC/YopD the translocon component and InvJ/YscP the needle molecular ruler were differentially expressed in ileum and liver respectively, reflecting the different need for each at specific infection stage. Moreover, the effectors SopA and SopB were detected in all tested tissues (127). In enteropathogenic *Escherichia coli* (EPEC), genes encoding for chaperones, several effectors, structural and regulatory proteins are clustered in various operons on the LEE Pathogenicity Island, while genes for the rest of effectors are scattered on the chromosome (*nle*). Transcription control of LEE genes is positively regulated by the master transcriptional activator Ler, and negatively by the GrlA and GrlR regulators. Although knowledge about the roles of *nle* gene products in virulence is rapidly expanding, their transcriptional control mechanisms are not well characterized. Recently, sequence and mutagenesis analysis revealed that they have their own transcriptional control mechanisms distinct from the LEE genes. Most of the *nle* genes posses a conserved *nle* regulatory

inverted repeat sequence (NRIR) which seems to be essential for regulation of gene expression for many of these effectors. This reflects the highly fine-tuned gene expression in EPEC which coordinates the functions of T3S proteins for this pathogen. In another illustrative example, three T3S transcription regulators VirF, VirB, and MxiE together with its co-activator IpgC are encoded on the virulence plasmid of *Shigella flexneri*. VirF controls transcription of VirB which in turn controls transcription of genes for T3S structural components, translocators, and chaperones, whereas MxiE/IpgC only controls transcription of effectors.

The process of substrate production in the *Yersinia* Ysc-Yop T3SS is thought to be tightly regulated. LcrF is the master positive transcriptional regulator of Yops in response to elevated temperature (see section 1.8.2). At a translational level, YopD together with its chaperone LcrH, and probably with the aid of LcrQ negatively regulate *yop* gene expression (see section 1.8.4). Recently, Deborah Anderson stated that T3SS gene expression in *Yersinia pestis* lacking the YopD translational negative regulator is differentially induced, with the effector *yop* genes being the most upregulated (54). Evidence suggests that YopD binds with different affinities to two 5'- AU rich regions upstream and downstream of start codons of target mRNAs accounting for this temporal gene expression of different T3S substrate classes. Consistent with this, our preliminary translational reporter analysis showed that Shine-Dalgarno sequence plus a short 5'- sequence immediately downstream of YopD start codon are required for optimal YopD expression (PAPER I). Moreover these differing sequences among representatives from different T3S substrate classes are responsible for creating differential gene expression (see section 3.1.4) Together, these findings suggest that a very early event in T3S temporal control occurs at the level of T3S gene expression.

1.10.4 Temporal T3S substrate secretion; fact or fiction?

Going through the different functional stages of the tightly controlled T3SS reveals how complicated is the protein export machinery. Several protein classes contribute in establishing this well orchesterated infection process most likely with its multiple stages taking place on time. A growing positive attitude towards the probability of temporal control of T3S substrate secretion was strengthened by several similar events from earlier stages during T3SS function. The well organized T3SS injectisome assembly (see section 1.10.1), followed by a substrate specificity switch (a first level of temporal control) (see section 1.10.2) and evidence of differentially expressed T3S gene products (see sections 1.10.3 and 3.1.4), all support the proposed substrate secretion hierarchy to act in concert giving rise to a functional T3SS. Aligned with this, prioritized translocator secretion (to be termed middle substrates) was suggested to form translocon pores that act as gates for passage of effector toxins (late substrates) to the host cell cytosol on time (a second level of temporal control). Multiple studies have focused on investigating the possible mechanisms lying behind such secretion hierarchy where translocators are prioritized over effectors. For instance, the T3S machinery might differentially recognizes the different substrate classes in the bacterial cytosol which in turn facilitate their ordered secretion. Moreover, the different substrates

themselves might harbour their own recognition signals that make them unique for temporal recognition and subsequent secretion via the injectisome. In the following sections we will have a closer look at aspects of substrate recognition mechanisms that could possibly control export via the Ysc-Yop T3SS in *Y. pseudotuberculosis.*

1.10.4.1 N-terminal T3S signals

The function of bacterial secretion systems is essentially based on the export of their protein substrates to the extracellular milieu. This happens either sequentially through the bacterial envelope passing by the periplasm or directly from the cytoplasm to the outside via an injection device (see section 1.4). In *Yersinia*, The Ysc-Yop T3SS belongs to this second type of protein export machineries and involves about 14 secreted Yop substrates that act together to disarm the eukaryotic host upon infection. These substrates are most likely recognized by the T3S machinery, which in turn facilitates their subsequent secretion. Although they share no consensus sequence, the extreme N-terminal peptides of many Yop substrates were believed to act as recognition signals for the Ysc-Yop T3SS (189, 245). This was initially identified when fusions of the first 15 and 17 amino acid residues from YopE and YopH effectors respectively to the *Bordetella pertussis* adenylate cyclase (Cya) were found sufficient to export Cya via the Ysc-Yop T3SS (273). More recently, Hans Wolf-Watz and collegues showed that introducing frameshift mutations to the first 11 amino acids of the YopE N-terminus, i.e. altering the peptide sequence while keeping the 5'- mRNA coding sequence intact, this drastically reduced YopE secretion levels (190). Interestingly, altering the 11 codons of *yopE* 5'- mRNA coding sequence without changing the peptide sequence, did not affect YopE secretion (190). This gave the first indication to a proteinaceous nature of the YopE N-terminal secretion signal. Furthermore, comparative analysis of amino acid composition for many signal peptides from several T3SS utilizing bacteria showed that they all share an amphipathic physical property (composed of 43% hydrophobic and 57% hydrophilic amino acids) (191). This was confirmed when YopE chimeras harbouring amphipathic synthetic signal peptides (composed of 7 alternate Serine/Isoleucine residues) were efficiently secreted while others with synthetic hydrophobic (mainly Isoleucine) or hydrophilic peptides (mainly Serine) were not (191). Consistant with this, replacing the YopD N-terminal secretion signal with an amphipathic 15 amino acids synthetic peptide also supported YopD secretion and effector translocation (PAPER I).

On the other hand, Olaf Schneewind and colleagues showed that frameshifting the first 10 amino acid residues of YopE or YopQ/YopK do affect its export via T3SS. Surprisingly, introducing these frameshifts in the context of the first N-terminal 15 amino acid residues from YopE and YopK restored normal secretion levels of neomycin phosphotransferase (Npt) reporter fused to these altered signals (246, 247). Moreover, altering the 5'- sequence of the *yopQ* first 10 or 15 mRNA codons with keeping the protein sequence unchanged always impaired Npt secretion (247). Additionally, fusions of an altered mRNA or peptide sequence of the 15 N-terminal residues of YopE could restore the secretion deficiency monitored when a similarly altered 7 YopE N-terminus sequences were fused to the to Npt reporter protein (246). Interestingly, a single nucleotide mutation in codon 3 of the *yopE* mRNA (still

maintaining the YopE N-terminal amino acid sequence) diminished secretion of the Npt reporter and YopE secretion when introduced in the context of the full *yopE* gene (246). Collectively, these findings challenge the proteinaceous nature of the Yops N-terminal secretion signal, rather proposing that both *yopE* and *yopK* 5'- mRNA harbours essential information required for their export via T3SS.

Table 6: T3SS mediated substrate export via N-terminal secretion signal peptides in *Yersinia* and other Gram-negative bacteria. Methods of analysis used: site directed mutagenesis(SM), deletion mutagenesis (DM), and translational reporter fusions (TRF). nd = not determined

Class	Secreted Substrate	Bacteria	Region	mRNA, protein , or both	Method of Analysis	Ref.
Structural	YscX	*Y. pseudotuberculosis*	1-15	nd	TRF,& DM	PAPER II
	EspA	*Escherichia coli*	1-20	nd	TRF	(217)
Translocators	YopD	*Y. pseudotuberculosis*	1-15	protein	TRF, & DM	(10)
	LcrV	*Y. pseudotuberculosis*	1-15	nd	DM, & SM	(41)
	SipB	*Salmonella enterica (Typhimurium)*	1-8	nd	TRF	(170)
	EspB & EspD	*Escherichia coli*	1-20	nd	TRF	(217)
	IpaC	*Shigella flexneri*	1-15	nd	DM	(141)
	PopD	*Pseudomonas aeruginosa*	1-20	nd	DM	(282)
Effector	YopE	*Y. pseudotuberculosis*	1-15	both	TRF, & SM	(190, 246)
	YopH	*Y. pseudotuberculosis*	1-15	nd	TRF	(273)
	YplA	*Yersinia enterocolitica*	1-20	protein	TRF, DM, & SM	(291)
	SopD	*Salmonella enterica (Typhimurium)*	1-10	nd	DM	(36)
	SopE	*Salmonella enterica (Typhimurium)*	1-15	protein	TRF, DM, & SM	(169)
	Cif	*Escherichia coli*	1-16	nd	TRF, & DM	(52)
	EspF, Map, & Tir	*Escherichia coli*	1-20	nd	TRF	(52)
Regulators	YopQ (YopK)	*Y. pseudotuberculosis*	1-15	mRNA	TRF, & SM	(13)
	YopR	*Yersinia enterocolitica*	1-11	mRNA	TRF	(30)
	YopN	*Yersinia enterocolitica*	1-12	nd	TRF & SM	(129)
	MxiC	*Shigella flexneri*	1-30	nd	DM	(38)
	SepL	*Escherichia coli*	1-20	nd	TRF	(318)

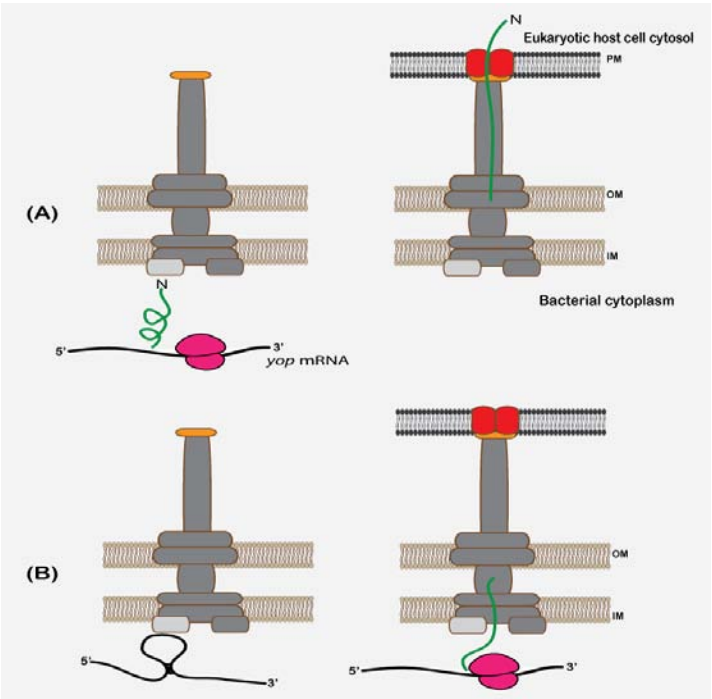

Figure 6: T3S via recognition of Yops N-terminal sequences. (A) Prior to host cell contact; synthesized Yops are recognized via their N-terminal peptides by components of Ysc-Yop T3S apparatus. This facilitates their export out of the bacterial cytoplasm as soon as host cell contact is established (B) Prior to host cell contact; *yops* mRNAs form stem-loop structures that hinder their translation. These structures are recognized by the Ysc-Yop T3S apparatus. Uppn host cell contact; co-translational secretion of the recognized Yops is initiated.

Presumably, this information facilitates a co-translational secretion of Yops upon recognition of their mRNAs by the Ysc-Yop T3SS (Figure 5) (12, 13, 284). The contradictory results concerning with Yop N-terminal secretion signals are based on protein or mRNA sequence could actually indicate that some substrates might require both signals; the bipartite nature of the signal being important for proper secretion control. To date, most analyses have investigated the nature of the N-terminal secretion signals of several *Yersinia* T3S effectors and regulators (Table 6). Hence, we initiated studies on other substrates from different classes. We revealed the necessity of the first 5 N-terminal amino acid residues for both YopD translocator and YscX structural component T3S dependent export (PAPERS I & II). Moreover, we showed that YopD export signal is more proteinaceous than being mRNA dependent (see section 3.1.2). Interestingly, *Y. pseudotuberculosis* harbouring chimeric T3S substrates, in which the N-terminal signal sequence of a substrate from a certain Yop class (translocators 'middle'/ effectors 'late') was exchanged with an equivalent sequence of another substrate from a different class, were less resistant to uptake by mouse macrophage-like cells. This could be consequence of compromised T3SS function due to less control on

temporal T3S, a feature most probably maintained by controlled substrate recognition via their N-terminal signal sequences (PAPER I).

1.10.4.2 Additional non N-terminal T3S signals

In addition to the established role of the T3S N-terminal secretion signals in controlling substrate export, recent studies highlighted the requirement for additional non N-terminal sequences to confer secretion of several T3S substrates. For example, deletion mutagenesis of the C-terminal region (residues 519-524) of the enterohemorrhagic *E. coli* (EHEC) T3S effector 'Tir' impairs its secretion (9).Moreover, the SipB translocator in *Salmonella enterica* (*S. Typhimurium*) possesses an N-terminal T3S signal peptide of 8 residues (Table 6) (170), but its N-terminal 160 amino acids region cannot be secreted via the SPI-1 T3SS unless it is fused to the SipB C-terminal amphipathic region (residues 300-593) (170). Furthermore, two predicted α-helical domains at the C-terminus of the *Salmonella* SopD effector (residues 200-220 & 268-302) were required to direct its secretion through the SPI-1 T3SS via interaction with the ATPase component InvC (a YscN homologue) (36). Additionally, the C-terminal domain (299-363 residues) of the IpaC (a YopD homologue) translocator of *Shigella* is required for its localization at the bacterial pole, a perquisite for its export via T3SS (162). Finally, *P. aeruginosa* translocators are constitutively secreted regardless of Ca^{2+} concentration, while effector secretion is triggered only when Ca^{2+} levels are depleted. This raised the possibility of the presence of T3S temporal control among these two substrate classes (57, 282). Although the N-terminal T3S signal of the PopD translocator is generally required for its efficient secretion (Table 6), two additional signal peptides were suggested to prioritize its secretion in the presence of Ca^{2+}. One signal is located directly upstream to the PopD chaperone binding domain (residues 32-45), while the extreme C-terminal peptide (residues 270-295) represents the second export signal (282). Moreover, efficient binding of the PcrH chaperone to PopD is essential for establishing its secretion in the presence of Ca^{2+}. *Pseudomonas* strains lacking any of these PopD export control elements are no longer able to secrete this translocator in the presence of Ca^{2+} and consequently can not intoxicate eukaryotic host cells. Presumably, this is a consequence of compromised translocator/effector temporal secretion control.

In *Yersinia*, several efforts to find such similar non N-terminal domains that may control Yops export via the Ysc-Yop T3SS were not successful. Although extensive mutagenesis of regions of the YopD translocator downstream of its N-terminal signal sequence detected residues and/or domains required for interaction with the LcrH chaperone and/or other T3S components, pore formation, translocation of Yop effectors, and presumably other uncovered *in vivo* functions, none of these phenotypes were a reflection of poor YopD secretion (69-71, 111, 223). On the other hand, secretion of the early substrate YopR requires a particular mRNA secondary structure located near to its 3' end (30). A translational fusion of the non-T3SS glutathione S-transferase enzyme (GST) to the C-terminus of YopR jams the secretion channel upon being recognized by the Ysc-Yop injectisome (272). This in turn blocks complete secretion of this hybrid protein as well as subsequent Yops secretion (272). YopR N-terminal peptide (residues 1-11) fused to GST was not sufficient to create this secretion

block (30). Fusions of randomly mutagenized YopR to GST revealed the need for a second signal between residues 131 and 149 for YopR to be recognized by the *Yersinia* T3S machinery (30). Furthermore, silent mutations where a mRNA stem loop structure in this region was disrupted again abolished Yops secretion block meaning that this mRNA motif must act as a second T3S signal for YopR (30). In conclusion, most probably information located at the C-termini of several T3S substrates is necessary for ensuring their orchesterated extracellular export via T3SS.

1.10.4.3 Chaperone dependant recognition

In addition to their role in conferring pre-secretory stability to their cognate substrates or in T3SS regulation, T3S chaperones are believed to have additional roles in guiding these various substrates for export via the correct cognate T3SS machinery (6, 101, 114, 228). Chaperone binding to its cognate substrate promotes structural conformational changes in the latter. Presumably, this exposes a recognition signal located nearby or at the chaperone binding domain (CBD) to the T3S machinery for recognition. Principally, three structurally different cytosolic T3S chaperone classes are thought to uniquely interact with at least four functionally different T3S secreted substrates. This reinforced the hypothesis that chaperone-dependent substrate recognition may further orchestrate substrate export in a timely manner (228).

In *Y. pseudotuberculosis*, YopE CBD (residues 23-78) wraps around its homodimeric chaperone SycE (a member of Class I chaperones) (252). Nuclear magnetic resonance analysis (NMR) showed that YopE binding to SycE induces conformational changes in its natively unfolded CBD (disorder-to-order transition) (251). Consequently, this resulted in complex tertiary structure where a YopE peptide is surface exposed (252). Alanine scanning mutagenesis of this region in YopE revealed the necessity of five amino acid residues for YopE T3S export (252). Probably this peptide acts as a signal recognized by a component of the T3S injectisome. While the *E. coli* (EPEC) Tir–CesT and *Salmonella* SptP–SicP effector-chaperone complexes are recognized by the EscN and InvC ATPase molecules respectively (see section 1.10.5.1), this is not yet proven for the YopE-SycE complex and ATPase YscN in *Yersinia* (4, 123, 251). Interestingly, parental *Y. enterocolitica* and *Yersinia* over-expressing other Yop effectors were not able to deliver YopE lacking its SycE CBD into eukaryotic host cytosol whereas *Yersinia* devoid of multiple Yops was able to do so (39). Hence, the presence of the SycE CBD enables *Yersinia* to compete for YopE delivary even if other Yop effectors are over-expressed, probably ensuring its timely controlled export (39).

The *Yersinia* translocators, YopB and YopD, share a common class II chaperone LcrH. In complex with YopD, LcrH is involved in negative regulation of Yops synthesis (see section 1.8.4) (11, 112). Whether LcrH controls the export of YopB and/or YopD translocators for export via the Ysc-Yop T3SS is not yet understood. Recently, a role of the the *P. aeruginosa* PcrH chaperone (a LcrH homologue) in orchesterating temporal T3S of its translocator PopD (a YopD homologue) was revealed (see section 1.10.4.2) (282). Most probably, PcrH binds to PopD translocator bringing its two signal peptides in close proximity to be further recognized

by the T3S apparatus. Recent crystallographic analysis showed that YopD binding to LcrH is very similar to PcrH-PopD from *Pseudomonas* and IpgC-IpaC from *Shigella* (262). Taken together, LcrH could well act as a secretion pilot guiding YopD and YopB for efficient export via the Ysc-Yop T3SS.

1.10.5 Yop substrate recognition via the T3SS machinery

It is essential for T3SS function in Gram-negative bacteria including *Yersinia* species to secrete sets of substrate classes across the bacterial envelope to the extracellular space. The various T3S machineries must recognize the different substrate classes in order to promote and organize their secretion. This happens via recognition of certain secretion signals intrinsic to the substrate and/or when in complex with their cognate chaperones. The exact molecular mechanisms of T3S dependent substrate recognition are still poorly understood. However, several studies have identified interactions between representative proteins of the T3S injectisome and several T3SS translocator and/or effector substrates. It is anticipated that this interplay would contribute to the export of these T3SS substrates which in turn mediate subsequent T3SS substrate function.

1.10.5.1 YscN; the system energizer

The common belief of Yop substrate passage through the T3S needle to the outside of the bacteria contradicts the fact that the inner diameter of the needle is tiny and makes it impassable for folded T3S substrates (62, 148, 302). Hence, there is a need for these substrates to be totally or at least partially unfolded prior to their T3S (302). This is stimulated by binding their cognate T3S chaperones. Nevertheless, this is still an energy consuming process. The T3S component YscN is a member of the T3SS family of ATPases and possesses two consensus ATP binding motifs (Boxes A and B) that confer its ATPase activity required for T3SS function (305).It assemble at the cytosolic side of the T3S injectisome forming a mulitmeric ring structures (probably hexameric) that provide the energy required for T3S substrate dissociation from their chaperones, maintaining their unfolded state as they are feed into the secretion channel and pass through to the extracellular environment (305).

Recent studies showed that YscN contributes in establishing T3S dependent pre-secretory substrate recognition (272). In *Salmonella*, the T3SS energizer InvC (a YscN homologue) binds to the substrate-chaperone complex SptP-SicP, bringing it to the base of the secretion machinery. This occurs regardless of its ATPase activity. This interaction induces dissociation of the complex, and unfolding of the SptP substrate turning it competent for secretion via the T3SS channel (4). Impassable YopR-GST hybrids in the bacterial cytosol were affinity co-purified with YscN but not with any other injectisome components (272). Interestingly, a similar GST fusion to a truncated YopR (lacking its first 15 amino acids N-terminal peptide) or a GST-YopR fusion neither blocked Yops secretion nor interacted with YscN (272). Moreover, a YopE-GST hybrid was blocked in the bacterial cytosol but did not affect secretion of other Yops (272). Collectively, these findings indicate that the T3SS ATPase YscN recognizes Yop substrates via their N-terminal secretion signals promoting

their Ysc-Yop T3S dependent export. Addtionally, the Yops secretion blockade created by the YopR-GST but not YopE-GST is evidence of temporal secretion of different Yop substrate classes, a proposed requirement for efficiently functional T3SS.

1.10.5.2 YscU cleavage and Yops export control

YscU, a homologue of the FlhB structural protein from the flagella, is an essential component of the Ysc-Yop T3SS export apparatus located at the bottom of the basal body of the *Yersinia* injectisome (271). Like FlhB, YscU is a large integral membrane protein that spans the bacterial inner membrane with four helices followed by its C-terminal domain exposed to the cytoplasm (8, 209). Together with YscP, it is required for controlling the Ysc-Yop T3SS dependent protein export via switching substrate secretion specificity from early substrates (Ysc needle proteins) to middle and late substrates (Yop substrates) (see section 1.10.2). This is believed to be promoted through a Ca^{2+} independent autoproteolysis event that occurs at the YscU C-terminal cytoplasmic domain (24, 184). The YscU cytoplasmic domain contains a conserved motif of four amino acids: Asparagine, Proline, Thereonine, and Histidine (NPTH) where the cleavage occurs specifically at Proline 264 (8, 184). This releases a large YscU extreme C-terminal peptide (called YscUcc) for secretion that then allows subsequent Yop secretion (118). Substituting the N263 or P264 in this motif to Alanine hinders the cleavage of YscU C-terminus and this in turn compromises the Ca^{2+} dependent regulation of Yops production and secretion (24). Interestingly, over expression of these YscU variants *in trans* in *Y. enterocolitica* abrogated secretion of YopB, YopD, and LcrV translocators but did not have a significant effect on secretion of other Yop effectors (271). Presumably, YscU cleavage confers a structure that can recognize translocator class substrates prioritizing their secretion over the effector Yops (a second level of secretion hierarchy). This was supported by the restored secretion of a chimeric LcrV translocator, having its N-terminal secretion signal replaced by the equivalent signal of the YopE effector (271). However, *Y. pseudotuberculosis* harbouring these YscU mutations *in cis* i.e. expressed under the control of native promoter in monocopy, showed a general defect in Yops production and secretion. This suggests that YscU generally blocks secretion of Yop substrates and its cleavage is a requirement to relieve this block (24).

1.10.5.3 Sorting T3SS substrates for timely export

A significant approach to understand the functional molecular interplay between various T3SS components is to investigate the multiple protein-protein interaction partners among these players. Jorge Galan and co-workers described how in *Salmonella enterica* a cytoplasmic large heterogeneous protein complex composed of the cytoplasmic T3S components SpaO, OrgA, and OrgB (homologues of YscQ, YscK, and YscL respectively) acts as a sorting platform that discriminates between translocators and effectors orchesterating their temporal export via the T3SS (181). The ATPase component InvC was also shown to interact with the SpaO platform, although it is not required for assembly or stability of this complex. In parental *Salmonella*, Liquid Chromatography and Mass

Spectrometry (LC-MS/MS) identified the SipB/C/D translocators (homologues of YopB/D, and LcrV) in isolation with the SpaO complex components, whereas no interacting effectors were detected (181). On the contrary, SpaO complexes analyzed from *Salmonella* devoid of these three translocators were loaded with the SipA, SptP, and SopE effectors. In either case, detection of the substrate class bound to the sorting platfom was possible only in the presence of their cognate chaperones (181). Interestingly, translocators and/or effectors were absent from SpaO complexes obtained from *Salmonella* lacking the regulatory protein InvJ (a YscP homologue) (181).

In conclusion, these data suggest that the SpaO sorting platform is sequentially loaded by substrate-chaperone complexes promoting secretion of translocators prior to effector toxins upon being dissociated from their chaperones by the help of InvC. These events are most probably preceeded by a substrate secretion specificity switch promoted by the InvJ regulatory molecule.

Similarly, interactions between YscQ orthologues from other T3SS utilizing bacteria with the secretion machinery were detected and shown to be essential for T3S assembly and substrate export control. For example, in enteropathogenic *Escherichia coli* (EPEC), a ternary complex of EscL, EscQ, and EscN (homologues of YscL, YscQ and YscN respectively) is present near to the inner membrane and is essential for secretion of translocator substrates (21). In *Yersinia*, genetic and biochemical analysis detected interactions between YscN, YscL, and YscQ proposing that they might build a ternary complex essential for T3SS assembly and function (29, 160). Furthermore, YscK was the only other injectisome component required for building up this complex (84). Furthermore, together with the cytoplasmic domain of YscD, this complex was shown to recognize and promote dissociation of the YopH effector from its SycH chaperone facilitating its export via T3SS (122).

1.10.5.4 The InvE protein family; regulation of temporal substrate secretion

Yet another mode to establish temporal control of substrate secretion involves the InvE family of proteins. The InvE protein family are representatives of regulatory T3S components required to ensure temporal export of middle and late T3S substrates, a second level of control that occurs upon a shift from early substrate secretion phase (2, 193). In contrast to early and late secreted proteins, *S. enterica* Typhimurium lacking *invE* are defective in translocator secretion (middle substrates). InvE is proposed to recognize translocator-chaperone complexes prioritizing secretion of translocators (Figure 7 A) (171, 177). Presumably, it exerts this function inside the bacterial cytosol, since a non-secreted GST-InvE hybrid restores the lost T3SS function in a null *invE* mutant (177). On the other hand, SepL from *E. coli* (EPEC and EHEC) interacts with SepD/Rorf6, an essential component for general secretion control, forming a membrane localized complex that promotes a switch from translocator secretion to effector secretion upon sensing depleted Ca^{2+} levels (81, 221). Recently, David Gally and co-workers showed that SepL interacts via its C-terminus with the Tir effector retarding its secretion and other effectors to allow prioritized translocon assembly (Figure 7 B) (290). MxiC, a third member of this family, has no influence on *Shegilla flexneri*

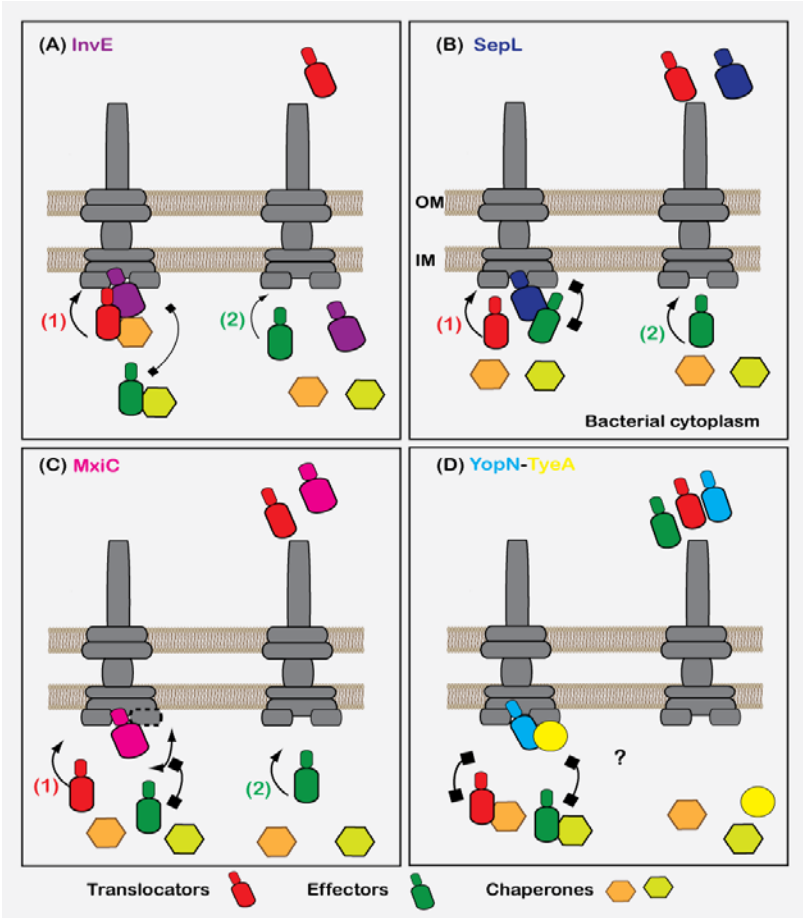

Figure 7: Role of the InvE protein family in prioritizing translocators T3S before effectors. (A) InvE (*S. enterica* Typhimurium) interacts directly with SipB, SipC in complex with SicA chaperone prioritizing their secretion before the SopA, SipA, and SptP effectors. (B) SepL (EPEC) interacts with the effector Tir delaying its secretion until after the EspA, EspB and EspD translocators. (C) MxiC interacts with the system energizer Spa47 creating a secretion block for the effectors IpaA, IpgD, IcsB, IpgB2, OspD1, and IpaH5 until after the translocators IpaB, IpaC, and IpaD (D) YopN-TyeA acts as a secretion plug causing a general block of both translocator and effector secretion prior to cell contact. (MxiC and SepL are secreted, while InvE restarts from the bacterial cytoplasm - The chaperone is attached to the substrate when the mechanism indicates recognition as a substrate-chaperone complex (e.g. InvE). On other occasions it might be recognition of substrate alone (e.g. SepL).- Lines with arrowheads indicate those substrates that are being advance for secretion, whereas lines with square symbols reflect those particular substrates whose secretion is being delayed)

injectisome assembly (197). Rather, it binds to the Spa47 ATPase (homologue of YscN) creating a cytosolic blockade that hinders secretion of several effectors until translocator secretion is achieved (Figure 7 C) (38). This blockade is relieved after MxiC secretion, events that are activated upon signal transmition to the bacterial cytosol via the T3SS needle

complex (see section 1.8.3) (197). Ultimately, regardless of utilizing different molecular mechanisms, members of this family significantly fine-tune temporal substrate secretion via T3SS of their respective bacteria.

1.10.5.4.1 The YopN/TyeA complex of *Yersinia*; an InvE family member

Rather than expressing a singular peptide, the Ysc-Yop T3SS in*Yersinia* species encodes for two proteins, YopN and TyeA, which show homology to the N-terminal and C-terminal regions of the InvE family members, respectively (224). YopN is a 32 kDa protein that is stabilized in the bacterial cytosol when the two chaperones SycN and YscB are bound to a region downstream to its N-terminal secretion signal (55, 76, 129, 159, 166). An earlier study proposed that surface localized YopN acted as an extracellular cap that prevented Yops expression and secretion at high Ca^{2+} levels (108). More recently, secreted YopN is thought to link the YscF needle to the translocon pore ensuring polarized translocation of Yop effectors to the eukaryotic host cell cytosol (55, 103, 194). Furthermore, YopN is translocated into the HeLa cell cytosol although the significance of this remains unknown (75). YopN translocation is counteracted by the 10 kDa TyeA protein prior host cell contact (55, 56, 75). Critically, TyeA binds to the YopN C-terminus (region 242-293) to form a 42 kDa heterodimer complex which is known for its role in establishing general control of Yops secretion (56, 103, 165). Several studies proposed molecular mechanisms by which this complex establishes such regulatory control (103, 108). Initially, a surface localized TyeA was proposed to act as a tether between secreted YopN and the translocon protein YopD to ensure selective translocation of Yop effectors to the host cell interior (155). Alternatively, other studies hypothesized that these proteins interact in the bacterial cytosol to perform this regulatory role (Figure 7 D) (56, 103). Presumably, cytosolic TyeA bind to both YopN and the T3S apparatus maintaining partially secreted YopN within the hollow T3S conduit. This in turn acts as a cytosolic plug that prevents Yops secretion prior to cell contact (103, 164). However, unlike other InvE family members, there exists no significant evidence for a role of the YopN-TyeA complex in promoting temporal export of one substrate class over another. However, some circumstansial evidence is consistent with this general idea. First, *Yersinia* lacking *tyeA* is no longer able to translocate YopH and YopE to the host cell interior while other effectors are still normally translocated (56, 155). Second TyeA was found interacting with the YopD translocator but not with effector substrates (56, 155). Finally, although in low levels, *Y. pestis* but not *Y. enterocolitica* produces and secretes a singular 42 kDa YopN-TyeA hybrid protein (similar to the InvE singular proteins).

1.10.5.4.2 The YopN-TyeA hybrid phenomenon

Frameshifting is common in both prokaryotes (including viruses) and eukaryotes. It either occurs as missense event producing a non-functional protein, or as translational control mechanism to regulate gene expression of functional proteins (programmed frameshifting) (18, 100). Principally, multiple elements such as certain DNA and mRNA sequences and/or architectural features, codon usage, and variations in tRNA levels are presumed to control

frameshifting (53, 140, 172, 185, 203, 264). Together, these features culminate in causing the ribosome to pause during protein translation which can alter the original reading frame giving rise to a different protein product. Interestingly, this mechanism is required for regulating gene expression of several T3S components. For example, the *Shigella flexneri* T3SS transcriptional regulator MxiE is produced by a frameshift that involves a slippage of the RNA polymerase during transcription (232). Consequently, this influences both transcription and translation of a downstream gene *mxiD* encoding a component of the T3S apparatus (231). Moreover, production of Spa13, Spa33, and MxiA T3SS components is fine-tuned by frame-shifts during transcription of their respective genes (230). Recently, genetic and biochemical analysis reasoned the production of an additional 42 kDa YopN-TyeA singular peptide in *Y. pestis* but not *Y. enterocolitica* as the result of a +1 frameshift event that occurs near to the 3' – end of *yopN* mRNA (102). In particular, it occurs at a UUU-UGG ribosomal pausing site at codons 278 and 279 of *yopN* mRNA (102, 264). Presumably, the frameshifting event in *Yersinia* is required for either producing a functional YopN-TyeA hybrid protein or to fine-tune the translation of the *tyeA* downstream gene. Both assumptions seem logical because the YopN-TyeA fusion protein shows high similarity to other singular functional proteins of the InvE family homologues (224). Curiously, ectopic expression of this hybrid restored general regulatory control on Yops secretion lost in *Yersinia* lacking both *yopN* and *tyeA* (102). Moreover, we show that *Y. pseudotuberculosis* predominantly producing the YopN-TyeA hybrid protein partially restored T3SS function *in vivo* (PAPER III). It is possible that this frameshifting event is a way to differentially express *yopN* and *tyeA*, expressed from the same poly-cistronic operon, as a mean to fine-tune expression of these components that are essential for optimal T3SS function (54). In conclusion, this process seems physiologically important for T3S function in *Yersinia*.

2 Objectives of this thesis

Explore the concept of T3S substrate export control; identify mechanisms to orchesterate differential expression and secretion of various substrate classes via the Ysc-Yop T3SS.

In particular:

- Dissect the contribution of N-terminal T3S signals in controlling the Ysc-Yop T3SS dependent substrate export in *Yersinia*.

- Investigate the role of YopN and/or TyeA in substrate trafficking through the T3SS injectisome.

3 Results and discussion

The *Yersinia* Ysc-Yop T3SS is multi-stage infection procedure. In brief, several of the Ysc proteins are secreted to build up a syringe-like device spanning both bacterial membranes and protruding extracellulary with the YscF needle (see section 1.7.1). Then a secretion switch from Ysc components to various classes of Yop proteins is necessary for further stages (see section 1.10.2). The hydrophobic translocators YopB and YopD are secreted to form a translocon pore in the host cell membrane (see section 1.9.1). This is aided by the hydrophilic LcrV translocator located at the distal tip of the YscF needle. Finally, a set of effector Yops are presumably translocated through the translocon pore reaching the host cell cytosol to perform certain roles that collectively compromise the host immune response for the benefit of *Yersinia*. In reality, to achieve this about 40 proteins have to function in concert, and many of them have to be secreted through a narrow 2 nm secretion channel. Hence, multiple regulatory elements are required for temporal and spatial regulation of injectisome assembly and substrate export. Probably each secreted substrate harbours structural information that facilitates its recognition by components of the injectisome which in turn promote its timely and ordered export.

3.1 YscX and YopD, representatives of secreted structural and translocator T3S substrates.

In this study we began to dissect this information for YscX and YopD, two essential components of the Ysc-Yop T3S machinery (69, 86, 91, 92, 154). YscX is a member of the *Yersinia* export apparatus located at the base of the injectisome and probably controls secretion of substrates required for needle assembly (YscF, YscI, and YscP), an early stage in T3SS function (86). *Yersinia* lacking full length *yscX* shows general loss of Yops production probably because of a defect in secretion that traps negative regulators (LcrQ, and YopD) in the cytoplasm causing a phenomenon known as feedback inhibition (PAPER II, Fig. 2) (86). On the other hand, YopD is a major multifunctional protein involved in negative regulation of Yops synthesis and translocon pore formation (92, 218), the latter function requires its efficient secretion outside the bacteria (91, 130). Absence of this major translocator prevents *Yersinia* from infecting their eukaryotic hosts, although they are constitutively producing Yops regardless of Ca^{2+} levels (223). Like most other *Yersinia* T3S substrates, both proteins are present in the bacterial cytoplasm in complex with their cognate non-secreted chaperones YscY, and LcrH respectively (77, 92). While the regulatory role of the YopD-LcrH complex in establishing a negative control loop for Yops synthesis was previously confirmed (11, 54), less is known about regulatory functions of YscX and YscY in governing T3SS activity. In the absence of YscY, YscX is no longer detectable in *Yersinia* (PAPER II), confirming the notion that YscY act as a cytosolic stabilizer for YscX (77). Interestingly, the YscY and LcrH chaperones together interact (40), giving another dimension of complexity to their regulatory roles in Ysc-Yop T3SS.

3.1.1 YscX and YopD exploit their N-terminal sequences as T3S signals.

Relying on published genetic information derived from many T3S secreted substrates from various bacteria; several computational prediction methods claim to identify T3S substrates on the basis of features located at their N-termini (15, 192, 313). In *Yersinia*, the extreme N-terminal regions of several Yop effectors are required for export of their respective substrates via the Ysc-Yop T3SS (60, 189). Thus far, most experimental efforts have focused on mapping such signal sequences for the effector Yops. On the other hand, less is known for translocator proteins and structural components. It is already established that secretion of the YopD translocator is an essential event preceding translocon pore assembly (91), yet it is still poorly understood if YscX secretion, as demonstrated by us and others (77) (PAPER II, Fig. 1), is essential for its T3S function in *Yersinia*. Our hypothesis is that they have secretion signals and these allow them to be distinctively recognized and secreted by the Ysc-Yop T3SS.

Hence, we sought to first determine the existence of N-terminal signal sequences that guide these substrates to be recognized and secreted by the Ysc-Yop T3SS. To ascertain this, we targeted the region encompassing the first ~20 amino acids from each substrate utilizing *in cis* deletion mutagenesis. Three truncated YscX variants showed reduced ($YscX_{\Delta 3-7}$) or non-detectable ($YscX_{\Delta 8-12}$, and $YscX_{\Delta 13-22}$) levels in cleared bacterial culture supernatants after growth in T3SS inducing conditions (at 37 °C in media depleted of Ca^{2+}) when compared to *Yersinia* harbouring native YscX protein (PAPER II, Fig. 4). Similarly, progressively smaller deletions in the equivalent region of the YopD N-terminus gave rise to variants that are abolished ($YopD_{\Delta 4-20}$) (223) and ($YopD_{\Delta 5-19}$) or severly deffective ($YopD_{\Delta 6-19}$ to $YopD_{\Delta 10-19}$) in secretion (PAPER I, Fig. 7). These YscX and YopD variants were resistant to endogenous proteases suggesting that the secretion defect is not due to poor stability (PAPER I, Fig. 1, and PAPER II, Fig. S1). To further confirm that, we replaced the first 15 residues of several confirmed T3S substrates with the equivalent region from YscX. Interestingly, YscX N-terminus supported *in vitro* secretion of LcrQ, YopD, and YopE, although general control of T3SS activity was lost for the $LcrQ_{YscX\text{-}Nterm}$ (PAPER II, Fig. 2). Similarly, $YopE_{D\text{-}Nterm}$ was secreted *in vitro* in similar levels to *Yersinia* secreting wild type YopE (PAPER I, Fig. 9). Hence, as is the case for other substrates from several T3SS utilizing bacteria, including *Yersinia* (Table 6), both YscX and YopD possess a T3SS secretor domain lying within the first ~20 N-terminal residues.

3.1.2 Composition of the YopD N-terminal T3S signal

A few years ago, a debate arose concerning the peptide versus mRNA nature of T3S signals from different T3SS secreted effector substrates (see section 1.10.4.1). To uncover the nature of the YopD translocator secretion signal, we strategically generated three YopD variants. The first two ($YopD_{Frame+1}$, and $YopD_{Frame-1}$) altered the YopD 15 N-terminal peptide sequence, while keeping the equivalent mRNA coding sequence intact, while the third ($YopD_{Scramble}$) changed the mRNA sequence but not the peptide sequence (PAPER I, Table. 2). After analyzing the *in vitro* T3S profile, only secretion of the $YopD_{Frame+1}$ was severely

affected (PAPER I, Fig. 2). This effect was specific to YopD, since secretion of other Yops (e.g YopE) in these mutants did not deviate from that of parental *Yersinia* (PAPER I, Fig. 2). While this did not absolutely rule out the mRNA signal hypothesis, it emphasized the presence of a YopD T3S signal peptide that promotes its export via the Ysc-Yop T3SS. Furthermore, the mRNA secondary structures for $YopD_{Frame+1}$ and native YopD were identical, which further supported the proteinaceous nature of the YopD N-terminal secretion signal (PAPER I, Fig. S1). Moreover, to map possible amino acid residues critical for a functional YopD T3S signal peptide, site-directed mutagenesis identified two Isoleucine residues at positions 3 and 5 that seemed important components of the YopD signal peptide (PAPER I, Fig. 3). Interestingly, the unadulterated secretion of the $YopD_{Frame-1}$ mutant was probably due to the retention of Isoleucines at positions 3 and 4 (PAPER I, Table 2), because when mutated the secretion efficiency dropped (PAPER I, Fig. 3) In conclusion, the YopD N-terminal T3S appears to be proteinaceous in nature with two important Isoleucine residues, rather than being dependent on a mRNA structural signal.

3.1.3 Minimal signal length for efficient YscX and YopD export.

Like several Yop effectors, we have shown that both YscX and YopD possess N-terminal T3S signals that guide them outside the bacteria via the Ysc-Yop T3SS. For YopD at least, this signal comprises its extreme N-terminal polypeptide sequence. Yet, an important aspect to determine is the sufficient length of each signal required for efficient secretion of its respective substrate. To achieve this, we constructed progressively larger translational fusions of the *yscX* and *yopD* 5'- ends, each encompassing their native Shine-Dalgarno (SD) sequences, to a *bla* gene encoding for a promoterless and signaless β-Lactamase reporter. We used this approach to verify whether YscX and/or YopD N-terminal peptides are sufficient to function as T3S signals independent of the context of full length protein. This was particularly important for analysis of the YscX N-terminal secretor domain because in most cases mutagenizing the YscX N-terminus *in cis* had a negative impact on general T3S function; suggesting that this segment has other essential roles in the Ysc-Yop T3SS (see section 3.1.6). Plasmids harbouring these fusion proteins were introduced *in trans* into parental *Y. pseudotuberculosis* to analyze their IPTG inducible production and secretion after growth in T3SS inducing conditions.

Unlike their shorter N-terminal sequences fused to β-Lactamase (Bla), the first 15 N-terminal residues of YscX and YopD were sufficient to promote secretion of large amounts of their reporter protein fusions (PAPER I, Fig. 11 and PAPER II, Fig. 6). However, overexposure of the immunoblot images enabled the detection of minimal levels of secreted $YscX_5$-Bla (PAPER II, Fig. 6) and $YopD_5$-Bla, but only when secreted from *Yersinia* lacking both native YopB and YopD proteins (PAPER I, Fig. 11). In fact, when expressed in parental bacteria, all the YopD-Bla fusions were poorly secreted, if at all. We interpret this to suggest that native YopB and YopD are secreted more efficiently, which in turn blocks entrance of the YopD-Bla fusions into the secretion channel. The reason for this could be the existence of other secretory elements downstream of the N-terminal signal of native translocators such as binding domains to the LcrH chaperone which better prioritize their secretion. Collectively,

these data show that an N-terminal peptide sequence between residues 5 and 15 acts as an optimal T3S signal for YscX and YopD.

3.1.4 Synthesis of T3S substrates; potential mechanisms of translational control

While investigating the length of YscX and YopD N-terminal T3S signal required for adequate export of their β-Lactamase fusions (see section 3.1.3), we noticed that the levels of accumulated protein fusions were proportional to the length of the appended N-terminal sequence (PAPER I, Fig. 11 and PAPER II, Fig. 6). This was a significant finding because these differences could not simply be explained by the protein turn-over since the fusions were all resistant to bacterial endogenous proteases (PAPER I, Fig. S7 and PAPER II, Fig. S5). Accumulated steady-state levels of $YopD_1$-Bla that include the YopD SD sequence plus AUG start codon, were dramatically less when compared to those of $YopD_{20}$-Bla (PAPER I, Fig. 11). Similarly, levels of $YscX_5$-Bla were less when compared to $YscX_{15}$-Bla (PAPER II, Fig. 6). These phenotypes highlight the potential for N-terminal sequences to establish translational control of their T3SS substrates.

If this is the case, then it also seems a realistic notion that mechanisms of translation control that serves to regulate cytoplasmic pools of T3S substrates could be a way to orchesterate secretion hierarchy; those substrates that accumulate to higher levels will be prioritized for secretion. To investigate this a little further, we examined the accumulated levels of analogous $YopE_{20}$-Bla and $YopD_{20}$-Bla fusions. Interestingly, less of the former fusion was produced (Figure 8). Surprisingly, swapping the SD sequences between constructs had a dramatic effect on production levels. When the native *yopE* SD sequence was used to translate *yopD*$_{20}$*-bla* mRNA, production of $YopD_{20}$-Bla decreased. Conversely, when the *yopD* SD sequence was used to translate *yopE*$_{20}$*-bla* mRNA, production of $YopE_{20}$-Bla increased (Figure 8). Since these constructs are driven by the same IPTG inducible promoter, in an identical plasmid back bone, we assume that changes in production occur at the translational level and not by alterations to transcriptional output. While this still needs to be verified, it is tempting to speculate that information in the 5'- untranslated region and also information immediately upstream of *yops* initiation codons directs the amount of Yops that are produced.

Naturally, much more work is needed to confirm this. However, from our preliminary data it seems that this could be a mechanism to generate greater production of the translocator substrate YopD compared to the effector substrate YopE, with obvious reperocussions to orchestrating substrate secretion. Significantly, this is not without precedent as numerous studies have shown a requirement of mRNA sequence and/or structural elements to control protein translation (115, 176). In actual fact, there exists examples in the Ysc-Yop T3SS where changing the levels of a particular component has a dramatic effect on T3SS activity. For example, YopK is a T3S substrate that controls the rate of Yop translocation by *Yersinia* (83). *Yersinia* lacking this protein hyper translocates Yop effectors into the host cell cytosol, presumably because they make larger translocon pores in host cell membranes (150).

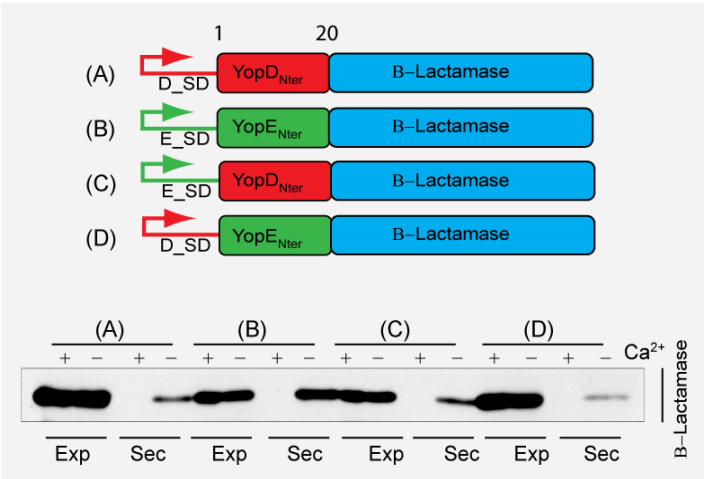

Figure 8: Translational reporter fusions and the *in vitro* sysnthesis and secretion profile of β – Lactamase. Translational fusions of (A) *yopD* SD plus YopD N-terminal 20 residues, (B) *yopE* SD plus YopE N-terminal 20 residues, (C) *yopE* SD plus YopD N-terminal 20 residues, and (D) *yopD* SD plus YopE N-terminal 20 residues to the signaless and promoterless β–Lactamase.

On the other hand, over expression of YopK results in production of smaller size translocon pores and this consequently reduces Yop translocation efficiency (150). Interestingly, YopK accumulates in different amounts in the cytoplasm of several *Y. enterocolitica* serotypes and *Y. pseudotuberculosis*. This is due to sequence differences in their *yopK* 5'- ends which result in different mRNA secondary structures (284). No doubt the effect of this is to alter the extent of Yop translocation capable by the different *Yersinia* isolates. Another example is the LcrQ early substrate that has a regulatory role in T3SS function. Together with the YopD-LcrH complex, it negatively regulates Yops synthesis in *Yersinia*. *Yersinia* lacking LcrQ constitutively produce and secrete Yop effectors in large amounts. On the contrary, over expression of LcrQ significantly affects production and secretion of YopE (311). Thus, mechanisms must be in place to fine-tune production of T3S proteins to ensure their individual levels are optimal to ensure efficient T3SS function.

3.1.5 Temporal T3S control; role of N-terminal T3S signals

There is no clear evidence for sequence or structural similarities between N-terminal secretion signals from different T3SS substrate classes (i.e. early, middle, and late). Our previous analysis showed that minimal amounts of secreted native YopD are sufficient to promote Yop effector translocation into eukaryotic cells (91). However, for several of our YopD variants altered in their N-terminal sequences the secreted YopD product was not sufficient to promote Yop translocation (PAPER I; Fig. 8). Perhaps the changes to the N-terminus altered the order of YopD export, which in turn caused an effector translocation deficiency.

Having in mind the various stage dependent functions of many Ysc and Yop components, we wondered whether temporal control of substrate T3S could also be contributed to by differential recognition of the N-terminal secretion signals by some cytoplasmic component of T3SS. We chose to investigate this a little further by generating several *Yersinia in cis* mutants which harbour chimeric T3S substrates where the N-terminus of a substrate from a certain class is exchanged by an equivalent region of another substrate from the same or different class (Figure 9). We hypothesize that replacing the N-terminus of a T3S substrate by another from a different class might prioritize or delay its secretion (Figure 10), while the probability of this occurring would be less if the N-terminus is replaced by another from the same class. As a result, altering substrate secretion order would be expected to affect T3SS functionality (Figure 10).

Initially, exchanging T3S N-terminal peptides among representatives from middle and late Yop substrate classes had a clear effect on T3S activity. Although the chimeras $YopD_{E\text{-}Nterm}$ and $YopE_{D\text{-}Nterm}$ were secreted in similar levels to their native counterparts upon inducing T3SS *in vitro* (PAPER I, Fig. 9), their effector translocation efficiency, as measured by *Yersinia* resistance to uptake and killing by J774-1 mouse macrophage-like cells, was clearly compromised (PAPER I, Fig. 10). Most probably, the reason for the normal *in vitro* secretion profile is the problem of the assembly of individual T3SSs being non-synchronous i.e. a heterogeneous bacterial culture will contain bacterial cells that have numerous T3SSs at various stages of development, a situation that can be overcome using a host cell contact dependant assay where only the function of a mature T3SS at the zone of bacteria-host cell contact are analyzed. Moreover, the generated early-middle and early-late chimeras, $YscX_{D\text{-}Nterm}$ and $YscX_{E\text{-}Nterm}$ respectively, were not detected in culture supernatant *in vitro* (PAPER II, Fig. 1). On the other hand YopD and YopE harbouring the YscX N-terminal peptide ($YopD_{YscX\text{-}Nterm}$ and $YopE_{YscX\text{-}Nterm}$) are still normally secreted *in vitro* (PAPER II, Fig. 1). In contrast to *Yersinia* harbouring $YscX_{D\text{-}Nterm}$, a strain that produced the $YopD_{YscX\text{-}Nterm}$ chimera could secrete YscF and assemble T3S needle structures (PAPER II, Fig. 3) (see section 3.1.6). Collectively, these data show that substrate secretion order can be compromised upon exchanging its N-terminal T3S signal by another of a different substrate class.

To further analyze our hypothesis, we generated a set of T3S chimeric substrates that have their N-terminal signals exchanged by another from the same class. As expected, in addition to maintaining its normal *in vitro* secretion (PAPER I, Fig. 9), *Yersinia* can translocate the produced $YopE_{H\text{-}Nterm}$ chimeric protein (i.e. YopE containing 2-15 residues derived from the YopH effector) efficiently to the eukaryotic host cell interior resisting phagocytosis (PAPER I, Fig.10). We interpret this finding to indicate that T3S N-terminal signals from the effector class share similar characteristics that make them functionally interchangeable. Conversely, *Yersinia* producing $YopD_{B\text{-}Nterm}$ (i.e. YopD containing 2-15 residues derived from the YopB translocator) was compromised in effector translocation and was easily internalized by phagocytic immune cells (PAPER I, Fig.10). We reasoned that this phenotype suggests that characteristics between T3S N-terminal signals from translocator proteins have specifically evolved unique features that do not make them functionally interchangeable. This might have something to do with YopD, but not YopB, exhibiting additional regulatory

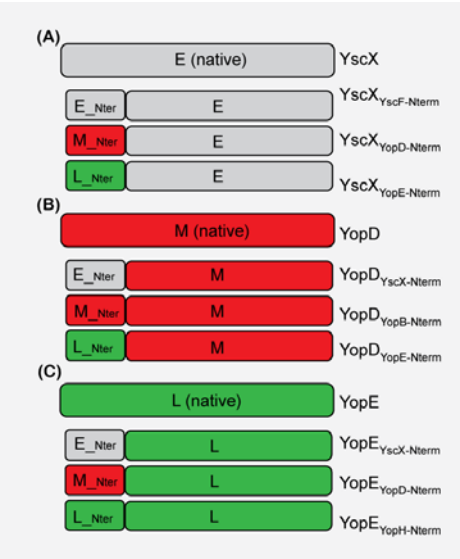

Figure 9: Chimeric Ysc-Yop T3SS substrates (A) The first 2-15 N-terminal residues of an early T3S substrate (e.g. YscX) is exchanged by equivalent regions from other early (E), middle (M), or late (L) substrates. (B) The first 2-15 N-terminal residues of a middle T3S substrate (e.g. YopD) is exchanged by equivalent regions from other early (E), middle (M), or late (L) substrates. (C) The first 2-15 N-terminal residues of a late T3S substrate (e.g. YopE) is exchanged by equivalent regions from other early (E), middle (M), or late (L) substrates.

functions. Applying the same approach with *Yersinia* producing $YscX_{YscF\text{-}Nterm}$ or $YscF_{YscX\text{-}Nterm}$ early substrates chimeras affected negatively the general T3SS activity. Consequently this abolished any possibility to investigate secretion control by these *Yersinia*. This was probably due to the fact that both the N-terminus of YscF and YscX have also evolved specific tasks in the assembly of the needle that extend beyond a standard secretor domain (see section 3.1.6). In conclusion, our data support to a large extent the exsistance of a temporal substrate T3S control which may be established through N-terminal sequences harboured by different T3S substrates.

3.1.6 YscX N-terminal peptide; a role other than as a secretion signal

Both YscX and its T3S chaperone YscY are important for Ysc-Yop T3SS activity. We showed that YscX is efficiently secreted via utilizing a 15 residue N-terminal T3S signal (see section 3.1.2). However, whether secretion is essential for YscX to perform its T3SS function(s), is still poorly understood. Although secretion of the ($YscX_{\Delta 3\text{-}7}$) variant is reduced, *Yersinia* producing this protein still efficiently secretes YscF and and assembles a T3S needle structure on the bacterial surface (PAPER II, Fig. 3). Furthermore, regulated production and secretion of both translocator and effector Yops is maintained *in vitro* (PAPER II, Fig. 4), a phenotype accompanied with Ca^{2+} dependant growth of this strain at 37 °C (PAPER II, Fig. S2). In parallel, we fused a sequence coding for a six His tag epitope directly upstream to the *yscX* start codon and ectopically expressed this fusion ($His_{(6)}$-YscX) in *Yersinia* devoid of its native *yscX.* Interestingly, this fully restored T3SS function *in vitro* (PAPER II, Fig. 5) regardless of the fact that this fusion was very poorly secreted. Moreover, replacement of YscX N-terminal 2-15 residues by any of the equivalent regions from other Ysc or Yop substrates rendered both the YscX variants and the T3SS non-functional. These data mirror our phenotypes with the $YscX_{\Delta 8\text{-}12}$ and $YscX_{\Delta 13\text{-}22}$ deletion variants. This is because all these

non-functional variants could not secrete and further assemble the T3S YscF needle. Hence, it is apparent that the YscX N-terminus contains non-redundant information required for both an early stage cytoplasmic function and then subsequent secretion of YscX. As this YscX N-terminal peptide seemed not to be essential for interaction with the YscY chaperone, we believe that it is most probably necessary for cytoplasmic-located YscX to interact with the cytoplasmic domain of YscV, a third member of a hetero-trimer complex required to promote secretion of T3SS early substrates (YscF, YscI, and YscP) to ensure needle complex assembly (86). Additionally, YscX secretion seems to be a secondary event. Perhaps it allows recycling of free YscY chaperone to engage with the LcrH chaperone, which may prioritize translocator secretion (40).

3.2 YopN and TyeA control T3S substrate export.

Recently, homologues to the InvE family, known for their role in ensuring translocator secretion before effector secretion, were reported within the Ysc-Yop T3SS of *Yersinia.* This was partitioned over two proteins YopN and TyeA (see section 1.10.5.4.1) (PAPER III, Fig. 1) that forms a 42 kDa complex known to control general T3S in *Yersinia*. Here we aimed to achieve a better understanding of the molecular mechanism by which the YopN-TyeA complex control substrate export in *Y. pseudotuberculosis*. Part of this goal was also to determine if this complex prioritizes YopD translocator secretion (middle substrate) before YopE effector secretion (late substrate).

To examine this, we used *in cis* deletion mutagenesis to generate *ΔyopN, ΔtyeA, and ΔyopN-tyeA* mutant *Y. pseudotuberculosis*. Like the two individual mutants, *Yersinia* lacking both YopN and TyeA showed unconditional constitutive production and secretion of the YopD translocator and the YopE effector *in vitro* regardless of Ca^{2+} levels. The SepL C-terminus which is homologous to TyeA (PAPER III, Fig. 1) was previously shown to delay effector secretion (see section 1.10.5.3) (290). Moreover, deletion of the InvE N-terminal 100 residues or the C-terminal last 52 residues, which are regions of homology to YopN and TyeA respectively (PAPER III, Fig. 1), inhibited secretion of SipB, SipC, and SipD translocators but did not hinder effector secretion (171). This differs from the general loss of control we encountered with our *Yersinia ΔyopN* and/or *ΔtyeA* mutants. We attempted to complement the lost T3S regulatory control in the *ΔyopN-tyeA* double mutant by ectopic expression of genes encoding for InvE from *S. enterica* Typhimurium, SepL from enteropathogenic *E. coli*, or MxiC from *S. flexneri*, but these trials were not successful (data not shown). This likely means that the Yop-TyeA complex act to control T3S substrate secretion via a different mechanism despite sharing some genetic homology. However, completion of this analysis should make sure of proper expression of these proteins in *Yersinia*.

Finally, *Yersinia* devoid of producing individual YopN or TyeA or both are no longer able to maintain polarized translocation of the YopE effector into the cytosol of HeLa cells during tissue culture infection. Distinct from parental *Yersinia*, all three mutants leak significant amounts of YopE into the extracellular environment (PAPER III, Fig. 6). This reduced translocation efficiency is also consistent with *Yersinia* lacking *ΔyopN* and/or *tyeA* being

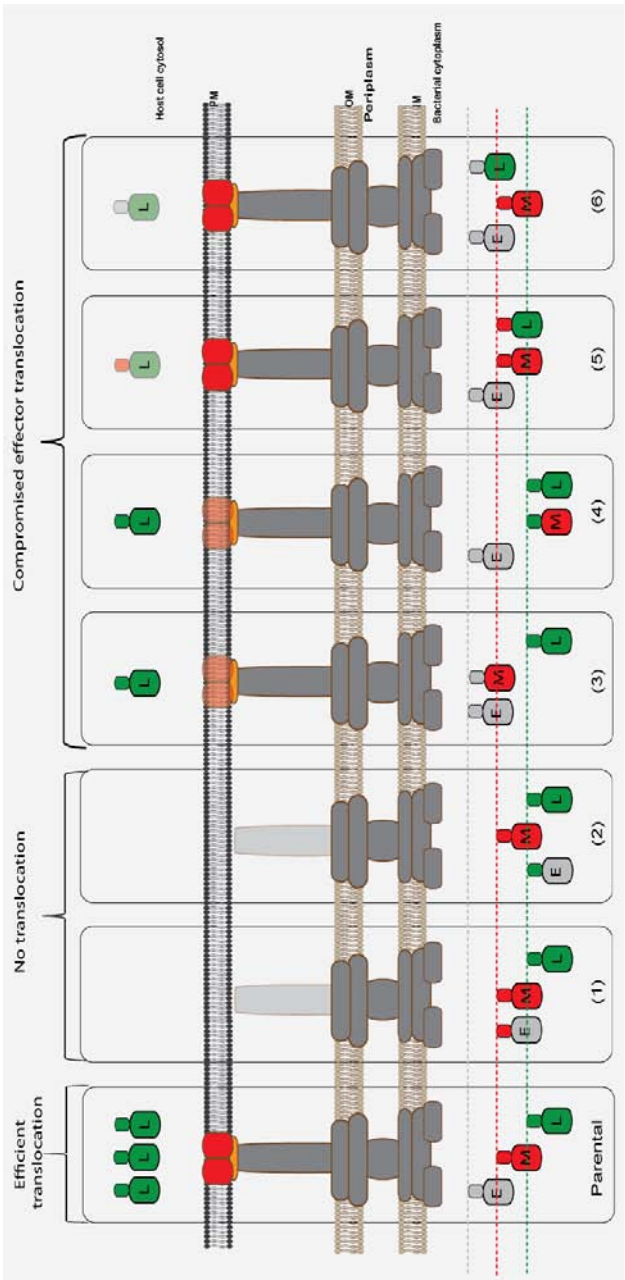

Figure 10: Effect of exchanging N-terminal T3S signals between different substrate classes on T3SS function. (1 & 2) If the N-terminal T3S signal of an early substrate is exchanged with that of a middle or late substrate, this will delay secretion of the early substrate and probably affect T3SS assembly and function. (3 & 4) If the N-terminal T3S signal of a middle substrate is exchanged with that of early or late substrate, its secretion will be prioritized or delayed respectively, and this can affect translocon pore assembly and effector translocation efficiency. (5 & 6) If the N-terminal T3S signal of a late substrate is exchanged with that of early or middle substrate, its secretion will be prioritized and effector translocation efficiency will be affected. Note that dashed lines indicate ordrer of secretion; grey refers to early secretion, red refers to middle secretion, and green refers to late secretion.

significantly compromised in their resistance to phagocytosis by mouse macrophage-like immune cells.

In conclusion these data suggest a general role of YopN-TyeA complex in controlling secretion of Yop effector and translocator substrates rather than having the ability to prioritize

the translocators over the effectors. This probably occurs by acting as a physical cytosolic plug of the Ysc-Yop secretion channel prior to host cell contact (103).

3.2.1 Production of a functional YopN-TyeA hybrid in *Yersinia.*

Y. pestis was recently shown to form a 42 kDa YopN-TyeA hybrid protein that was probably triggered by a translational +1 frameshift occuring at a UUU-UGG site (codons F_{278}, and W_{279}) near to the 3'- end of *yopN* mRNA. This sequence is known to induce ribosomal pausing during protein translation (264). In contrast, *Y. enterocolitica* cannot form a hybrid because sufficiently different *yopN* sequence means that a premature stop codon would always form in the event of a +1 frameshift by the ribosome. We showed that *Y. pseudotuberculosis* possessing *yopN* sequence identical to that of *Y. pestis*, could also naturally produce and secrete a YopN-TyeA hybrid via T3SS (PAPER III, Fig. 3). This occurs also as a result of a +1 frameshifting event, since introducing a *yopN* sequence from *Y. enterocolitica* to *Y. pseudotuberculosis* ensured that only singular YopN and TyeA polypeptides could be produced (PAPER III, Fig. 2 and Fig. 3). Interestingly, when we targeted the F278 and W279 residues for mutagenesis, none of these mutants lost the ability to produce the 42 kDa hybrid protein (PAPER III, Fig. 9) This indicated that the ribosome pausing site represented by these two codons is not the sole element that induces the +1 frameshift.

Several studies proposed additional features downstream to the ribosomal pausing site that can contribute to induce frameshifting, such as mRNA secondary structures and sequence length and position of internal Shine-Dalgarno sites (53, 140, 172, 185). Other external factors can also affect ribosomal pausing and frameshifting, such as codon usage, relative tRNA abundance, and mRNA binding molecules e.g. polyamine organic compounds (144, 203, 264). The contribution of polyamines to this process, presumably through their strong interactions with mRNA, is fascinating in light of their requirement for maintaining functional T3SS in several bacteria (163, 320).

3.2.1.1 The YopN-TyeA hybrid; biological relevance for T3SS

Although the YopN-TyeA hybrid was functional *in vitro (102)*, it was not clear if the YopN-TyeA hybrid protein could function *in vivo* to support Yops translocation or virulence. To assess its biological importance, we used an *in cis* mutagenesis approach to engineer *Yersinia* strains to either produce predominantly the YopN-TyeA hybrid by introducing +1 frameshifts in *yopN* after codon 278 or 287. To avoid any possibility of producing any residual singular TyeA, into these two *Yersinia* strains we also disrupted the *tyeA* SD sequence (PAPER III, Fig. 2) (Figure. 11). Significantly, the four engineered 42 kDa YopN-TyeA fusions were abundantly produced, stable, and efficiently secreted *in vitro* (PAPER III, Fig. 4 and Fig. 5). Moreover, these *Y. pseudotuberculosis* bacteria could all maintain controlled production and T3S of both the YopD translocator and the YopE effector (PAPER III, Fig. 5). This was true also for control bacteria unable to produce the hybrid (PAPER III, Fig. 3). We then

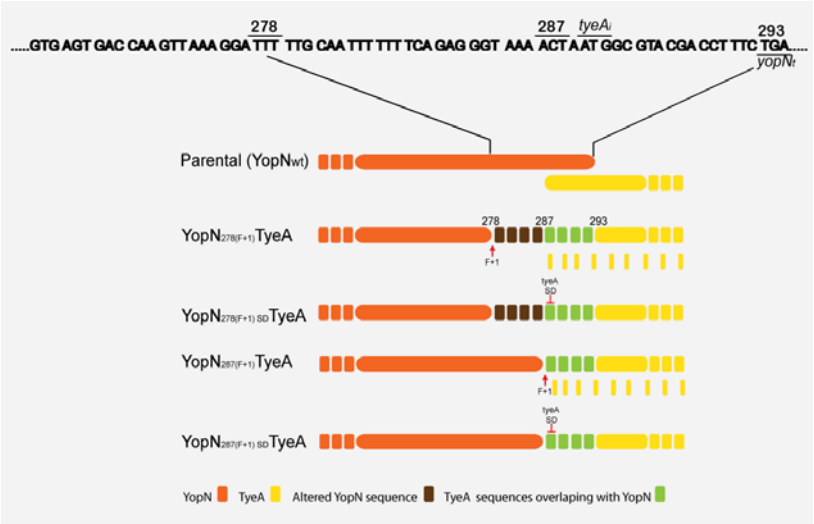

Figure 11: Generation of *Y. pseudotuberculosis* predominantly producing the YopN-TyeA hybrid protein. $YopN_{278\ (F+1)}$ TyeA: a +1 frameshift by removal of 'T' directly after *yopN* codon 278. $YopN_{278\ (F+1)SD}$ TyeA: the same +1 frameshift mutant with the *tyeA* SD site disrupted. $YopN_{287\ (F+1)}$TyeA: a +1 frameshift by removal of 'A' directly after *yopN* codon 287. $YopN_{287(F+1)SD}$ TyeA: the same +1 frameshift mutant with the *tyeA* SD site disrupted.

wondered if predominant production of the YopN-TyeA hybrid in *Yersinia* would maintain efficient effector translocation upon host cell contact. To assess this we measured the ability of these mutants to resist phagocytosis when exposed to J774-1 macrophage-like immunecells. Only *Yersinia* possessing an active T3SS can deliver effector toxins into these immune cells to resist internalization (known as 'anti-phagocytosis'). Interestingly, all the four mutants producing the YopN-TyeA hybrids showed the same level of resistance as parental *Yersinia* throughout the duration of the infection.

The ability of *Yersinia* to intoxicate host cells depends on how efficient it can direct the delivery of effector Yops towards the host cell interior, a property defined as 'polarized translocation'. This is measured by analyzing the ratio of YopE released into the extracellular space (taken from cleared supernatant) to the total amount of YopE found in whole cell lysate derived from both bacteria and the infected HeLa cell monolayers. In contrast to parental *Yersinia* and both the $YopN_{287\ (F+1)}$TyeA and $YopN_{287(F+1)SD}$ TyeA mutants, the two $YopN_{278\ (F+1)}$ TyeA and $YopN_{278\ (F+1)SD}$ TyeA mutants showed a slight increase in amounts of YopE in the supernatant fraction, reflecting a little defect in polarized translocation by these *Yersinia*. Crucially, the *yopN* sequence in these two mutants deviates significantly from native sequence between residues 279 to 287, and this probably causes this phenotypic effect. Interestingly, the reduction in polarized translocation did not appear to affect the ability of these mutants to be anti-phagocytic.

Since *in vitro* assays sometimes lack the sensitivity to identify defects in T3S activity (69, 70), we investigated the virulence of these YopN-TyeA hybrid producing strains *in vivo* using a murine infection model. We sought to measure the ability of these strains to compete with parental *Yersinia* by establishing oral co-infections and measuring their competitive index 'CI'

in relation to that of parental *Yersinia*. The 'CI' of each strain was calculated using colony forming unit (CFU) counts from collected spleens 4 days post-infection (PAPER III). Interestingly, the four strains showed CI values ranging from 0.04 to 0.22 compared to a 0.83 of parental *Yersinia.* This reflects the inability of these strains to restore full T3SS function by sole production of YopN-TyeA hybrids, a phenotype which was not obvious from *in vitro* analysis.

3.2.2 Influence of YopN C-terminus on the Ysc-Yop T3SS function

We reasoned the slight loss of control on polarized translocation of YopE into HeLa cells by the $YopN_{278\ (F+1)}$ TyeA and $YopN_{278\ (F+1)SD}$ TyeA mutants (PAPER III, Fig. 6) could be due to their different YopN sequence in the region between residues 279 to 287 (PAPER III, Fig. 2). To better understand the functional importance of this YopN C-terminal region we designed a series of *in cis Yersinia* mutants that predominantly produce singular YopN and TyeA proteins having the C-terminus of YopN altered after codons 278 or 287 (Figure 12).

3.2.2.1 The YopN C-terminus impacts on *in vitro* T3SS function.

Interestingly, almost all these YopN variants were stable against intrabacterial proteases similar to native YopN. The notable exception was the YopN variant truncated after codon 278 ($YopN_{279STOP}$) (PAPER IV, Fig.1). This was probably due to an altered structure that made it extremely sensitive to degradation. Next, we wondered if these variants still maintain regulatory control of T3SS. To do so, we examined their *in vitro* LCR growth characteristics at elevated temperature. Like parental *Yersinia*, strains producing $YopN_{288(scramble)293}$, or $YopN_{288STOP}$ variants maintained a 'CD' growth phenotype. On the contrary, *Yersinia* producing the $YopN_{279(F+1),\ 287(F\_1)}$, $YopN_{279(F+1),\ 287STOP}$ or $YopN_{279STOP}$ were all unable to grow at 37°C regardless of Ca^{2+} levels. This 'TS' growth phenotype resembles that of the null *yopN* strain (PAPER IV, Fig. 2). This was expected for *Yersinia* producing unstable $YopN_{279STOP}$ but not stable $YopN_{279(F+1),\ 287(F\_1)}$, $YopN_{279(F+1),\ 287STOP}$ proteins, which means that the latter two YopN variants seem affected in their function. We wondered if these altered LCR growth phenotypes affected their ability to produce and secrete T3S proteins required at multiple stages of T3SS assembly and/or function. Interestingly, crosslinking with the bacterial impermeable BS^3 crosslinker showed the ability of all these bacteria to produce, secrete and finally polymerize the YscF early T3S substrate at the bacterial surface (PAPER IV, Fig. 3). This means that they are capable of forming the T3S needle and completing the assembly of the T3S apparatus.

To address if they could control synthesis and secretion of other classes of T3S substrates, we checked their *in vitro* Yops synthesis and secretion profiles. Interestingly, all the mutants could produce and secrete YopN, although the stable $YopN_{279(F+1),\ 287(F\_1)}$ and $YopN_{279(F+1),\ 287STOP}$ as well as unstable $YopN_{279STOP}$ showed an uncontrolled constitutive production and secretion regardless of Ca^{2+} levels in the media (PAPER IV, Fig. 4). This in turn altered their production and secretion of both YopD translocator (middle substrate) and YopE effector (late substrate). Conversely, parental *Yersinia* and both $YopN_{288(scramble)293}$ or

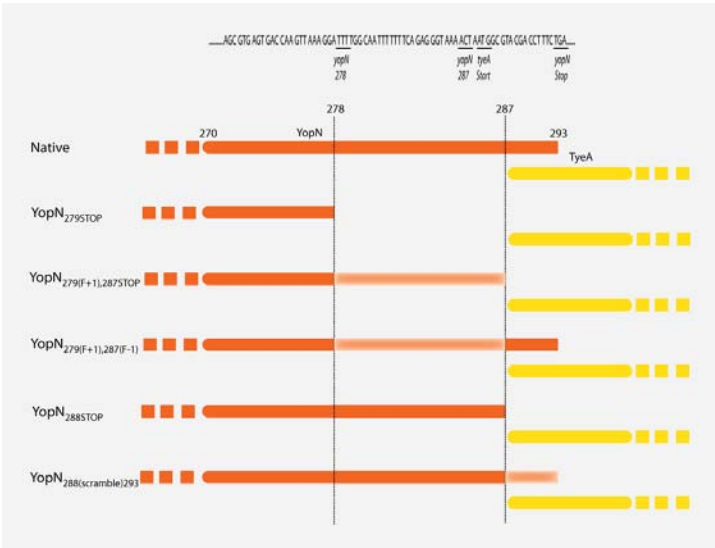

Figure 12: YopN variants produced upon mutagenesis of YopN C-terminus: $YopN_{279STOP}$ is produced via introducing a 'TAG' stop codon after *yopN* codon 278. $YopN_{279(F+1),287STOP}$ has its region 279 to 287 altered by adding a +1 frameshift after codon 278, and lacks its last 6 residues by introducing a 'TAG' stop after codon 287. $YopN_{279(F+1),287(F-1)}$ is a full length YopN having its sequence 279 to 287 altered via +1 frame-shifts after codon 278, and then restoration of the original frame with a -1 frameshift after codon 287. $YopN_{288STOP}$ is a truncated YopN produced by introducing a 'TAG' stop codon after *yopN* codon 287. $YopN_{288(scramble)293}$ is a full length YopN having its last 6 residues altered in a way that does not change the overlapping *tyeA* sequence.

$YopN_{288STOP}$ mutants maintained tight regulatory control over Yop synthesis and secretion (PAPER IV, Fig. 4).Collectively, these analyses confirm that YopN C-terminal region between residues 278 and 287 is essential for its stability and *in vitro* T3S function while the region between residues 288 and 293 is of less importance. To determine their T3SS functionality during eukaryotic host cell contact *in vitro*, we measured the ability of these mutants to resist phagocytosis upon infecting J774-1 mouse macrophage-like immune cells. Similar to parental *Yersinia*, strains producing $YopN_{288(scramble)293}$ or $YopN_{288STOP}$ could resist phagocytosis even after 6 hours post-infection. However, *Yersinia* producing $YopN_{279(F+1),\ 287(F\_1)}$ or $YopN_{279(F+1),\ 287STOP}$ started to show signs of defective resistance after 6 hours post-infection, whereas *Yersinia* producing the unstable $YopN_{279STOP}$ was severely compromised early on in the infection, phenocopying the null *yopN* mutant. In conclusion, it seems that the YopN C-terminus (residues 278 to 287) is essential for its T3S activity during eukaryotic host cell contact.

3.2.2.2 Influence of YopN C-terminus on *in vivo* T3SS function.

In vitro analyses showed significant reduction of T3SS function for *Yersinia* producing $YopN_{279(F+1),\ 287(F\_1)}$, $YopN_{279(F+1),\ 287STOP,}$ and $YopN_{279STOP}$ variants. This could not be

confirmed *in vivo* because of their sensitivity to elevated temperatures. However, the normal growth profile of bacteria producing $YopN_{288(scramble)293}$ or $YopN_{288STOP}$ permitted virulence analysis *in vivo.* To assess this, we again applied a mouse co-infection approach where the ability of these strains to compete with parental *Yersinia* in mice was measured. Both strains showed a CI score of 0.55 and 0.44 respectively compared to the 0.83 of parental *Yersinia*. This indicates that altering the YopN six C-terminal residues does not have any dramatic impact on its T3S function *in vivo*, as was predicted by our *in vitro* analysis (see section 3.2.2.1)

3.3 Summary

In order to build an active T3SS, coordination between multiple functional stages is a necessity. To achieve this, controlled export for many T3S components across the bacterial cell envelope is required. In this study we aimed to understand the molecular mechanisms by which secretion of Ysc and Yop substrates is controlled by the T3SS in *Y. pseudotuberculosis*.

Interestingly, the level of control seemed to be initiated at the stage of protein translation. We showed that specific features at the 5'- end of *ysc* and *yop* mRNA contribute to efficient production of their cognate substrates. Moreover, these sequence elements ensure differential levels of production among substrates from different classes (Fiure 13). Perhaps, this is because some T3S components are dedicated for performing multiple functions at different stages or at different locations during T3SS assembly and activity. For example, our data showed that sufficiently produced YscX, a member of the export apparatus, was required at the early stage of T3SS assembly inside the bacterial cytoplasm (Figure 13 'steps 1 and 2'). These functions were aided by features located at its N-terminus. This same segment also promotes YscX subsequent secretion (Figure 13 'step 3'); a step that is yet has no known functional relevance. Nevertheless, YscX might be a signal that is coupled to the completion of polymerized YscF at the bacterial surface (Figure 13 'step 2'). At this stage, it is well known that a switching signal is triggered by YscU cleavage and cross-talk with the molecular ruler YscP. This event induces secretion of middle translocator and late effector Yops (Figure 13 'steps 6 and 7'). YopD is a multi-functional pore-forming T3S translocator, which also inhibits synthesis of Yop effectors prior to host cell contact. The 5'-end of *yopD* appears to induce its production in larger amounts than the equivalent region of yopE encoding YopE effector toxin, is able to do. We assume that this can contribute to prioritizing YopD secretion before YopE (Figure 13 'steps 6 and 7'). Furthermore, a combined analysis of early, middle, and late substrates N-terminal T3S signals suggest that they may prioritize secretion of YscX before YopD that is before YopE (Figure 13 'steps 3, 6, and 7').

It is yet not known how the T3S apparatus recognizes YscX, YopD, and YopE N-terminal T3S signals to facilitate their temporal secretion. However, previous studies in *Yersinia* showed a role for the YscN ATPase in recognition of T3S substrates via their N-terminal sequences. Moreover, prioritized translocator secretion before effectors is promoted by the InvE family of proteins from several Gram-negative bacteria. Hence, we proposed a similar role for the YopN-TyeA complex, an InvE family member in *Yersinia*. However, our

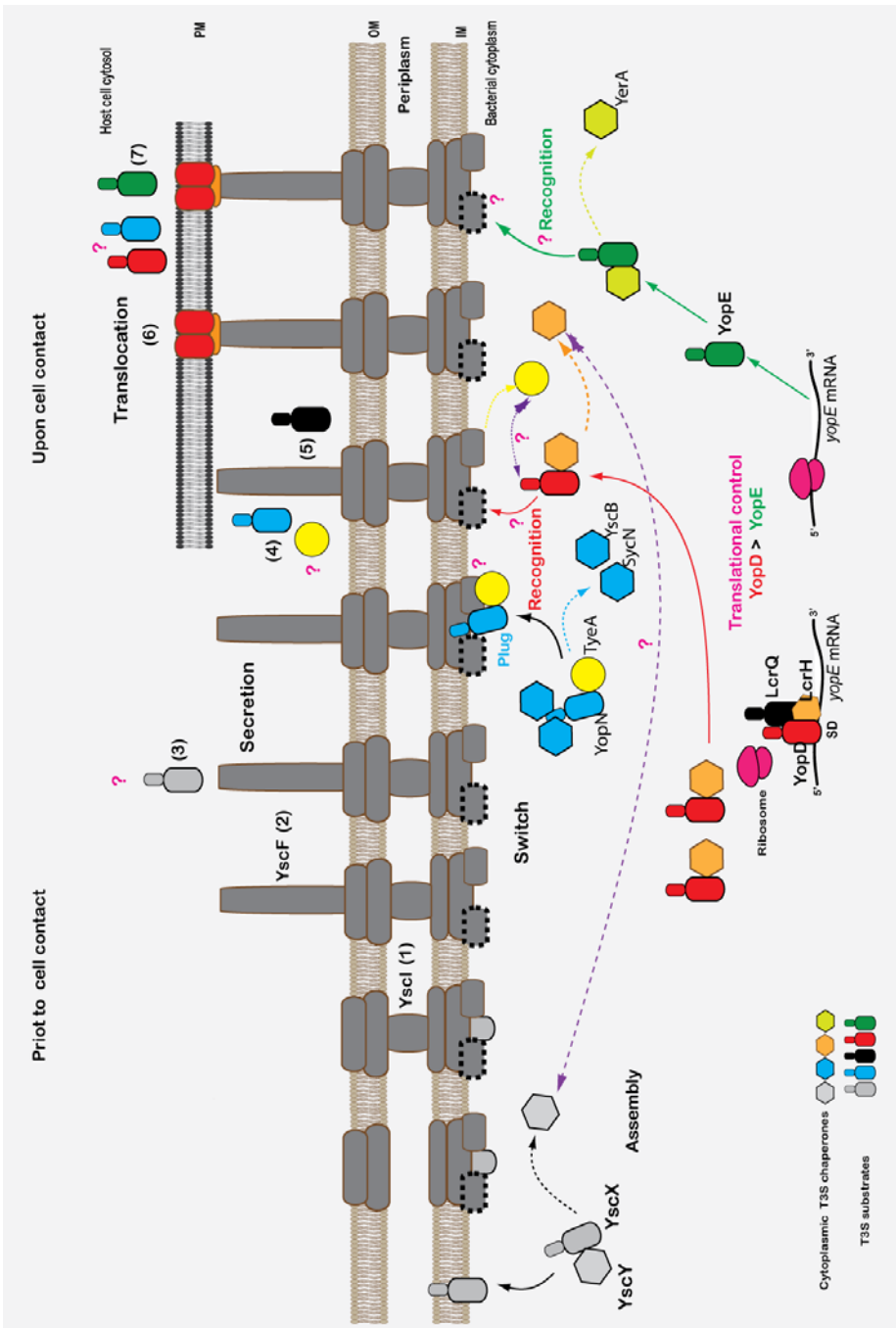
Priot to cell contact
Upon cell contact
Host cell cytosol
PM
OM
Periplasm
IM
Bacterial cytoplasm
Translocation
(6)
(7)
(5)
(4)
(3)
Secretion
YscF (2)
YscI (1)
Switch
Assembly
YscX
YscY
Plug
Recognition
YopN
TyeA
YscB
SycN
Translational control
YopD > YopE
Ribosome
LcrQ
LcrH
YopD
SD
yopE mRNA
YopE
YerA
Recognition
Cytoplasmic T3S chaperones
T3S substrates

Figure 13: Model representing factors that may contribute to temporal T3S control.
Prior to cell contact: YscX (a subunit of the T3S export apparatus) is piloted to the base of the T3S machinery by its cognate chaperone YscY. The export apparatus promotes secretion of YscI 'step1' and YscF 'step2' to complete injectisome assembly. At some point, YscX is expected to be secreted 'step3', although the importance of this for T3SS function is unknown. A substrate secretion specificity switch occurs upon interplay between YscU and YscP. This signals that the injectisome is competent for the secretion of translocator and effector substrates. The relationship between the YscU/P switching signal and YopN T3S control is unclear. Nevertheless, YopN is piloted to the base of the secretion apparatus by the help of its chaperones SycN and YscB. TyeA binds to both the base of the secretion apparatus and to the YopN C-terminus to ensure its partial secretion into the T3S channel. This acts as a secretion plug preventing secretion of Yops. YopD-LcrH-LcrQ are bound to the 5'- end of *yops* mRNA preventing their translation by ribosomes.
Upon cell contact: The YopN-TyeA complex is dissociated allowing full secretion of YopN and relief of the plug 'step 4'. LcrQ is released from its complex and secreted 'step 5'. LcrH pilots YopD to the T3SS to promote its T3S 'step 6'. Once secreted, YopD (together with YopB) form translocon pores in the host cell membrane. YopE and other effectors are synthesized and piloted by cognate chaperones, such as YerA - to the secretion machinery 'step 7'.
It is conceivable that information located with the 5'- end of *yopD* and *yopE* mRNA induce production of larger amounts of YopD relative to YopE, and this can prioritize YopD secretion before YopE.
Component(s) of the T3S machinery (indicated as a rectangle bordered by a black dashed line) must recognize substrate N-terminal secretion signals and/or substrate-chaperone complexes to promote temporal substrate T3S.
Interactions between YscY-LcrH and YopD-TyeA are indicated by dashed lines in violet. To date however, they have no known role in T3S activity.

analysis found no support for this role by YopN-TyeA. Rather, we concluded that it has general control on secretion of both YopD (middle) and YopE (late) substrates. Presumably, they establish this role upon interaction with the cytoplasmic side of the injectisome, but further analysis to confirm this are required.

Significantly, our data support the early-middle-late substrate secretion phenomenon. We hypothesize that prioritized secretion of YscX followed by YopD then YopE is logical, since YopE translocation into the eukaryptic cell cytosol, requires a pre-formed needle complex aided by YscX, followed by YopB-YopD translocon pore at the host cell membrane. We do not believe that N-terminal T3S signals are the only required element for substrate T3S. Other factors such as chaperone dependent secretion signals, and further recognition mechanisms by the T3S injectisome are most likely involved. To investigate these will fall within the scope of our future analysis of hierarcheal T3S of Yops.

Main findings in this thesis

- The N-terminal domains of YopD and YscX, T3SS components in *Y. pseudotuberculosis,* possess molecular features reminiscent of T3S signals.

- The Ysc-Yop substrate N-terminal T3S signals are essential elements required for establishing efficient and timely control of their secretion.

- N-terminal secretory domains also maintain translational control of their respective substrates.

- The YscX N-terminal secretor domain contributes other significant T3S functions with respect to injectisome assembly and substrate-chaperone interaction.

- *Y. pseudotuberculosis* produces a 42 kDa YopN-TyeA hybrid protein which is biologically significant for Ysc-Yop T3SS function.

- Dissection of the YopN C-terminus revealed a domain required for maintaining its regulatory functions.

Future prespectives

The focus of this thesis has been to provide better understanding to the concept of temporal control of Ysc and Yop substrate export by the T3SS of *Yersinia*. Multiple elements were examined, some of which provided new insight into the temporal control phenomenon at different stages of T3SS function. However, several questions still remain concerning how these elements might act together to establish such control.

We revealed that features located at 5'- ends of *ysc* and *yop* mRNA sequences impacted on the level of their accumulated translation product. As yet however, we do not know what these are. Hence, future studies should focus on comparing features of the mRNA secondary structure within the 5' untranslated regions derived from different *ysc* and *yop* genes to determine differences that may influence the efficiency of ribosome binding and translation initiation. In the same way, comparisons between the different Shine-Dalgarno sequences and also rare codon usage immediately upstream of the various initiation codons should also be investigated. These are strategically important studies given that the relative accumulation of substrates in the bacterial cytoplasm could provide a mechanism to promote temporal T3S - substrates in higher concentration likely to be recognised by the T3SS first to provide to them a competitive advantage for secretion.

Studies described herein also identified the importance of N-terminal domains of YscX and YopD in ensuring their T3S in *Yersinia*. Initially, more emphasis was placed on determining the composition of the YopD N-terminal signal sequence. This study indicated that YopD contains a unique secretor domain, although pin-pointing exactly what those differences were still remained elusive. However, we know even less about the characteristics of the YscX secretor domain. Future work could use site-directed mutagenesis to better characterize this region. It would be anticipated that at least some of the isolated mutants would help to address the actual role of secreted YscX, while others would be useful to better define the apparent non-redundancy of this sequence for YscX function in T3SS assembly, which we believe to be independent of YscX secretion. Furthermore, this work will have general added benefit, because very few studies of this nature have actually been performed on any early T3S substrate.

This thesis also presents data suggestive that N-terminal secretion signals of different T3S substrate classes are capable of influencing temporal T3S control. We studied a series of mutants producing chimeric substrates in which the native secretor domain had been exchanged with the equivalent region from another substrate. The secretion efficiency was measured indirectly using the immune cell killing assay. Ideally, future work should establish methodology that would allow live microscopic monitoring of substrate secretion in real-time from a single bacterium.

Establishing T3S control is quite obviously a complex process, and is expected to require multiple elements that act in concert. While we have focused here on N-terminal T3S signals, additional signal domains located elsewhere in the protein sequence as well as substrate-chaperone complexes most likely act as recognition signals. Such features were recently confirmed to prioritize the T3S of PopD (a YopD homologue) in *P. aeruginosa.*

Related to this, our laboratory has unpublished evidence that upon target cell contact the T3S chaperone LcrH confers secretion specificity to both YopB and YopD, ensuring that they are secreted via the correct cognate T3SS. Given that free LcrH has also been shown to interact with additional Ysc-Yop T3SS components, YscY and YscO, defining the influence of LcrH on temporal secretion control should be a priority.

The existence of T3S signals implies that they must be interpreted by some type of recognition and sorting platform associated with the T3SS. In our efforts to investigate recognition mechanisms by the T3SS machine, we chose to first focus our attention on the InvE protein family, which are known to prioritize the T3S of translocator substrates before effector substrates. Although YopN-TyeA complex of *Yersinia* is homologous to members of this family, we could not yet identify a role for this complex in orchestrating the secretion of the YopD translocator before the YopE effector. Yet circumstantial evidence still points in this direction. One notable example is the stated interaction of TyeA with YopD. We aim to investigate this molecular interplay with a view to determining if it influences T3S control in any way.

We determined that YopN and TyeA can be naturally synthesized as a 42 kDa YopN-TyeA hybrid that is largely functional. Production of this hybrid is induced by a +1 frameshift at the 3'-end of *yopN*. However, mutagenesis of the presumed ribosomal pausing site did not abolish frameshifting, meaning that additional features must be involved that act together to promote the +1 frameshifting event. Future work will mutagenize the 3'- end of *yopN* in a way that alters the mRNA secondary structure or changes the *tyeA* SD sequence length and position to determine if either feature affects YopN-TyeA hybrid production. In parallel, we will utilize a frameshifting assay based on the generation of translational reporters fused to regions of *yopN-tyeA* possessing these elements. Such an assay will enable the efficiency of frameshifting to be easily measured. Finally, we aim to investigate the influence of polyamine synthesis and transport on production of YopN-TyeA hybrid and consequently on T3SS function in *Yersinia*. This is motivated by the fact that polyamine compounds are known to induce frameshifting in both prokaryotes and eukaryotes and recent findings showed that these mRNA binding compounds also affect T3SS function in some bacteria.

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