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Coordinating type III secretion system biogenesis in *Yersinia pseudotuberculosis*

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To my fathers

Late Indra Gurung

Suk Gurung

Out beyond ideas

of wrongdoing and rightdoing,

there is a field.

I'll meet you there.

- *Rumi*

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Abstract

Various Gram-negative bacteria utilize type III secretion system (T3SS) to deliver effectors into eukaryotic host cells and establish mutualistic or pathogenic interactions. An example is the Ysc-Yop T3SS of pathogenic *Yersinia* species. The T3SS resembles a molecular syringe with a wide cylindrical membrane-spanning basal body that scaffolds a hollow extracellular needle with a pore-forming translocon complex crowned at the needle tip. Together they form a continuous conduit between bacteria and host cells that allow delivery of effector proteins.

Dedicated actions of cytoplasmic chaperones, regulators and components of the cytoplasmic complex orchestrates hierarchical assembly of T3SS. On the basis of secretion hierarchy, proteins can be categorized as 'early' needle complex proteins, 'middle' translocators and 'late' Yop effectors. However, how the system recognizes, prepares and mediates temporal delivery of T3S substrates is not fully understood. Herein, we have investigated the roles of YscX and YscY (present specifically in the Ysc family of T3SS), as well as YopN-TyeA (broadly distributed among T3SS families) to provide a better understanding of some of the molecular mechanisms governing spatiotemporal control of T3SS assembly.

Despite reciprocal YscX-YscY binary and YscX-YscY-SctV ternary interactions between the member proteins, functional interchangeability in *Yersinia* was not successful. This revealed YscX and YscY must perform functions unique to *Yersinia* T3SS. Defined domain swapping and site-directed mutagenesis identified two highly conserved cysteine residues important for YscX function. Moreover, the N-terminal region of YscX harboured an independent T3S signal. Manipulating the YscX N-terminus by exchanging it with equivalent secretion signals from different T3S substrates abrogated T3S activity. This was explained by the need for the YscX N-terminus to correctly localize and/or assemble the 'early' SctI inner adapter and SctF needle protein. Therefore, N-terminal YscX performs dual functions; one as a secretion signal and the other as a structural signal to control early stage assembly of T3SS.

In Ysc-Yop T3SS, YopN-TyeA complex is involved in the later stage of T3SS assembly, inhibiting Yops secretion until host cell contact is achieved. Analysis of the YopN C-terminus identified a specific domain stretching 279-287 critical for regulating Ysc-Yop T3SS activity. The regulation was mediated by specific hydrophobic contacts between W_{279} of YopN and F_8 of TyeA.

In conclusion, this work has provided novel molecular mechanisms regarding the spatiotemporal assembly of T3SS. While the N-terminal region of YscX contributes to the early stage of T3SS assembly, the C-terminal region of YopN is critical for regulating Ysc-Yop activity at a later stage of T3SS assembly.

Abbreviations

5’ UTR - 5’-untranslated regions
ATP - Adenosine-5’-triphosphate
BACTH – Bacterial adenylate cyclase-based two-hybrid
BHI – Brain heart infusion media
Bla - Beta-lactamase
CBD - Chaperone binding domain
CD - Calcium dependent
CI - Calcium independent
C-ring - Cytoplasmic ring
Cryo-EM – Cryogenic electron microscopy
CSR – Carbon storage regulator
C-terminus - Carboxy-terminus
Cys – Cysteine amino acid
DNA - Deoxyribonucleic acid
EHEC - Enterohemorrhagic *Escherichia coli*
EIEC - Enteroinvasive *Escherichia coli*
EPEC - Enteropathogenic *Escherichia coli*
F-T3SS – Flagellar T3SS
GST - Glutathione S-transferase
HNS – Histone-like nucleoid structuring protein
HR - Hyper sensitive response
IM - Inner membrane
IPTG – Isopropyl β-D-1-thiogalactopyranoside
LCR - Low calcium response
LPS - Lipopolysachharide
M-cell – Microfold intestinal epithelial cell
MLN - Mesenteric lymph node
mRNA - messenger RNA
MS - Membrane spanning
NF-T3SS – Non-flagellar T3SS
N-terminus- Amino-termius
OM - Outer membrane
PAI - Pathogenicity island
PG - Peptidoglycan
Rcs – Regulator of capsule synthesis
RBS - Ribosome binding site

rRNA – Ribosomal RNA
RNA – Ribonucleic acid
Sct – Secretion and cellular translocation
Sec – General secretion pathway
SD - Shine Dalgarno sequence
SPI - *Salmonella* pathogenicity island
T3SS - Type III secretion system
Tat - Twin arginine secretion system
TEM - Transmission electron microscopy
TM - Transmembrane
TPR - Tetratricopeptide repeat
TS - Temperature sensitive
UTR – Untranslated region
WHO - World Health Organization
Y2H – Yeast two-hybrid
Y3H – Yeast three-hybrid
Yop - *Yersinia* outer protein
Ysc - *Yersinia* secretion

Papers in this thesis

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

I. Gurung, J.M., Amer, A.A.A., Francis, M.K, Costa, T.R.D., Chen, S., Zavialov, A.V., and Francis, M.S. (2018). Heterologous complementation studies with YscX and YscY protein families reveals a specificity for *Yersinia pseudotuberculosis* type III secretion. *Front. Cell. Infect. Microbiol.* 8:80. Doi: 8:8010.3389/fcimb.2018.00080

II. Gurung, J.M., Zavialov, A.V., and Francis, M.S. (2020). In search of key YscX elements critical for *Yersinia pseudotuberculosis* type III secretion. (Manuscript)

III. Gurung, J.M., Amer, A.A.A., Chen, S., Diepold, A., and Francis, M.S. (2020). Type III secretion assembly in *Yersinia pseudotuberculosis* is reliant upon an authentic N-terminal YscX secretor domain. (Submitted manuscript)

IV. Amer, A.A.A.*, Gurung, J.M.*, Costa, T.R.D., Ruuth, K., Zavialov, A.V., Forsberg, Å., and Francis, M.S. (2016). YopN and TyeA hybrophobic contacts required for regulating Ysc-Yop type III secretion activity by *Yersinia pseudotuberculosis*. *Front. Cell. Infect. Microbiol.* 6:66. Doi: 10.3389/fcimb.2016.00066 (*contributed equally)

Papers not included in this thesis

V. Thanikkal, E. J.*, Gahlot, D. K.*, Liu, J., Fredriksson Sundbom, M., Gurung, J. M., Ruuth, K,. Francis, M. K., Obi, I. R., Thompson, K. M., Chen, S., Dersch, P., and Francis, M. S. (2019). The *Yersinia pseudotuberculosis* Cpx envelope stress system contributes to transcriptional activation of *rovM*. *Virulence* 10, 37-57

1. Introduction / Background

1.1. Bacterial evolution – unfolding the past

Since the origin of life billions of years ago, bacteria were one of the earliest and predominant cellular life forms on the planet earth. Indeed, fossil records have captured the existence of bacteria dating back 3.45 billion years (1). Over the course of time, bacteria have penetrated all possible ecological niches defining the limits of life. Therefore, it is not surprising that the presence of bacteria must have had fundamental impact on the evolution of various life forms, not just because of the ability to cause diseases but the ability to socialize in different ecological niches. Once dubbed as the generic loner, the view regarding its habitat has been fundamentally challenged in recent years reporting stable inter- and/or intra-communities. Fortuitous exchange of genetic materials among members in such bacterial communities has been a key influence on evolutionary success. Moreover, genetic changes that refine existing functions in response to bacterial niche and genetic novelties that occur by random chances combined together have also contributed as an evolutionary force. This has resulted vast bacterial diversity that is most evident in terms of metabolic strategy, but also in terms of morphology, ubiquitous habit and other variations.

Comparative phyologenetic trees largely based on 16S rRNA sequences have studied evolutionary relationship of bacteria to all of life forms. However, molecular and genetic advances provide new phylogenetic approaches to portray evolutionary relatedness (2-4). Importantly, the tree not only illuminates bacteria as a separate domain of life but also portrays them as a dominant branch that has accumulated extensive diversification. The most famous tree published by Woese and co-workers based on 16S rRNA sequences identified 12 main bacteria phyla, which has dramatically expanded over time with the advent of new genomic sampling (4,5). Of the different evolutionary clades, one discrete cluster that emerges from analysis of these trees belongs to proteobacteria or purple-bacteria as first circumscribed by Woese and co-workers. The group consist of several sub-clusters of which γ-protobacteria is an important one. This is because (a) it encompasses one of the most abundant divisions among the prokaryotes, and (b) it comprise a large family of important agricultural and clinical pathogens (6). Genus *Yersinia* constitutes one important member of γ-protobacteria based on the phylogenetic analysis.

1.2. The *Yersinia* genus

Yersinia is a genus of bacteria in the class γ-protobacteria, order Enterobacteriales and family Yersiniaceae (7). Discovered in the late 19th century, the first species of *Yersinia* was initially grouped in the genus *Pasteurella*. Since then the nomenclature and taxonomy has undergone a massive transformation. While Van Loghem in 1944 proposed a new genus *Yersinia* (in the family Enterobacteriaceae), in honor of Alexandre Yersin, who was one of the first to isolate the plague causing bacteria *Yersinia* (*Pasteurella*) *pestis* (8-11), recent phylogenetic analysis based on conserved signature indels has mapped the genus in the novel family Yersiniaceae (7). However, in the map of *Yersinia* history, Shibasaburo Kitasato should also be credited for isolating the *Yerisinia* bacillus in the same year as Alexandre Yersin (12).

The genus *Yersinia* consists of 18 known species that can be broadly classified into pathogenic and non-pathogenic species (11). Three of the well-characterized pathogenic species are *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica*. These species cause disease in mammals including humans. The other *Yersinia* species are environmental strains commonly found in soil and aquatic environment. They are not extensively studied, and are considered non-pathogenic due to a lack of classical virulence associated properties. However, there are studies suggesting *Y. ruckeri* as a fish pathogen responsible for large economic losses in aquaculture, and *Y. entomophaga* as an insect pathogen (9,13).

Considerable effort has been invested to understand how pathogenic *Yersiniae* made an evolutionary leap from the non-pathogenic lineage. One aspect is the acquisition of DNA encoding pathogenic attributes that mediate host cell attachment and resistance to host-mediated killing. These attributes include the Ysc-Yop Type III Secretion System (T3SS) and functional surface receptors proteins like Ail and Inv. However, another aspect is key loss of functional competence, such as motility, through DNA loss or incorporation of nonsense mutations (14-18).

Despite being allied by these virulence properties, pathogenic *Yersiniae* reveal radically distinct disease outcomes. While *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogens that cause self-limiting gastrointestinal disease transmitted through fecal-oral route, *Y. pestis* can cause bubonic, septicaemic and pneumonic plague (detail in section 1.4) transmitted through infected fleas from rodent reservoir (19-21).

1.3. Decoding Plague – the roles of *Y. pestis*

Y. pestis, the etiological agent of the infamous plague has ravaged human populations throughout history. Paleogenetic studies have documented its involvement as an infectious agent in humans for the last 5000 years (22,23). However, three important pandemic waves have forever marked history. Justinian plague was the first chronicled pandemic in the 6th century (year 541 to 767), and was focused around the Mediterranean Sea. The second infamous pandemic 'Black death' occurred in 14th century (year 1346 and through the 18th century), and responsible for killing 50% of the European population of the time. It is suggested to have disseminated from East Asia to Europe via trade routes. The third and 19th century modern pandemic is suggested to have travelled to China which then via infected marine ships in Hong Kong spread globally to India, Africa and the Americas (24-26).

Despite devastating human populations, the primary hosts of *Y. pestis* are wild rodents of various species, which serves as a natural reservoir. Infectious cycles of disease between rodent to rodent and rodent to human are facilitated by blood sucking species specific flea vectors. In this process, one of the most efficient flea vectors is *Xenopsylla cheopis. Y. pestis* has limited ability to survive freely in the environment. Therefore, its ability to exploit vector resources in establishing proliferation and maintaining a cyclic flea-mammal transmission is fundamental for its pathogenicity (16,27,28). Following ingestion of a blood meal from a bacteremic host, the flea vector can deploy bacterial transmission in two different ways. The first mode of transmission is referred to as early phase transmission. In this mode, residual bacteria that contaminate the mouthparts can be transmitted in the subsequent feeding with the highest transmission during the first few days of a previous infected blood meal. The second mode of transmission involves the ability of *Y. pestis* to form biofilm in the proventriculus, the foregut region between oesophagus and midgut. Formation of the biofilm physically impedes ingested blood from reaching the midgut, consequently starving the fleas. This causes the flea to aggressively seek out a new blood meal, and forcing regurgitation of blood containing bacteria in the new flea bite site. Indeed, the blocked flea transmission mode is the dominant paradigm for *Y. pestis* transmission by fleas (16,27,29-31).

Following a flea bite, *Y. pestis* is dislodged into the dermis of mammalian hosts from where it rapidly migrates towards the nearest lymph nodes. Bacteria start to replicate invoking an immune response and tissue damage that produces

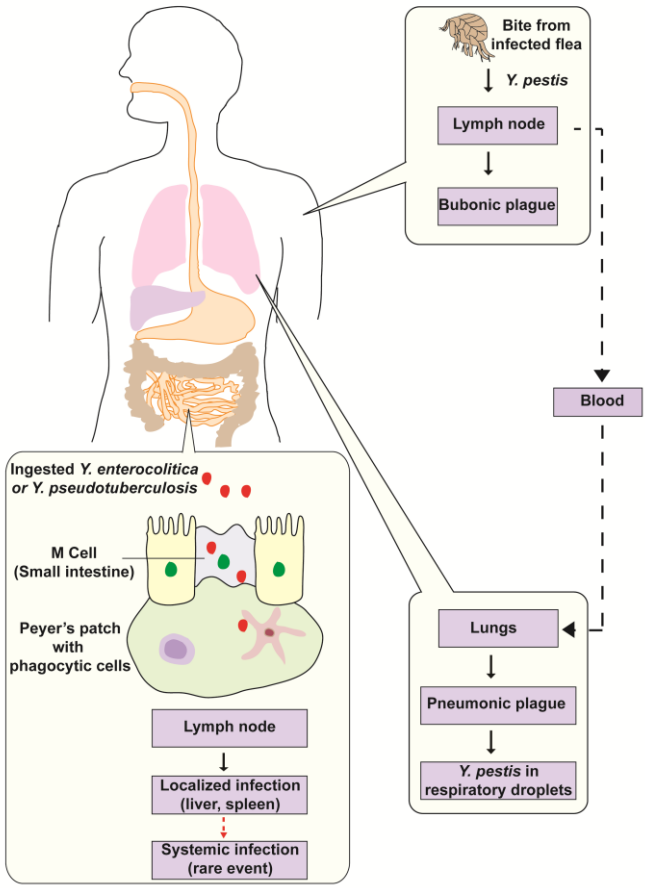

Figure 1. Lifestyles of pathogenic *Yersinia* species. Enteropathogenic *Yersinia* species, *Y. pseudotuberculosis* and *Y. enterocolitica* are commonly found in different environmental habitat like water, soil and plants. Different domesticated and wild animals also serve as reservoir of enteropathogenic *Yersinia*. They are transmitted via fecal-oral route by ingestion of contaminated food and water. Upon ingestion, they cross the intestinal epithelial barrier through M-cells and colonize underlying lymphoid tissues. Rodents of various species serves primary reservoir of *Y. pestis*. Transmission of *Y. pestis* to human relies on infected flea vectors, which carry the bacteria from rodent hosts. Following flea bite, *Y. pestis* is disseminated into lymph nodes to from bubonic plague. In certain occasions, *Y. pestis* can spread to lungs and cause pneumonic plague that mediates transfer of bacteria from person-to-person by respiratory droplets. The figure is inspired from the study by Heroven, A. K. *et al*. (32)

characteristic swelling, termed buboes, and upon which derives the name bubonic plague. Cellular damage within lymph nodes promotes dissemination of bacteria into blood and colonize deeper tissues like liver and spleen to cause septicaemic plague. Alternatively, deeper bites that discharge bacteria directly into the bloodstream can also result into septicemic plague. Eventually, the bacteria can traffic to the lung to establish pneumonic plague. This stage of the disease can facilitate direct aerosolised human-to-human transmission of *Y. pestis* and is responsible for promoting rapid spread underscoring onset of plague epidemics (Figure 1) (27,33,34).

Cumulative consequence of *Y. pestis* pathogenicity, the flea vector and widespread prevalence of wild life rodents reservoir are critical to the development of plague foci. However, other factors like climate, socio-economic condition, and host susceptibility also contribute to the plague landscape. In addition to these attributes, genome plasticity of *Y. pestis* to occupy new ecological niches as well as acquiring resistance to conventional antibiotics used in the clinic, will further make it challenging to eradicate plague (25,35). Plague eradication is further challenged by the lack of a licensed vaccine.

1.4. Pathogenesis mediated by enteropathogenic *Yersiniae*

Y. pseudotuberculosis and *Y. enterocolitica*, also termed enteropathogenic *Yersiniae*, possess a distinct inoculation route and disease outcome compared to *Y. pestis.* This is despite all three being pathogenic to humans, and all encoding some common virulence determinants. Found ubiquitously in nature associated with food, animals and abiotic environments, *Y. enterocolitica* and less frequently *Y. pseudotuberculosis* cause self-limiting gastroenteritis (also termed yersiniosis) transmitted via contaminated food or water (19,20). Upon ingestion, enteropathogenic *Yersinia* pass through the gastrointestinal tract and infect the terminal ileum of the small intestine. Bacteria can penetrate the intestinal epithelial barrier and colonizes the underlying lymphoid tissues, such as Peyer's patches (36). An important bacterial component in this process is invasin, an outer membrane adhesin expressed at lower temperature, such as the refrigeration temperature used for food storage. Invasin binds to β-1integrins of epithelial microfold (M)-cells (37-39). However, Ail and other adhesins are also important in this process (40). The translocation of *Yersinia* in the Peyer's patch induces local chemokine production that induces the recruitment of polymorphonuclear lecukocytes (PMNs) and monocytes to the infection site. Recruitment of these immune cells may lead to tissue disruption. Bacteria respond to this hostile

environment by inducing the expression of YadA, another surface adhesin-like molecule that contributes resistance to phagocytosis and promotes further dissemination into lymph nodes. In a healthy individual, most infections are self-limiting as the immune system can control and eliminate the invaders. However, immunocompetent individuals can develop severe systemic infections and spread to deep tissues like spleen, liver or lungs leading to mortality rate as high as 50% (Figure 1) (20,41). Sub-lethal doses of *Y. pseudotuberculosis* in mice can also disseminate to cecal lymphoid follicles and establish an asymptomatic, persistent infection (42,43). However, in some people, persistent *Yersinia* infections might also lead to development of postinfectious sequelae, such as reactive arthritis (44,45).

Although feared much less than the highly virulent plague-causing *Y. pestis*, enteropathogenic *Yersiniae* induced yersiniosis is still a disease of global burden. Although not routinely monitored, yersiniosis is notifiable in various national databases including the European surveillance system. According to the annual surveillance of 2016, the EU/EAA member countries confirmed 6918 cases of human yersiniosis and was the third most commonly reported zoonosis (European Centre for Disease Prevention and Control, https://ecdc.europa.eu/). In particular, enteropathogenic *Yersinia* is a very relevant biological hazard within the meat processing industry. Effective measures to control *Yersinia* infections are challenged constantly by three important factors; 1) the presence of *Yersiniae* in a wide range of natural reservoirs including soil, water, animals and birds, 2) the ability to thrive at refrigerated temperature of ~4° C, and 3) presence of multidrug resistant *Yersinia* species. Therefore, we must not neglect enteropathogenic *Yersiniae*; they are important pathogens and infection outbreaks need to be comprehensively monitored (19,46,47).

1.5. Tracing the molecular evolution of *Y. pestis*

Phylogenomic analysis of the genus *Yersinia* based on single-nucleotide-polymorphism (SNP) analysis within a set of core genes separate *Y. enterocolitica* and *Y. pseudotuberculosis*-*Y. pestis* clusters to different branches on the *Yersinia* evolutionary tree (11,15). Studies have suggested that *Y. pestis* evolved from its progenitor *Y. pseudotuberculosis* within the last 10,000 years (33). Evolutionary biologists attribute the radical transition from a mild enteropathogen to an intimidating flea-borne pathogen to limited gene acquisition and larger inactivation of ancestral genes. Important gene gain events in *Y. pestis* are the acquisition of two novel plasmids; a ~100 kb pMT1 plasmid that encodes

Yersinia murine toxin (Ymt) and the F1 capsule, along with a ~9.5 kb pCP1 plasmid that encodes plasminogen activator (Pla) (11,14,48,49). Acquisition of Ymt, a phospholipase D, is a significant event in the evolutionary pathway as it enables bacterial survival and reproduction in flea midgut, and this ultimately facilitates flea-mode transmission (28). Gain of the *pla* gene is another landmark event in the pathogenesis of *Y. pestis*. The Pla protein is a plasminogen activator, and has an important role in bacterial dissemination, which promotes systemic infections. *Y. pestis* acquired Pla very early during divergence from *Y. pseudotuberculosis*. This event was sufficient to acquire an ability to survive in the respiratory environment and cause pneumonic plague. Over time, modern strains acquired a different Pla variants defined by unique single amino acid substitution mutations. Such isolates can more efficiently cause invasive infections (33).

Loss of ancestral genes is also critical in shaping the evolution of *Y. pestis* pathogenesis. Compared to its progenitor, the genome of *Y. pestis* lacks almost 10% of coding potential associated with virulence and metabolism (11). Some of the genes lost include those encoding for invasin and YadA. These help *Y. pseudotuberculosis* to efficiently colonize the gastrointestinal tract, and attribute that is no longer required by *Y. pestis*. Moreover, loss-of-function mutations that enhanced cyclic-di-GMP-mediated biofilm formation and survival in the flea gut has consequently helped *Y. pestis* to adapt to flea-borne transmission. The genetic basis for this event was attributable to a specific loss of two phosphodiesterases, PDE2 and PDE3 as well as the RcsA sensor kinase phosphorelay protein, which would normally negatively regulate biofilm formation (16,17,50,51). Importantly, *Y. pestis* has also lost flagella-related motility during its transition to a vector-borne pathogen from a gastrointestinal pathogen (14,52)

While acquisition of novel genetic elements and selective inactivation of common genetic features has been fundamental for *Y. pestis* to adapt to different hosts niches, differential regulation of pre-existing genes is a vital strategy for all *Yersinia* to facilitate niche adaptation. For instance, Rcs phosphorelay signal transduction pathway that involves core components (RcsC, RcsD, RscB) and auxillary components (RcsA, RcsF) acts in response to extracytoplasmic stimulus and regulates important cellular processes such as biofilm formation, T3SS, flagella biosynthesis and motility (18,53). The Rcs system has been shown to positively regulate Ysc-Yop T3SS in *Y. pseudotuberculosis* and chromosomally encoded Ysa-YsP T3SS of *Y. enterocolitica* (54,55). Although *rcs* loci is present in all three pathogenic *Yersinia* species, *Y. pestis* encodes a non-functional *rcsA*

allele and a *rcsD* pseudogene (56,57). Therefore, maintenance of an intact Rcs phosphorelay pathway by enteropathogenic *Yersinia* could have been an adaptation strategy linked to the route of infection. This is further strengthen by the fact that presence of RcsA strongly repress the ability of *Y. pestis* to form flea biofilm (57). Therefore, conversion of *rcsA* to a pseudogene have helped *Y. pestis* to adopt flea-mode of transmission.

1.6. *Y. pseudotuberculosis* – a template for understanding bacterial pathogenesis

Y. pestis has left its trace in history as a notorious plague causing bacteria. Moreover, with the increasing reports of antibiotic resistance problems and its possible use as a biological warfare agent, *Y. pestis* still possess an important threat to humankind. Different animal models involving rats, mice and guinea pigs have been used to study *Y. pestis* pathogenesis and plague development (58). However, attempt to understand plague biology using *Y. pestis* is often limited due to different challenges. One important challenge to consider is the likely degree of high human exposure to the bacteria via direct contact. The high degree of genetic relatedness between *Y. pseudotuberculosis* and *Y. pestis* has implications for the understanding of plague biology. Despite different disease outcome, the two bacteria that cluster in the same evolutionary clade share common virulence properties like tropism for lymphoid tissue, plasmid encoded Ysc-Yop T3SS delivery of effector proteins into host cell cytosol to prevent phagocytic killing, and resistance against complement mediated killing (20,59). In addition, *Y. pseudotuberculosis* can efficiently infect mice and mimic pathophysiology analogous to human infections by *Y. pestis*. Thus, *Y. pseudotuberculosis* can be used as a surrogate model of *Y. pestis.* This minimizes the risk of handling pathogenic plague bacillus and also contributes understanding of plague and its etiological agent.

Although much has been learned about plague biology using mice as a model organism, it is often restrained by economical and ethical considerations. However, *Y. pseudotuberculosis* has offered flexibility in the use of alternative animal models like *Caenorhabditis elegans* that are genetically tractable and easier to handle. Moreover, its anatomical simplicity and transparency provides opportunities for direct visualization of infecting bacteria. A *C. elegans* – *Y. pseudotuberculosis* model is used extensively to study biofilm formation, which is so important for *Y. pestis* to colonize the flea digestive tract (60,61). Although *Y. pseudotuberculosis* does not readily colonize fleas, biofilm formation might be

a significant strategy to survive predator grazing in soil. Moreover, the ability of *Y. pseudotuberculosis* to form a biofilm at the anterior surface of the nematode is dependent on the *hmsHFRS* operon for exopolysaccharide production, and this parallels *Y. pestis* biofilm formation (62,63). Owing to the fact that *C. elegans* is reared at a temperature of ~ 25° C similar to that of flea, the *C. elegans* - *Y. pseudotuberculosis* model can be surrogate to dissect either biofilm-dependent or independent pathogenesis of *Yersinia* at molecular level.

Interestingly, *Y. pseudotuberculosis* is employed as representative bacteria to understand pathogen evolution. As discussed above, three *Yersinia* species are human pathogens and have been studied extensively, while other environmental *Yersinia* species are non-pathogenic to humans. Crucially, representative strains of almost all species of *Yersinia* have been fully sequenced. This allows comprehensive comparative genomic analysis between them. Thus, it has offered an excellent model to study how certain species can emerge from non-pathogenic bacteria to become successful pathogens (11).

Owing to the rich genetic information available in public databases, and in combination with established genetic tools, we have utilized *Y. pseudotuberculosis* as a model bacteria to study bacterial pathogenesis, its evolution and a comprehensive understanding of bacterial-host interactions. This knowledge can be expanded for therapeutic purposes to other bacterial families as many virulence factors are conserved. A noteworthy achievement of *Y. pseudotuberculosis* as a model organism was the identification of T3SS functions, a dedicated virulence mechanism shared by many Gram-negative bacteria including the notable clinical pathogens *Pseudomonas*, *Salmonella*, *Shigella* and *Bordetella*.

1.7. Type III Secretion System – a central player in bacterial pathogenicity

Bacteria have evolved different mechanisms to communicate with their host, the environment or target bacterial cells. One of the specialized mechanisms, known as protein secretion system, involves transport of cytoplasmic protein cargos across the bacterial cell envelope to its destined place. The type III secretion system (T3SS) is arguably one of the best characterized of these protein secretion systems utilized by human pathogens like *Chlamydia* spp., *Pseudomonas aeruginosa*, enteropathogenic *Escherichia coli*, *Salmonella* spp., *Shigella* spp., or *Yersinia* spp., plant pathogens like *Pseudomonas syringae*,

Erwinia spp., or *Xanthomonas* spp., and symbionts like *Rhizobium* spp. Also termed injectisomes, these nanomachines are evolutionary related to the bacterial flagellum. T3SS is a multi-mega Dalton apparatus comprised of more than 20 different structural proteins that forms an envelope-embedded multi-ring basal body, a needle that protrudes from the bacterial cell surface and a translocon-bound needle tip complex. Collectively, this assembled nanomachine mediates the passage of immune-modulatory effector proteins into target host cells directly in one-step or via a two-step process. Although the structure of T3SS is highly conserved across bacterial species, the arsenal of effector proteins it delivers is unique to the pathogen. Upon delivery, the effector proteins modulate host cell functions to the benefit of the bacteria. For instance, this could involve prevention of phagocytosis (*Yersinia*), invasion of nonphagocytic cells (*Salmonella*) or acquisition of nutrients (*Chlamydia*) (59,64-67).

T3SS in human pathogenic *Yersinia* is critical for causing disease (59). Often termed Ysc-Yop T3SS, it is encoded in a common 70 kb virulence plasmid, called pCD1 in *Y. pestis*, pYV in *Y. enterocolitica* and pIB1 in *Y. pseudotuberculosis*. *In vitro* expression of Ysc-Yop T3SS is induced at 37º C under low Ca^{2+} condition and *in vivo* induction is initiated by surface contact with target host cell. Adhesins like invasin, YadA, and Ail mediate docking to the target host cell, and this brings the injectisome in close proximity to the host cell plasma membrane. Upon cell contact, it is assumed that one or moresignals are received by the translocation pore components at the needle tip and transmitted to bacterial cell via the needle to induce T3SS. Eventually, this triggers the delivery of at least six effector proteins, commonly termed 'Yops' into the host cell cytosol. Inside the target host cells, the effector proteins work in concert to rearrange the host cell cytoskeleton, prevent phagocytosis, limit inflammation and eventually promote systemic spread (68-71).

Although the virulence plasmid encoded T3SS is a critical asset of pathogenic *Yersinia*, other attributes also determines the fate of *Yersinia* as a successful pathogen. Infection of the host organism is a multi-factorial event and other virulence determinants can attribute to the magnitude and extent of infection. Indeed, adhesin function is an important prerequisite for T3S dependent translocation of Yop effectors into target host cells. The virulence plasmid encodes a major adhesin YadA. In addition to mediating bacterial docking onto host cells by binding to the extracellular matrix proteins – fibronectin, laminin and collagen, YadA also contributes to serum resistance and bacterial autoagglutination (51,70,72). The fact that a Δ*yadA* null mutant of *Y.*

enterocolitica is avirulent in mouse infection model signifies its importance as a virulence determinant (37). Interestingly, despite these critical functions, YadA is inactive in *Y. pestis* due to a frame-shift mutation (50). Two other important adhesins that promote attachment and invasion into host cells include chromosomally encoded invasin and Ail. While invasin is an inactive pseudogene in *Y. pestis*, Ail is present in all three species of human pathogenic *Yersiniae*. Invasin is a key adhesin in the initial phase of infection. Maximal expression occurs at lower temperatures around~26º C at pH 8 or host body temperature of 37º C at pH 5.5. This could prepare *Yersinia* for infection prior to oral uptake and also promote β-1 integrin mediated transcytosis across the intestinal epithelial layer (50,73). Ail is expressed in all three pathogenic *Yersiniae*. In addition to its host cell binding capacity, Ail also confers bacterial cell invasion and resistance to serum killing. Ail is apparently a dominant adhesin of *Y. pestis* that has been demonstrated to bind to extracellular matrix component fibronectin ensuring efficient T3S delivery of effector proteins into target host cells (41,70,74). In human pathogenic *Yeriniae*, spatio-temporal regulation of adhesin expression is achieved via the concerted action of various important regulators like VirF, YmoA, RovA and HNS (71).

1.7.1. T3SS origin and divergence

The T3SS has a number of proteins homologous to the flagellum indicating an evolutionary relationship between the two systems. Although the flagellum is a dedicated organelle for motility, representative functions analogous to T3SS include the presence of a core secretion apparatus that facilitates export of hook and filament components, the ability of the filament to polymerize, and devoted substrate-chaperone complexes that regulate spatio-temporal assembly. Moreover, various studies have provided evidence of cross talk between the two systems in terms of regulatory overlap and reciprocal exchange of secreted substrates, which is indicative of a common ancestry pathway (75-77). Herein, the term non-flagellar T3SS (NF-T3SS) will be used to distinguish the T3SS injectisome from the flagellar-T3SS (F-T3SS).

The metagenome data explosion has befitted discovery of additional T3SSs in a highly diverse group of bacteria. Distribution of NF-T3SSs has been reported in all four (α, β, γ, δ) classes of the phylum proteobacteria and some sequenced species from the phylum chlamydiae. Although the NF-T3SS was initially described as a devoted mechanism to cause disease in higher organism; it has a broader capacity to maintain a host-bacteria symbiotic interaction (for example,

to form nodules on leguminous plants roots) or in environment survival (for example, to resist protozoan grazing) (77-79). Phylogenetic analysis has indicated acquisition of NF-T3SS to favour a billion year old interaction between the two kingdoms of life, the bacteria and the primordial eukaryotes, and therefore it must have appeared after the first eukaryotes (80). On the other hand, the flagellated bacteria have existed for roughly three billion years before the appearance of the first eukaryotes. It is logical to assume that the need for motility and chemotaxis to find food in the primordial soup precedes the need to communicate with eukaryotes, which would point towards F-T3SS as the ancestor. However, the origin of the first T3SS has been a subject of dispute. Early phylogenetic analysis showed F-T3SS and NF-T3SS evolved independently of each other from a common ancestor (81). However, with the growing number of genomic sequences, it became apparent that genes encoding T3SS are either present in an extrachromosomal plasmid or tightly clustered as a high pathogenicity island in the chromosome with an unusual GC content compared to the bacterial genome (82,83). Moreover, a striking difference exists between 16s rRNA based bacterial phylogeny and NF-T3SS-based phylogenetic relationships implying that NF-T3SSs evolved via genetic transfer events (77,84). Furthermore, NF-T3SSs have limited distribution of Gram-negative bacteria whereas F-T3SSs are widely distributed in Gram-positive and Gram-negative bacteria (85). Taken altogether, a F-T3SS is the likely ancestral system in the evolutionary roadmap of NF-T3SSs. Indeed, a recent evolutionary study argued that the modern NF-T3SS evolved from the flagellum by a series of gene deletions and subsequent acquisition of components from other cellular systems (78). The study argues that the ancestor of the NF-T3SS utilized a common T3SS structural core to primarily export the flagellar proteins and dictate bacterial movement. Over time, the system adapted to export proteins that necessitate bacterial-eukaryotic interactions through two specific events. Firstly, loss of flagellum specific genes and acquisition of injectisome specific genes resulted in a T3SS intermediate, which is still evident in *Myxococcus*. The second key event was the recruitment of a secretin from a different molecular apparatus that allowed contact dependent NF-T3SS. Eventually, this allowed dramatic diversification of the NF-T3SS into eight different families specific to different eukaryotic hosts including animals, plants and protozoa (Figure 2) (Table 1) (77,78,85).

Although exponential increase in available genomic data has revealed fascinating glimpses into T3SS evolution, complementing this line of study are

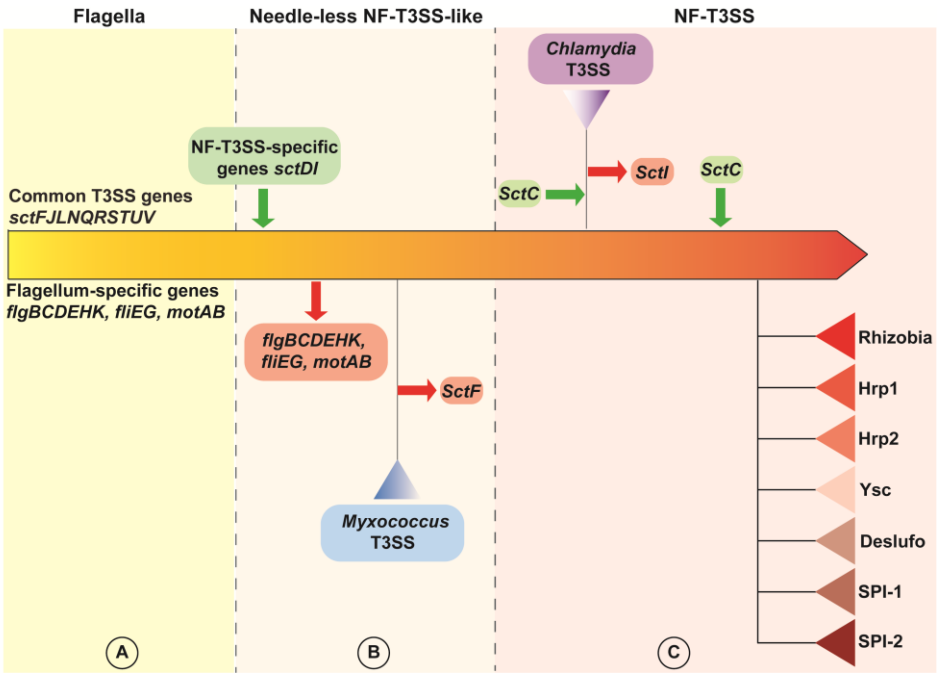

Figure 2. Proposed schematic for the evolution of T3SS. The figure is adapted from study by Abby, S. S. *et al*. (78). Common genes to the NF-T3SS and flagellum existed in the flagellar ancestor (A). A series of loss of the flagellum-specific genes and gain of the NF-T3SS-specific genes *sctD* and *sctI* generated early lineage of needle-less injectisome-like T3SS still evident in *Myxococcus* (B). Acquisition of SctC secretins from multiple cellular machineries permitted the emergence of ancient contact-dependent NF-T3SS (C), which possessed the ability to deliver effector proteins into eukaryotic host cells. Finally, NF-T3SS quickly evolved to adapt to specific host cells giving rise to different T3SS sub-families. In the figure, green arrow defines gene acquisition, red arrow defines gene loss and coloured triangles represents different T3SS sub-families.

in-depth genetic-based structure-function studies of T3SSs.Considering this, I and others have utilised an experimental genetic approach to explore the evolutionary functions of YscX-YscY member proteins from the Ysc T3SS family (Paper I and Paper II). By performing functional interchangeability among member proteins in *Yersinia* background, we have uncovered novel functions that are conserved and/or unique among YscX-YscY member proteins in the Ysc T3SS family. Thus, our studies have added value to the phylogenetically grouped T3SS families.

Table 1 Classification of T3SS families based on phylogenetic analysis

	Species	System	Potential functions
Ysc	Pathogenic *Yersinia* spp.	Ysc	Block phagocytosis
	Pseudomonas aeruginosa	Psc	
	Aeromonas salmonicida	Asc	Reduce pro-inflammatory response
	Aeromonas hydrophila	Asc	
	Vibrio harveyi	Vsc	
	Vibrio parahaemolyticus	Vsc	
	Bordetella pertussis	Bsc	Trigger apoptosis
	Desulfovibrio vulgaris	Dsc	
Inv-Mxi-Spa (SPI-1)	*Salmonella enterica*	SPI-1	Trigger bacterial uptake by non-phagocytic cells
	Shigella flexneri	Inv-Mxi-Spa	
	Yersinia enterocolitica	Ysa	
	Burkholderia pseudomallei	Bsa	
	Yersinia ruckeri	Inv-Mxi-Spa	
	Chromobacterium violaceum	Inv-Mxi-Spa	
Ssa-Esc (SPI-2)	*Y. pestis*	?	Attachment and effacement lesion
	Y. pseudotuberculosis	?	
	E. coli (EHEC)	Esc	
	E. coli (EPEC)	Esc	Intracellular survival
	Salmonella enterica	SPI-2	
	Citrobacter rodentium	Ssa	
	Chromobacterium violaceum	?	
	Erdwardsiella tarda	?	
Hrp1	*Pseudomonas syringae*	Hrp1	Induce hypersensitive response in resistant plants and disease in non-resistant plants
	Erwinia amylovora	Hrp1	
	Vibrio parahaemolyticus	Hrp1	
Hrp2	*Xanthomonas campestris*	Hrp2	
	Burkholderia pseudomallei	?	
	Ralstonia solanacearum	Hrp2	
Rhizobiales	*Rhizobium spp.*	?	Establish symbiotic relationship with leguminous plants
	Mesorhizobium loti	?	
Chlamydiales	*Chlamydia trachomatis*	?	Intracellular survival
	Chlamydophila pneumonia	?	

1.7.2. What is the point of encoding multiple heterogeneous T3SSs?

Many bacteria harbour more than one T3SS often belonging to different phylogenetic clades. Most notable examples of T3SS co-existence is the SPI-1/SPI-2 (for *Salmonella* Pathogenicity Island) T3SSs in *Salmonella enterica*, T3SS-1/T3SS-2 of *Vibrio parahaemolyticus* and Ysc-Yop/Ysa (for *Yersinia*

secretion apparatus) T3SSs in *Y. enterocolitica* (86-88). Carriage of multiple T3SSs begs many important questions; (1) Why do bacteria need heterogeneous T3SSs? (2) How is substrate targeting demarcated between different T3SSs? (3) Is there a regulatory cross talk between the various systems? Although a thorough understanding requires astronomical work, various studies have tried to address these questions. One likely explanation is that the different T3SSs provide selective advantage to bacteria in colonization and transmission of different hosts. For example, *Burkholderia pseudomallei* is the etiological agent of melioidosis, an infectious disease in humans and animals. It encodes three T3SSs designated T3SS-1, T3SS-2 and T3SS-3. The T3SS-3 is a member of the Inv-Mxi-Spa family of T3SS and is required for full virulence in hamsters and mice (89,90). Interestingly, T3SS-1 and T3SS-2 share homology with Hrp2-T3SS family of various plant pathogens and has been shown to be important for infection of tomato plants (91,92). This raises the possibility of *B. pseudomallei* to have evolved over time to survive in alternative hosts, and represents an example of multiple T3SS acquisition for adaption to different ecological niches. Similarly, pathogenic *Yersiniae* encodes both the well-characterized plasmid-encoded Ysc-Yop T3SS, and also contains another chromosome encoded T3SS gene cluster. *Y. pestis* and *Y. pseudotuberculosis* harbour a representative of the Ssa-Esc family of T3SS that is not well-characterized and likely degenerated. On the other hand, *Y. enterocolitica* (biotype 1B) also encodes a chromosomal Ysa-T3SS that belongs to the Inv-Mxi-Spa family, which is expressed at relatively lower temperature of ~26º C. The Ysa-T3SS is implicated in initial intestinal colonization in mice and in infection of *Drosophila* S2 cells, indicating its possible role in allowing *Y. enterocolitica* (biotype 1B) to adapt to interactions with alternative host (86,93,94). Additionally, the presence of different T3SS within the same bacteria could also be important for mediating specific aspects of bacterial pathogenicity within the same host. *V. parahaemolyticus*, a pathogen for human and marine animals, comprises two sets of T3SSs that mediates distinct aspect of pathogenicity. Chromosome 1 encoded T3SS (T3SS-1) evolutionary related to Ysc-T3SS is responsible for cytotoxicity and chromosome 2 encoded T3SS (T3SS-2) evolutionary related to Inv-Mxi-Spa-T3SS mediates intestinal colonization and enterotoxicity in animal models (88,95,96). Another well-studied example is the two SPI T3SSs of *S. enterica*. While SPI-1 T3SS facilitates cell invasion, SPI-2 T3SS enhance intracellular survival and disseminated infection (87,97).

Overall, as NF-T3SSs have evolved in bacterial species that made contact with eukaryotes, heterogeneity in NF-T3SS could be a consequence of diverse niche adaptation. Indeed, a clear distinction exists between different families of T3SS and the nature of eukaryotic hosts (Table 1). Precise NF-T3SS association with eukaryotes could be determined by specificity in substrate recognition and spatial and temporal regulatory control. However, in many cases, the specificity of the interaction can be facilitated by the diversification of T3S proteins. YscX/YscY uniquely present in Ysc T3SS (Paper I) and YopN/TyeA that can exist as two independent proteins in the InvE-family member (Paper IV) could represent examples of diversification to allow NF-T3SS adaptation to specific niches.

1.7.3. Conserved architecture of T3SS

Expression of T3SSs are energetically expensive and comes at a cost to bacterial growth and fitness. Therefore, T3SS expression must be tightly controlled in both time and space. The assembly of T3SS is a hierarchical process that involves build-up of distinct sub-parts that eventually forms a complete and coherent functional structure. Outlined below is a description of the coordinated assembly of the different sub-parts (Figure 5), which is also highlighted in Table 2. A T3SS is a syringe-like structure that spans the bacterial envelope (see section 1.7.3.1.), and also contains a soluble protein complex attached to the cytosolic face of the inner membrane (see section 1.7.3.3.) and an extracellular needle that protrudes from the bacterial surface (see section 1.7.3.4.) that eventually terminates in a needle-tip structure (98-100). Upon contact with target host cell, the apparatus delivers intracellularly in one or two steps a series of species-specific effector proteins (commonly termed 'Yops' in the Ysc-Yop T3SS of human pathogenic *Yersinia*) (101,102). Hierarchical and temporal assembly of T3SS is controlled at multiple check points involving transcriptional, post-transcriptional, translational as well as post-translational mechanisms of control (see section 1.8.3. and section 1.9.3.) (103). This ensures step-wise assembly beginning with the formation of basal body, followed by the cytosolic complex and the deployment of protein components forming the needle complex, which all occurs prior to the deployment of the effector proteins.

As the core structural component of T3SS machinery is conserved among diverse families, this thesis has attempted to use a universal Sct (Secretion and Cellular Translocation) nomenclature to unify different T3SSs. Where no Sct exists for *Yersinia*-specific proteins, the standard Ysc nomenclature is used (Table 2.) (104-106).

Table 2. A summary highlighting the major constituents of T3SS with their proposed functions.

Subunit	Units (Ysc-Yop T3SS)	Unified nomen-clature	Proposed functions
Cytosolic complex	YscK	SctK	Localized beneath the inner membrane ring and the export apparatus.
	YscQ	SctQ	
	YscL	SctL	Facilitates recognition of substrate/chaperone complex and hierarchical secretion of T3S substrates (see section 1.7.3.3.).
	YscN	SctN	
	YscO	SctO	SctN ATPase energizes T3SS (see section 1.9.2.4.).
	YscX	-	YscX-YscY-YscV controls export of early substrates SctI and SctF (see section 1.9.3.1.).
	YscY	-	
Export apparatus	YscR	SctR	Embedded in the inner membrane and forms an entry portal for T3S substrates (see section 1.7.3.2).
	YscS	SctS	
	YscT	SctT	SctU undergoes autocleavage and controls substrate-switching (see section 1.9.3.2.)
	YscU	SctU	
	YscV	SctV	SctV assembles as a nanomeric ring and recognizes chaperone/substrate complex (see section 1.9.2.6.).
Membrane spanning complex	YscC	SctC	SctC forms an outer membrane ring (see section 1.7.3.1.).
	YscD	SctD	YscW pilots YscC for proper targeting and assembly (see section 1.7.3.1.).
	YscJ	SctJ	SctD that forms larger ring in the inner membrane. (see section 1.7.3.1.).
	YscW	-	SctJ, a lipoprotein, forms a smaller ring encapsulated within SctD ring (see section 1.7.3.1.).
Needle complex	YscI	SctI	The inner adapter formed by ~ 6 SctI serves to anchor needle filament (see section 1.7.3.4.1.)
	YscF	SctF	The needle complex composed of > 100 SctF copies serves as a conduit for translocators and effectors secretion (see section 1.7.3.4.).
Translocators	YopD	SctB	Assembles as a hetero-oligomeric complexes at the needle tip.

	YopB	SctE	SctA forms a needle tip complex that scaffolds insertion of hydrophobic translocators SctB and SctE into eukaryotic host cells membranes (see section 1.7.3.4.3).
	LcrV	SctA	
Substrate-switch regulators	YscP	SctP	SctP in association with SctU is involved in needle length control (see section 1.9.3.2.).
	YopN-TyeA	SctW	SctW, commonly known as gatekeeper proteins, are involved in substrate switching from middle to late substrates. In *Yersinia*, YopN-TyeA forms a cytosolic plug that prevents Yop proteins secretion prior to host cell contact (see section 1.9.3.3.2.).

1.7.3.1. The bacterial envelope spanning complex

Assembly of T3SS starts with formation of the membrane-spanning complex, which is a socket-like structure embedded in the bacterial cell envelope. It is composed of SctC, SctD and SctJ (YscC, YscD and YscJ equivalent in the *Yersinia* Ysc-Yop T3SS) and forms a series of membrane spanning rings with a wide base and neck-like region (Figure 3) (107-109). SctC is a member of secretin family also present in type II secretion systems and type IV pilus systems. High-resolution cryo-electron microscopy has demonstrated SctC to form concentric rings stacked in the outer membrane as a massive β-barrel pore composed of 15-16 monomers. The highly conserved C-terminal region is membrane embedded and the more variable N-terminus forms a periplasmic neck. In addition, the SctC C-terminus also harbours a domain that associates with its cognate pilotin, and this interaction mediates proper targeting and assembly of the secretin (110-113). The periplasmic N-terminal domain of SctC couples with the C-terminus of SctD. SctD is an integral inner membrane protein with a single trans-membrane segment, a C-terminus ring forming periplasmic domain and a N-terminal cytoplasmic domain. SctJ is anchored to the inner membrane through a N-terminal lipidation event, while the C-terminus harbours a transmembrane helix. SctD and SctJ exhibit intimate two ring packing with 24 monomer stoichiometry. SctD forms larger outer ring that encapsulates the inner SctJ ring and together they provide a protective environment for the inner membrane export apparatus (SctRSTU) within (109-111,114-116).

1.7.3.2. The export apparatus

The inner membrane export apparatus comprises SctR, SctS, SctT, SctU and SctV (equivalent to YscR, YscS, YscT, YscU and YscV in the *Yersinia* Ysc-Yop T3SS). It is a critical sub-structure of the T3SS basal body incorporated at the centre of the membrane spanning rings (Figure 3). Historically, it was characterized as the integral inner membrane proteins. However, recent electron microscopy structure of the complex revealed that the core components did not adopt typical integral inner membrane topology (117,118). Among the SctRSTUV cohort, SctR, SctS, and SctT consist of transmembrane helices with substantial periplasmic parts. A recent cryo-electron microscopy study of SctU from F-T3SS showed its transmembrane helices wrap around the base of SctRST (119). On the other hand, not much is known regarding the localization and arrangement of transmembrane helices of SctV. However, both SctU and SctV contain large cytoplasmic domains involved in substrate switch control and T3SS-mediated protein secretion (see section 1.9.3.2. and section 1.9.2.6) (120-122). SctRST that forms the core of an export gate adopts a helical structure with a likely 5:4:1 stoichiometry and traverses the inner membrane to localize largely in the bacterial periplasm (114,117,123,124). SctRST represents a strategically important structure of the basal body, facilitating numerous inter- and intra-molecular interactions with different T3S components of the membrane rings (SctC, SctJ) or the inner adapter (SctI). Indeed, it the helical topology of SctRST could be important for nucleating subsequent helical arrangement of the inner adapter and needle filament (114,125,126).

SctU, an essential component of the inner membrane export apparatus, consist of four predicted transmembrane domain and a cytosolic C-terminal domain that contains a conserved NPTH domain. Autocleavage within the NPTH domain is important for substrate switching to transition from early substrates that make the inner rod and the needle to later substrates required for translocon assembly (see section 1.9.3.2) (127-130). The other central player, SctV, constitutes eight predicted transmembrane helices integrated into the inner membrane, a large cytosolic C-terminal domain ($SctV_C$) that assembles into a nanomeric ring, and a relatively small linker domain that tethers SctVc to the inner membrane (121,131-133) . Studies have highlighted a role for SctVc in recognizing T3S substrates for temporal secretion. This is discussed in detail below (see section 1.9.2.6.).

Altogether, the inner membrane export apparatus represents a focal point to couple the inner and outer membrane T3S components into a coherent structure

that spans the entire bacterial envelope. Several direct associations as revealed in various T3SSs is best evidenced of this (114,125,134). First, SctR with both the SctC secretin and the SctI inner adapter. Second, SctS with the SctD component of the inner membrane ring. Third, SctV with the SctJ component of the inner membrane ring.

1.7.3.3. The cytosolic complex

Located at the peripheral cytoplasmic face of the T3S basal body is a set of proteins that forms a large mutimeric structure termed the cytosolic complex. The cytosolic complex of the NF-T3SS is composed of five conserved proteins; SctK, SctL, SctN, SctO and SctQ (equivalent to YscK, YscL, YscN, YscO and YscQ in the *Yersinia* Ysc-Yop T3SS) (Figure 3). Since this cytosolic complex selects T3S substrates for secretion in a hierarchical order, it is also referred to as the sorting platform. In addition, the complex is also involved in signal transduction and overall assembly of the T3SS (66,135-137). Although member proteins of the cytosolic complex from NF-T3SS share homology with the F-T3SS constituents, the functional and topological appearance that they portray can be different. For instance, FliG and FliM/FliN (SctQ equivalent in NF-T3SS) involved in torque generation and rotational switching form a robust C-ring structure in flagella (138-140). In contrast, recent *in situ* cryo-electron tomography (cryo-ET) studies of *Salmonella* and *Shigella* injectisome have revealed the cytosolic complex to exhibit a distinct cage-like structure enclosed by six peripheral pods (made up of SctK and SctQ) that emerge from the basal body and eventually converge into a central six-spoke wheel-like structure (formed by SctN, SctO and SctL). The cytosolic complex couples to the injectisome base via SctK and and the cytoplasmic SctD domain (Figure 3) (100,141,142). A pool of untethered and stable complexes also exist free in the cytoplasm. These components can undergo dynamic exchange with the assembled complex already tethered to the basal body (143-147).

SctQ, one of the core component of the cytoplasmic complex, and conserved among all known injectisome, is the homologue of flagellar C-ring proteins FliM and FliN involved in flagellar rotation (139,143). Although injectisome rotation is a subject of debate, SctQ governs coordinated export of T3S export suggesting divergent functional adaption of the F-T3SS (135). SctQ is encoded from a single reading frame in most injectisome systems. However, it is synthesized as two separate polypeptides, $SctQ_{Full}$ (homologous to FliM) and $SctQ_{Small}$ (homologous to FliN) because of an internal translation initiation site (Preliminary data, Paper

III). Both the variants are important for proper assembly of the cytosolic complex and thereby is central for a fully efficient NF-T3SS (143,148-150).

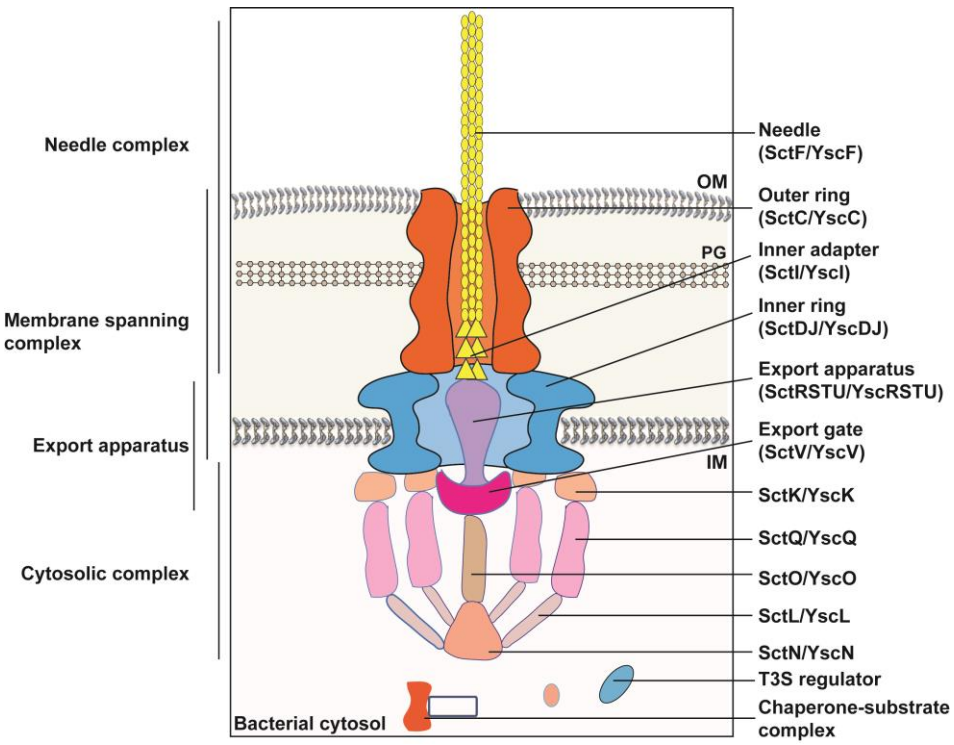

Figure 3. Structural overview of NF-T3SS. The cartoon structure of T3SS depicts four major sub-structures of needle complex, membrane complex, export apparatus and cytosolic complex with their relative localization in inner membrane (IM), peptidoglycan layer (PG) and outer membrane (OM). The needle complex that protrudes out into the extracellular environment from the bacterial cell surface is composed of more than 100 copies of SctF and ~ 6 copies of SctI. The membrane complex consists of protein stacks that traverse the bacterial outer and inner membrane. It comprises of an outer membrane ring (SctC) and inner membrane ring (SctD, SctJ) with a likely stoichiometry of 15:24:24. Embedded in the inner membrane are five highly conserved proteins (SctR, SctS, SctT, SctU, SctV) that serves as an entry portal for T3S substrates. While SctRSTU organize with a likely 5:4:1:1 stoichiometry, SctV assembles into a nanomeric ring. The cytosolic complex composed of SctK, SctQ, SctL, SctN and SctO is located beneath the inner membrane rings and the export apparatus. It is arranged as a six-fold rotational symmetry based on cryo-electron tomography; however, precise stoichiometry of the cytosolic components is not known. In the figure, the different components of T3SS are color-coded and labelled as unified nomenclature proposed by Hueck, C. (104) and standard nomenclature of the Ysc-Yop T3SS.

SctN exhibits structural conservation with the β-subunit of F_0F_1 ATPase suggesting it to be an ATPase that energizes the T3SS (151,152). Although it is not the only source of energy (see section 1.9.2.4.), SctN activity enhances substrate secretion efficiency byT3SSs (153). Assembled as a hexameric ring below the SctV export gate in the injectisome, SctN binds chaperone-substrate complex that in an ATP dependent fashion uncouples the substrates and prepares them for export (100,141,154,155). This is important as the catalytically inactive SctN mutant is severely compromised in T3SS (156).

SctO is important for T3SS but the basis of its significance is not clear. Although it represents a structural component of the cytosolic complex, it is also secreted to the extracellular milieu, and thereby represents an early secreted substrate (157,158). Based on the interactions with free unbound T3S chaperones, SctO and its flagellar orthologue have been suggested to recycle the chaperones for subsequent delivery to their cognate substrates. Recycling of chaperones could be favoured in association with the SctN ATPase activity (159,160). Interestingly, the topological resemblance of SctO as a stalk that interacts directly with SctN and the SctV export gate has led to the possible role of SctO in recruiting the cytosolic complex to the basal body (100,161).

SctK is the least studied component of the cytoplasmic complex and does not have a clear homologue in the F-T3SS. In the *in situ* cryo-ET injectisome studies, it represents the most proximal structure of the cytosolic complex that accommodates with the cytoplasmic domain of SctD. T3SS is abrogated in a Δ*sctK* null mutant, which could arise from its need to assemble a functional cytosolic complex, for instance, by direct interaction with SctQ and SctL. However, the precise mechanism of SctK mediated T3SS biogenesis is yet to be elucidated (142,161,162).

SctL, another critical component of the cytosolic complex, has been demonstrated to bind to SctN and repress its ATPase activity (151). In addition, SctL also interacts with the peripheral structure SctQ that could be important for the recruitment of SctN ATPase complex (142,144). This is in line with assembly of the *Salmonella* SPI-1 T3SS where recruitment of SctK, SctQ, SctL precedes the assembly of SctN-SctO complex (145).

In addition to these core cytosolic components that are conserved between different families of T3SS, localization and functional study of *Yersinia*-specific YscX reveals T3S behaviour that is suggestive of an additional cytosolic complex

component in the Ysc-Yop T3SS (Paper III). Furthermore, this could be a conserved feature in other bacteria harbouring a version of the Ysc family of T3SSs (Paper I).

1.7.3.4. The needle complex

The transition from basal body assembly to needle complex assembly requires extensive conformational change to allow export of T3S substrates. However, exactly what these changes are is still a focus of intensive study. In paper III, I and others have made an effort to investigate the role of YscX and YscY in T3S substrate recognition and secretion following the basal body assembly. The first T3S proteins exported are necessary for needle assembly and are categorized as 'early substrates'. These represents the SctI inner adapter protein, SctF needle protein and SctP needle-length control protein (66,163-165). In most T3SS, these early substrates do not have known cognate chaperones for their efficient delivery. However in the Ysc-T3SS family, SctF has two dedicated chaperones (YscE and YscG in *Yersinia*, PscE and PscG in *Pseudomonas*, AscE and AscG in *Aeromonas*) required for maintaining a stable cytoplasmic pool of monomeric SctF and facilitating its secretion (166-168). Within this specific Ysc-Yop T3SS family, additional proteins like YscX and YscY are also required optimal expression and assembly of YscI and YscF (Paper III) (169,170).

1.7.3.4.1. The inner adapter

The SctI inner adapter is represented by a short filament within the basal body scaffold in both F-T3SS and NF-T3SS (Figure 3). In F-T3SS, the flagellar rod is assembled from four to five different T3S exported substrates, however it does not have a true homologue of SctI (85). The inner rod in NF-T3SS is assembled from around 6 copies of SctI that docks above the SctRST core complex via direct interaction with SctR and SctT (114,171). Recent cryo-EM structural analysis identified a helical fold of 6 SctI monomers assembled over a SctR-SctT hexamer, while at the same time interacting with SctF that protrudes from the top. The hexameric and helical arrangement of components within the export apparatus served as a template for modelled assembly of the inner adapter and needle filament (111,114,171,172). Overall, assembly of SctI is tailored to serve as an 'adapter' that firmly anchors the base structure to the needle filament (114,173). In line with this, Paper III in addition to a recent study by Wagner and co-authors (171), showed that assembly of the inner adapter must precede assembly of the needle filament.

1.7.3.4.2. The needle filament

The needle filament that protrudes extracellularly from the bacterial cell surface anchors to the core export apparatus via the inner adapter structure (Figure 3). It is formed by helical assembly of more than 100 copies of SctF to produce a filament with an inner diameter of around 20 Å that provides a conduit to permit passage of folded substrates (101,174-176). A similar helical arrangement is observed also for the extracellular flagella filament, although flagella filament subunits exhibit only weak similarity to SctF (85,175). High resolution structures of needle complexes from different injectisomes has shown that the N-terminus of SctF is pointed outwards to decorate the needle surface, while the highly conserved C-terminus that is mostly polar in nature is faced inwards towards the lumen (174,175). Some have speculated that electrostatic repulsion between the T3S substrate and the internal surface of the lumen could facilitate passage of folded substrates (177). Indeed, electron microscopy images of a substrate-trapped injectisome has provided empirical evidence of the needle as a physical conduit to permit substrate export (101).

The needle assembles at the distal end of the inner adapter and polymerizes its subunit at the top of the growing needle (167). Subsequent polymerization of the needle induces structural rearrangement in the basal body that leads to opening of the secretin gate (111,114). A similar mechanism of filament assembly from the tip in F-T3SS imposes the need for a distal filament cap protein (178,179). Although no direct homologue of the cap protein is present in NF-T3SSs, a structurally analagous cap protein OrgC is evident in SPI-1 that promotes efficient needle assembly (180). The needle finally merges into a tip complex formed by a hydrophilic translocator protein, such as SctA (LcrV in *Yersinia*), which is thought to perform roles in host cell sensing and translocon pore assembly (see section 1.7.3.4.3.) (68,181-184). These hydrophilic translocator proteins are termed ‘middle substrates’. In light of this, the needle could serve as a mechanical device to transduce signals back from host cell cytosol to the bacterial cytoplasm in order to reprogram T3SS (177).

1.7.3.4.3. The translocon pore

The final step in the assembly of T3SS is formation of the translocon pore that permeates host cell membrane (Figure 5G) (185). The translocon pore, a hetero-oligomeric complex of hydrophobic translocators SctB and SctE (equivalent of YopD and YopB in the *Yersinia* Ysc-Yop T3SS) are also termed ‘middle

substrates' that assembles following deployment of SctA complex (186,187). The assembly and the pore forming activity of translocator proteins can be studied by bacterial infections of sheep erythrocytes followed by measurement of haemolytic activity (188,189). By utilizing this approach, Montagner, C. *et al*. purified heteropolymeric translocator complex of 500 to 700 kDa from membranes of *Yersinia* infected sheep erythrocytes and estimated a stoichiometry of approximately 2.4 YopD proteins per YopB (187).

Biogenesis, function and regulation of translocon pore is a complex process that is not fully understood. Although the hydrophobic translocators alone could form pores in membranes of lipid vesicles in *P. aeruginosa in vitro* (186), both of them were required by the bacteria for efficient haemolytic activity (190). Indeed in *Shigella*, it has been proposed that insertion of one of the translocators SctE facilitates recruitment of the second translocator SctB, which results in formation of the translocon pore and subsequent effector secretion (175,191). Several triggering signals have been proposed that induces transition to the next stage of T3SS effector secretion. In *Shigella*, it has been demonstrated that interaction of SctB with host intermediate filament induces conformational change of the translocon pore that is important for stable docking of the T3SS needle. The host cell signal is then transduced to the T3SS base to trigger secretion of effectors (192,193).

Each bacteria secrete effectors unique in functions and numbers that closely reflect their need to form symbiotic or pathogenic interactions with the eukaryotic hosts. The Ysc-Yop T3SS of *Yersinia* injects 6 effector proteins commonly referred to as 'Yops' (194,195). While inert inside the bacteria cell, Yop effectors are highly potent and target wide range of signalling pathways inside host cell. YopE, YopH, YpkA and YopT are early effectors that thwart host response by altering actin cytoskeletal structure and inhibit phagocytosis (195,196). The second set of effectors YopM and YopJ might be translocated later and target immune cells to downregulate immune responses (194). Overall, Yop effectors modulate host cell function that is beneficial for *Yersinia* survival inside its host.

1.8. Sensing the signals – a mechanism to govern T3SS

The promiscuous expression of T3SS genes and the wasteful delivery of effector proteins is energetically expensive. This can cost bacterial fitness in terms of growth and survival. Therefore, bacteria have evolved sophisticated mechanisms to maintain spatial and temporal control of T3SS. One mechanism

is the ability to sense specific environmental and host signals. Signal sensing and integration allows the bacteria to dictate when and where T3SS expression occurs, and this allows bacteria to efficiently survive in the environment or colonize a host.

1.8.1. Temperature

A prerequisite for pathogenic *Yersiniae* to succeed in host colonization is the ability to sense and respond to temperature (18,64). Upon encountering the mammalian host temperature of 37º C, the bacteria interpret this stimulus to regulate the expression of different virulence factors, including activation of T3SS genes (197). The virulence plasmid borne LcrF, an AraC-type transcriptional activator, serves as a master regulator of T3SS genes. Several studies have shown that the C-terminal helix-turn-helix motif of LcrF binds directly to the promoter regions of the *yopE*, *yopH* genes, the *yopBD-lcrGVH* and *virC* (*yscA* to *yscL*) operons that encode T3SS structural, regulatory and effector proteins, as well as the YadA adhesin (198).

The *lcrF* gene is encoded in a bicistronic operon with *yscW*. Transcription of *lcrF* is controlled from a promoter sequence upstream of *yscW-lcrF* operon by the thermo-labile transcription factor YmoA (198,199). When bacteria is at a lower temperature of ~25º C outside the mammalian host, expression of lcrF is repressed at both transcriptional and translational level. At the transcriptional level, YmoA homodimers and/or YmoA-HNS heterodimers interact specifically with sequences downstream of the *yscW* promoter and repress the *yscW-lcrF* transcription (197,200-202) (Figure 4). This leads to diminished *lcrF* mRNA output. Moreover, translation of any *lcrF* mRNA transcripts is impeded due to the presence of a thermo-labile stem loop structure in the 5'-untranslated region that masks the Shine-Dalgarno sequences from ribosomal access (203). However, at higher temperature such as encountered in mammalian hosts, DNA topology of the promoter region in *lcrF-yscW* operon undergoes a conformational change (202,204). Consequently, this prevents YmoA binding and free YmoA is rapidly degraded by both ClpP and Lon proteases (205). Subsequently, RNA polymerase gain access to the l*crF* promoter and enhance its transcription. Moreover, elevated temperature also melts the stem loop structure of *lcrF* mRNA allowing access to ribosome and efficient translation (205). Accumulated LcrF then binds to the promoter region of various *ysc* and y*op* genes leading to their activation that defines T3SS assembly (198). In this way, concerted actions of YmoA and RNA

thermometer recognize the temperature sensing mechanism to regulate LcrF mediated T3SS expression.

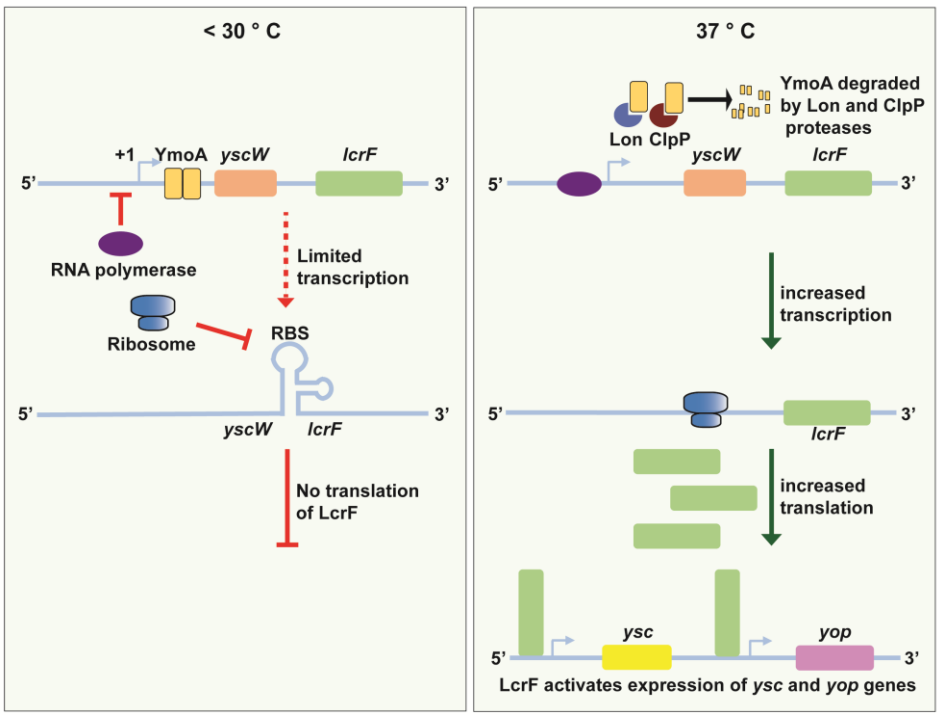

Figure 4. Thermoregulation of LcrF mediated Ysc-Yop T3SS control. At moderate temperature (< 30 ° C), transcription of the *yscW-lcrF* operon is inhibited by binding of YmoA downstream of the *yscW* promoter. Moreover, post-transcription of *lcrF* mRNA is inhibited due to the stem-loop formation in the 5'-untranslated region of l*crF* mRNA, which mask the Shine-Dalgarno sequence from access to ribosomes. At elevated temperature, promoter region of *yscW-lcrF* operon undergoes a topological change that diminishes YmoA binding, which is then rapidly degraded by ClpP and Lon proteases. Moreover, elevated temperature melts the stem-loop structure of *lcrF* transcripts, allowing enhanced translation. LcrF binds to promoter region of many *ysc* and *yop* genes to activate their expression. The figure is adapted from Chen, S. *et al*. (18).

However, several studies have proposed different mechanisms that antagonizes LcrF activity. Recently, Li *et al*. identified LcrQ to diminish LcrF activity although the authors could not detect any direct LcrQ-LcrF association (206). Likewise, YopD translocator protein was also suggested to repress LcrF synthesis (207). Although different mechanisms have been proposed, one the the YopD mediated repression mechanisms of LcrF is achieved by influencing the

components of Csr systems and bacterial degradosome components, PNPase and RNase E (208).

1.8.2. Calcium

Yersinia species carrying a functional Ysc-Yop T3SS require millimolar amounts of calcium ions to grow at typical host temperature of 37º C, but not at lower temperature of ~27º C encountered in the environment or during flea colonization (209-211). This phenomenon was termed low-calcium response 'Lcr'. The calcium growth dependency of *Y. pestis* was associated with virulence in mouse infection model (211). Indeed, the coupling of calcium dependency and virulence is linked to the presence of the virulence plasmid that encodes Ysc-Yop T3SS. Bacteria growth *in vitro* at 37º C in laboratory medium chelated of Ca^{2+} ions induces Ysc-Yop T3SS to release massive amounts of effector proteins into the culture supernatant. (212,213). The need of Ca^{2+} for growth at 37° C can be explained by two reasons. Firstly, *Yersinia* sequester most of its protein translation energy for synthesizing T3SS proteins in the absence of Ca^{2+} that ultimately leads to growth cessation (214). Secondly, Ca^{2+} is needed to minimize the toxic effect of Na^{+} that is accumulated during upregulation of T3SS (215).

Although the temperature dependent calcium need for *Yersiniae* growth is not fully understood, it has been instrumental in identification and characterization of many T3S genes. In general, mutations in the virulence plasmid encoded T3S genes lead to three distinct growth phenotypes. The first set of mutants are calcium-dependent (CD) isolates that maintains growth at 37º C in presence of calcium (Paper III) (216-218). The second set of mutations that permit bacterial growth at 37º C irrespective of calcium availability are called calcium-independent (CI) phenotype. This occurs under conditions that do not activate T3SS and therefore constitutively repress Yops expression and secretion (219). Alternatively, mutations that derail the activity of positive regulators, like LcrF, can also present with this phenotype. Finally, a third set of mutations generate a phenotype referred to as temperature-sensitive (TS). These are the T3S mutants of negative regulators like LcrQ, YopD or LcrH that constitutively express T3SS. Consequently, the mutants display growth cessation at 37º C independent of the presence or absence of calcium (Paper IV) (220-223). It has long been considered that activation of T3SS at 37º C in the absence of calcium mimics host cell contact. For example, some suggest that the T3SS can sense the low calcium environment of the host cell cytosol (224,225). Yet, others consider it is highly likely that the effects of Ca^{2+} are an *in vitro* artefact (226,227). Nevertheless,

whether Lcr is a true manifestation of *Yersinia* contact with host cell needs further investigation. However, calcium-dependent T3SS activation has been an excellent tool to identify and study the regulatory components of Ysc-Yop T3SS *in vitro*.

1.8.3. Post-transcriptional control of the Ysc-Yop T3SS – an overview

The Ysc-Yop T3SS in *Yersinia* is subject to negative feedback control, which ensures Yops expression only when the T3SS is functional. This is believed to occur at the post-transcriptional level and is facilitated by a concerted action of three main players; YopD, LcrH and LcrQ (YscM1 and YscM2 in *Y. enterocolitica*) (223,228,229). The translocator YopD in complex with its T3S chaperone LcrH binds to AU-rich sequence elements of 5'-untranslated region (UTR) of *yops* mRNAs (207,223). The binding to target mRNAs prevents access of ribosome to initiate translation and consequently the untranslated mRNAs are subject to degradation by cellular RNases (208,230). LcrQ is another critical anti-activator whose absence allows constitutive expression of Yop proteins at 37º C (231,232). Based on the observation that overexpression of LcrQ in Δ*lcrH* or Δ*yopD* is not sufficient to restore derepression of Yops production, LcrQ has been proposed to repress target mRNAs expression in complex with LcrH and YopD (233). This is also supported by the fact that YscM1 together with LcrH-YopD binds to the ribosome to block translation of the *yop* genes (234). When T3SS is induced with *Yersinia*-host cell contact or calcium depletion *in vitro*, the SycH T3S chaperone binds to LcrQ and guides it for secretion with dedicated cooperation from the inner membrane export component YscV (235-237). This triggers dissociation of LcrH-YopD complex from 5'-UTR of *yop* mRNAs allowing access for ribosomes to initiate translation. Finally, LcrH escorts YopD for secretion via an active T3SS.

This is the current accepted regulatory model. However, other key observations indicate further complexity. For example, although LcrH-YopD binding is necessary, it might not represent an exclusive mechanism to control Yops expression. This stems from the study in which LcrH in association with YscY provides an additional regulatory layer of Yops expression control that is independent of YopD (223). How exactly YscY binding to to LcrH facilitates regulatory control of Ysc-Yop T3SS is not known (223,238,239). However, mapping the YscY-LcrH binding sites and analysing the significance of interacting amino acids in T3SS activity by site-directed mutagenesis can shed some light on the regulatory aspect of YscY-LcrH complex. In addition, LcrQ

appears to repress the transcription of T3SS genes by inhibiting the role of the transcriptional activator, LcrF (206). The mechanisms for this activity is unclear as a direct LcrQ-LcrF interaction has not been described, and LcrQ does not appear able to compete with LcrF for binding to target DNA binding sites within *ysc* and *yop* regulatory promoter regions.

1.9. Orchestrating an ordered biogenesis of T3SS

Proteins that constitute the Ysc-Yop T3SS can be broadly categorized into four distinct groups: the structural components, the effector proteins, cytoplasmic chaperones and the system regulators. All the proteins work together in a highly coordinated manner to assemble a coherent nano-machine that spans three cellular membranes, bacterial inner membrane, bacterial outer membrane and eukaryotic host cell membrane (105,240). Biogenesis of the T3SS is triggered by specific signals (37º C, -Ca^{2+}) or by bacterial contact with a host eukaryotic cell (see section 1.8.1. and section 1.8.2.) (239). However, in the absence of the triggering signals, the amount of intrabacterial Yop proteins is strictly controlled by feedback inhibition (see section 1.8.3.). Progression of T3SS assembly occurs in a hierarchical fashion to ensure first the deployment of needle complex proteins (early stage), followed by the deployment of translocators (middle stage) and then finally the delivery of effector proteins inside host cell (late stage) (164). While the core structural components are conserved across different T3SS families, the effector proteins and system regulators can be species-specific (77). One example of protein components present specifically in Ysc T3SS family are YscX (Paper I to III) and YscY (Paper I). While the above sections have provided an overview of the evolutionary origin and general architecture of T3SSs, the coming chapters will discuss the molecular mechanisms that control temporal and spatial assembly of T3SSs.

1.9.1. A snapshot of step-wise assembly of the Ysc-Yop T3SS

In *Yersinia*, Ysc-Yop T3SS assembly of the basal body occurs in an 'outside-in' fashion. This means that it nucleates with the assembly of SctC secretin in outer membrane guided by YscW lipoprotein and then assembly progresses inward (Figure 5A) (113,161). Assembly of SctC secretin is followed by recruitment of SctD in the inner membrane. This serves as a template for the biogenesis of SctJ oligomeric inner ring, which altogether form a precursor to project assembly of the remaining T3SS components (Figure 5B) (161). This contrasts with 'inside-out' model proposed for *Salmonella* SPI-1 T3SS or in F-

T3SS. In these examples, assembly initiates at the inner membrane exports apparatus and the inner membrane ring and progresses outward (105,241).

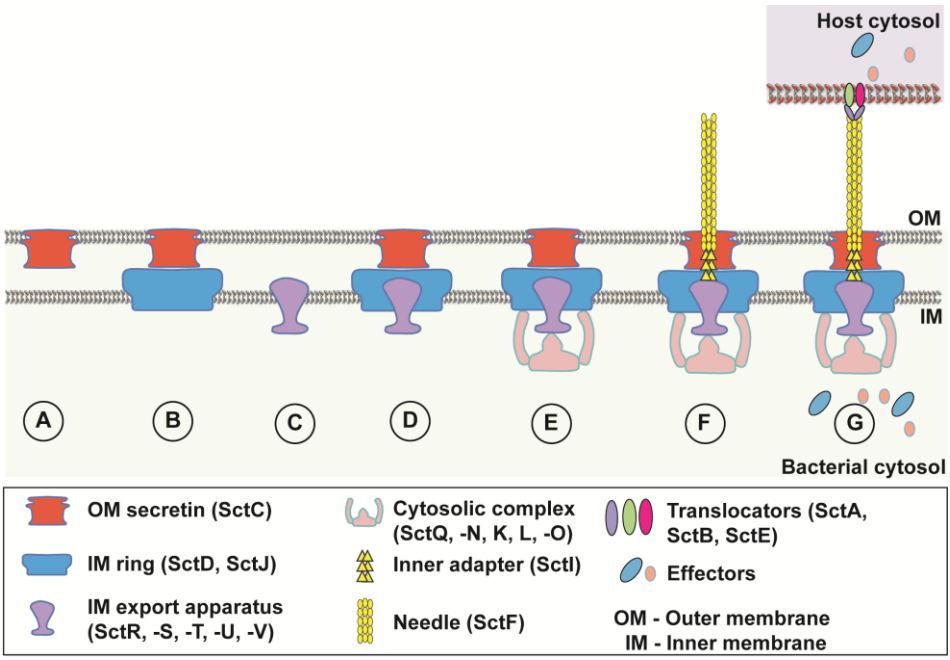

Figure 5. Schematic diagram illustrating a step-wise assembly of the Ysc-Yop T3SS. A unified 'Sct' nomenclature has been used for different T3SS components. Assembly of the Ysc-Yop T3SS assembly occurs in an 'outside-in' fashion that nucleates with assembly of the 'SctC' secretin (A). This is followed by the formation of the SctD and SctJ inner membrane ring. In the Ysc-Yop T3SS, SctD bridges with outer membrane secretin and scaffolds SctJ assembly (B). The inner membrane export apparatus assembles independently and requires the presence of SctR, SctS and SctU for the assembly of SctV (C). SctV ultimately merges in the assembly pathway via attachment to SctJ (D). This leads to subsequent of the cytosolic complex (E). The system is then competent for export of 'early substrates' that makes the inner adapter and the needle filament (F), followed by 'middle' translocators and 'late' effectors (G).

In *Yersinia* Ysc-Yop T3SS, assembly of the inner membrane export apparatus occurs independently of other T3S components (Figure 5C) (134). SctRST mediate the recruitment and assembly of SctV oligomerization. The structure eventually merges with the membrane spanning rings through SctV-SctJ association. Interestingly, SctU is not required for the assembly of SctV or its interaction with SctJ (134). However, most knowledge of the step-wise assembly of the inner membrane export apparatus is derived from detailed studies of

Salmonella SPI-1 T3SS and F-T3SS. In these cases, assembly of the export apparatus nucleates from five SctR monomers, followed by subsequent recruitment of one SctT monomer and four SctS monomers. Together, this complex represents the core complex unit. SctU then incorporates into the core complex, followed by SctV, which eventually completes the export apparatus (Figure 5D) (117,125).

The cytosolic complex (see section 1.7.3.3) is a highly dynamic structure whose step-wise assembly is not completely understood (143,144,146,147). Based on the study by Diepold *et al*. (161), its assembly in *Yersinia* T3SS occurs after the assembly of the outer and inner membrane rings, but is independent of inner membrane export apparatus assembly (Figure 4E). Based on the fact that SctQ and SctN assembly required the presence of each other and additional components, the authors suggested the cytosolic complex to be a single complete structure requiring all components to assemble (161). However, studies from *Salmonella* T3SS suggest otherwise. Cryo-electron tomography revealed visible cytosolic densities in Δ*sctN* and Δ*sctO* mutants while it was absent in mutants devoid of SctK, SctL and SctQ (100). This indicated that SctK, SctL and SctQ represent core components while SctN and SctO are accessory in the cytosolic complex assembly. In support to this, another independent study revealed that SctQ is needed for stable expression of SctK and SctL indicating it to play central role in coordinating the cytosolic complex assembly (149).

Following the assembly of the basal body, the inner membrane export apparatus and the cytosolic complex, the apparatus is competent to permit the passage of T3S substrates, firstly the export of needle filament components followed by distal pore forming translocators (Figure 4F and 4G). However, in specific Ysc-Yop T3SS, the export of early substrates requires the presence of additional components like YscX and YscY that are unique to Ysc-T3SS family (Paper III) (67,157,169,242).

1.9.2. Temporal targeting and recognition of T3S substrates

The coordinated switch from one stage to the next stage of T3SS assembly (see section 1.9.1.) must be monitored by different regulatory mechanisms. This is to ensure that proteins with specific functions are secreted in a strict hierarchical order. Recently, there have been many developments in our understanding of the regulatory proteins involved in an ordered build-up of a functional T3SS. Here, we discuss some of them that involves targeting of the

T3S substrates (see section 1.9.2.1. and section 1.9.2.3.), specific recognition of the substrate cargo (see section 1.9.2.4. and section 1.9.2.6.) and the mechanism for substrate specificity switching (see section 1.9.3.).

1.9.2.1. The role of N-terminus as a type III secretion signals

Bacteria have optimised numerous mechanisms to ensure spatial and temporal transport from the cytoplasm of the various subsets of protein cargo. This orchestrate control is necessary because the exported proteins perform multiple key functions essential for bacterial fitness. Broadly, protein cargo destined for export harbour a canonical secretion signal either at their N- or C-terminus. Two well-known machineries that export into or across the inner membrane proteins with distinctive N-terminal secretion signals are the twin-arginine translocation (Tat) machinery and the Sec secretion system, respectively. The Sec system recognizes a well-defined signal sequence comprised largely of a positively charged amino terminal, a central hydrophobic core followed by a polar carboxyl terminus, the Tat system recognizes a similar secretion signal that is marked with a twin-arginine motif (243-245). On the other hand, different secretion systems that export into or through the outer membrane, including T3SSs, recognise proteins that do not have so well-defined consensus secretion sequences.

A signal that guides proteins to the T3SS resides at the N-terminus (246-250). This conclusion stemmed initially from reporter fusion assays. Appending the first 15 or 17 amino acids residues of YopE or YopH, respectively, to signalless adenylate cyclase mediated T3S-dependent secretion of the fusion protein (251,252). Additionally, fusion of the first 15 amino acids of YopE and YopQ to a neomycin phosphotransferase (Npt) reporter was sufficient to promote secretion of the reporter protein in a T3SS-dependent manner (253,254). These results implied that the N-terminus contains a genuine secretion signal necessary for T3SS recognition and transport. Significantly, in the studies involving Npt fusions, frameshift mutations that altered the reading frame of the secretion signal while essentially keeping the mRNA sequence unchanged did not impair secretion This was unexpected, and led to a novel hypothesis that the T3S signal of the secreted protein is actually encoded in the 5’ end of mRNA sequence (253,254). The mRNA secretion signal hypothesis gained further attraction following observations that synonymous mutation of *yopQ* mRNA encompassing first 10 codons or a single codon 3 in *yopE* secretion signal without altering the native peptide sequence abrogated secretion signalling (254,255). Moreover, the notion was not restricted to substrates of the Ysc-Yop T3SS, since similar

observations were observed also for T3S substrates from plant pathogens and F-T3SS (250,256), and also with the report that 5'- untranslated RNA derived from *Salmonella* SPI-1 T3SS effector proteins could translocate adenylate cyclase reporter into host cells in Hfq-dependent fashion (257). Altogether, these observations lend themselves to a co-translational model of type III secretion. According to the model, the 5' mRNA sequences of substrate cargo forms a stem-loop structure recognized by discrete components of T3SS to couple translation with secretion (253,254). However, specific components of the T3S complex that recognizes a functional 5'-mRNA secretion signal has not been identified. Moreover, no consensus sequence or secondary structure that distinguishes functional mRNA secretion signals from a non-functional have not been determined (258).

On the other side, studies exist that directly challenge the mRNA signal secretion hypothesis. In short, studies have shown that integrity of a functional secretion signal tolerates drastic changes in mRNA coding sequence. For example, studies by Hans Wolf-Watz and colleagues showed that substantial changes in the first 11 codons of *yopE* mRNA without changing the primary amino acid sequences still maintained the secretion competence of the T3S signal sequence (259). However, frameshift mutation that altered the first 11 amino acid composition of YopE N-terminus, while keeping the mRNA sequence intact, led to dramatic reduction in secretion (259). Similar changes in *Salmonella invJ* mRNA codons 4 to 7 without affecting the actual coding sequence had no consequence in InvJ secretion (258). In many instances however, the T3S signals could endure frameshift mutation implying a high degree of degeneracy if they are proteinaceous in nature. Indeed, an elegant study examined the secretion competency of a vast repertoire of synthetic secretion signals appended to YopE. This identified an amphipathic N-terminal amino acid sequences as an efficient secretion signal, while highlighting strict hydrophobic or hydrophilic character as poor secretion signal (260). Similar evidences of an amphipathic N-terminal secretion signal were also reported in other T3S proteins (249).

1.9.2.2. Diversified secretion signals – what is the relevance?

Numerous studies report the varied molecular composition of secretion signals from diverse T3S substrates, and it turns out that they locate to the N-terminus as either amino acid sequence or mRNA sequence (246,247). The reason for this considerable degeneracy within the secretion sequence could be to provide flexibility for the T3S machinery to process proteins for temporal and spatial

secretion. Given that T3SS assembly occurs in a hierarchical fashion, with needle proteins being targeted for coordinated secretion prior to translocators and effectors (240), it is conceivable that N-terminal signal sequences of different substrates might be integrated with information that drives their ordered secretion (249,261).

Degeneracy of type III secretion signals can offer a number of advantages. First, T3SSs are ancient machineries. Diversity of secretion signals might reflect their optimisation to niche-specific environmental adaptations of respective T3SSs. In fact, theoretically an ability to endure diversity makes signal sequences an excellent evolutionary target. For instance in paper II, we observed that PscX (direct homolog of YscX in *P. aeruginosa*) unlike YscX was not a type III secreted substrate despite conserving other known functions of YscX. Therefore, multiple secretion signals could be an indicator of what worked best at different time under different situations. Second, subtle differences between signal sequences could drive substrate-specific functionalities. Compelling evidence in support of this idea is reported in paper III. Substituting the N-terminal region of YscX spanning 2 to 15 codons with equivalent secretion signals from other T3S substrate rendered it non-functional for T3SS. Interestingly, the N-terminal region of YscX could functionally substitute the YopD N-terminus, but not the YscF or LcrQ equivalent. This demonstrates that various secretion signal could be tailored to perform functions specific to a T3S protein. Third, any given T3S substrate is often demarcated with multiple functions. Multiple layers of information could be encoded in a single secretion sequence to maximize the functionality of substrate.

Finally, one can think of proteinacious and mRNA signals working in tandem. Consider that a mRNA encoded secretion signal that couples translation with secretion could provide a quick route for protein transport. However, it might not be an optimum method to secrete a large quantity of proteins simultaneously. This is because ribosome clustering at the T3SS base could complicate access for successive protein transport. In such situations, amino acid mediated secretion signals could be poised to secrete proteins at large quantity after their synthesis in the bacterial cytosol. In this context, a correlation between the length of 5' coding sequence and translation of T3S substrate has been described (249). Consistent with this, Paper III reports that appending longer N-terminal sequences of YscX substantially improved reporter protein expression. Hence, coupling of translation rate and secretion competency might be an unexplored

mechanism to fine tune an ordered T3SS biogenesis. Altogether, T3SSs exploit multiple secretion signals to impart precise control on its biogenesis and function.

1.9.2.3. The role of chaperones

One ubiquitous feature shared by different T3SSs in temporal secretion of substrates is the presence of customized T3S chaperones. T3S chaperones prevent premature degradation of the cognate substrates, maintain the substrate in a secretion competent state, and exert secretion specificity by escorting the substrate to the correct secretion apparatus (164,262). T3S chaperone function requires binding to a motif within the substrates that lies immediately downstream of N-terminal secretion signal sequence. Most often, this motif is referred to as the chaperone-binding domain (CBD). Chaperones of the NF-T3SSs are classified into at least three groups based on structural similarity and chaperone-substrate specificity. Class I T3S chaperones (~ 10-15 kDa) bind effector proteins and are further sub-divided according to their specificity to one effector (class Ia) or to several effectors (class Ib). Class II T3S chaperones (~ 15-20 kDa) possess a characteristic tetratricopeptide repeat structure and bind to the hydrophobic translocators, while class III chaperones bind to needle proteins (164,262,263). T3S chaperones usually function as a homodimer, but in some cases two different proteins can come together and function as a heterodimer.

The mechanism by which a substrate-chaperone complex is recognized by the component of the T3SS apparatus is not well understood. However, a chaperone-substrate complex can interact with members of the cytosolic complex, the SctN ATPase, the SctW gatekeeper protein and the cytosolic domain of SctV, a export apparatus member protein. In turn, the cytosolic complex has been proposed to recognize and orchestrate temporal secretion of the various T3S substrates (see section 1.7.3.3.). In particular, the T3S ATPase likely facilitates this process by engaging with, and dissociating chaperone-substrate complexes, as well as feeding the unfolded substrate into the secretion portal (see section 1.9.2.4.). Yet other observations suggest variations on this theme. For example, translocator-specific chaperones interact with the SctW gatekeeper protein, which facilitates an ordered secretion of T3S proteins (264). Moreover, recent reports suggest that SctV recognizesT3S chaperone-substrate complexes. In fact, structural studies by Kalodimos and colleagues demonstrated that binding of chaperone to its cognate substrate exposes a sequence motif recognized specifically by SctV during F-T3SS activity (265,266).

Although YscX is not a translocator, the cognate YscY T3S chaperone consists of predicted tetratricopeptide repeats that normally defines the class II T3S chaperone group (Paper I) (267). By recognizing a motif encompassing residues 70 to 90 of YscX, YscY maintains an intrabacterial stable pool of this substrate prior to its secretion (Paper III) (242). YscX and YscY are required for each other to interact with SctV (Paper I and III). Hence, YscX-YscY complex formation might be critical to allow a conformational change that exposes specific recognition signals to engage with SctV. This notion is entirely consistent with the previously mentioned study reported by Kalodimos and colleagues (265). In this way, the tripartite complex together coordinates to recognize and mediate temporal export of SctI and SctF (Paper III) (see section 1.9.3.1.). Following the completion of the needle assembly, YscX is secreted, while free YscY is re-cycled to engage with other YscX molecules or to cross talk with LcrH (223,268).

1.9.2.4. The ATPase in recognition and fuelling T3SS

An active T3SS can translocate several hundred substrates per second from a single injectisome (269). Moreover, the limited size of the injectisome needle channel can only permit passage of folded substrates during secretion (101,174). A conserved ATPase, SctN, catalyses substrate unfolding and its rapid entry into the injectisome portal (151,152). High-resolution structural studies of SctN have revealed structural and functional similarity to the β-subunit of F_0F_1-ATPase, and have visualized hexameric ring assembly below the SctV export gate (155,270). SctN can recognize a substrate-chaperone complex, induce chaperone release and unfold the substrate for secretion in an ATP hydrolysis dependent manner (106,154). This is important as loss of function mutations of SctN impair T3SS (152). The ATPase interacts with its negative regulator SctL and another cytosolic component SctO. Based on the structural analogy of SctO with the γ-subunit of F_0F_1-ATPase, SctO may coordinate with SctN to energize T3SS. Moreover, SctO takes on a stalk-like appearance in the lumen between the SctN ring and the SctV ring, and this presumably facilitates passage of substrates from SctN to SctV (155,271). It has also been proposed that SctO assists SctN to capture released chaperone for recycling (159).

Energization of NF-T3SS is still a matter of controversy. SctN-dependent ATP hydrolysis may not be the sole energy source for protein secretion in NF-T3SS. Treatment of *Y. enterocolitica* with a proton gradient uncoupler abolished Ysc-Yop T3SS implying the importance of proton motive force (PMF) in T3SS (272). Rietsch and co-authors addressed the issue more directly in *P. aeruginosa*,

which produces a NF-T3SS within the Ysc-Yop phylogenetic clade. In particular, they showed the involvement of SctO in regulating PMF-dependent export of T3S substrates (273). This would argue the need for multiple co-existing energy sources to drive NF-T3SS. This might involve a scenario where SctN unfolds substrates and feeds them into the inner membrane export apparatus, while subsequent threading of substrate through the needle channel requires a PMF driven mechanism. In addition, passage of proteins through the needle channel might also be facilitated by electrostatic repulsion between the substrate cargo and inner lumen of the needle channel as well as the potential energy gained from substrate unfolding prior to secretion (274).

Interestingly, the F-T3SS utilizes PMF as the predominant energy source to export flagellar substrates. Indeed, PMF alone can power the export of flagellar substrates in absence of T3S ATPase. Moreover, the flagellar assembly defect observed in ATPase lacking mutants can be corrected by secondary mutations that increase substrate production or increase the magnitude of PMF (132,275,276). On this basis, it is conceivable to think that PMF is the main driving force of secretion, while ATPase activity might perform secondary functions, for instance in chaperone release and unfolding of substrates to initiate the export process (153).

1.9.2.5. YscX as a sensing mechanism? A brief commentary

Bacteria have adapted their T3SSs in response to the distinct ecological niche they encounter. It is possible that each biological system has been optimized to use specific ions according to the environmental condition to couple energy mechanism and drive T3SS (277). While *Salmonella* and *E. coli* utilizes H^+ as the coupling ion to fuel F-T3SS mediated protein export, marine *Vibrio* spp. such as *V. alginolyticus* employ Na^+ as the coupling ion (132,179). Our preliminary data regarding a biochemical study of YscX-YscY has shown low pH to be crucial for stabilizing purified YscX-YscY in solution. Hence, it would be interesting to investigate if a purified complex of YscX-YscY homologues from marine *Vibrio* spp. (e.g.: *V. harveyi* and *V. parahaemolyticus*) and/or marine *Aeromonas* spp. (e.g.: *A. salmonicida* and *A. hydrophila*) also exhibit pH dependent stability or if other ions like Na^+ found predominantly in marine environments have greater roles. Moreover, the ability of YscX to interact with YscV, whose equivalent in F-T3SS possesses an ion channel activity, suggests that T3SS specificity of YscX may rely on its ability to activate T3SS by coupling to H^+ ion channelling. Recently, it has been shown in *Y. enterocolitica* that low extracellular pH is

detected by the inner membrane protein SctD and is relayed to the bacterial cytosolic complex to inhibit T3SS (278). As a functional Ysc-Yop T3SS requires an active inner membrane localized YscX (Paper III), it is tempting to speculate the role of YscX in transducing information regarding H^+ activity. It is probable that YscX-YscY engaged at the base of the T3S apparatus changes its activity in response to the concentration of H^+ ions, which influences the active status of Ysc-Yop T3SS. So coming back to the observation that YscX-YscY member proteins from other bacteria did not function in the *Yersinia* background (Paper I), perhaps these YscX homologues have evolved to interpret different ions in the coupling to T3SS activation. Whether H^+ dependency of YscX is a biologically relevant activity or is an artefact observed during YscX-YscY purification will require further investigation.

1.9.2.6. The role of SctV in T3S substrates recognition

SctV (termed YscV in *Yersinia*), a component of the inner membrane export apparatus, is indispensable for *Yersinia* T3SS, and shares sequence homology with member proteins in both NF-T3SS and F-T3SS (121,279,280). In particular, the N-terminal segment of SctV is highly conserved among family members, and likely forms eight transmembrane helices for integration into the inner membrane (281-284). Functional interchangeability was evident between the N-terminus of SctV ($SctV_N$) member proteins from *Y. pseudotuberculosis* and *S. typhimurium*, indicating that this transmembrane segment serves a general role in T3SS. On the other hand, the cytoplasmic-located SctV C-terminus ($SctV_C$) was not functionally interchangeable, suggesting that it is needed to recognise unique elements within any given T3SS (285). Structural and cryo-EM studies of $SctV_C$ revealed four conserved structural domains (SD1 – SD4) that oligomerize into a nanomeric ring and a small linker domain between $SctV_N$ and $SctV_C$ that tethers $SctV_C$ to the basal body (133,286-288). The cytosolic nanomeric ring is localized below the inner membrane spanning ring and above the hexameric ATPase ring (100,133). It therefore serves as a focal point to receive substrates for secretion following substrate-chaperone recognition by the ATPase. In addition, the cytosolic $SctV_C$ domain recognizes different classes of chaperone-substrate complex for hierarchal secretion through direct interaction (289). Most direct evidence for this role comes from studies in F-T3SS, where a number of studies report on a tripartite SctV-substrate-chaperone complex (265,266,283). At least within F-T3SS, a recent high resolution structural study highlighted a hydrophobic cleft formed between SD1 and SD2 as a recognition motif (265). Furthermore, amino acid residues spanning SD2 directly interacted with different

classes of chaperone-substrate complexes (290). In addition, truncation of the SD4 allowed F-T3SS to randomly export different T3S substrates irrespective of their class, i.e.: without a defined secretion hierarchy (289). Moreover, atomic force microscopy combined with mutagenesis revealed the role of the linker domain between $SctV_N$ and $SctV_C$ in substrate specificity switching (291). Collectively, these studies support the role of $SctV_C$ and the linker domain in recognition and ordering of substrates for type III secretion.

So, how are the different T3S substrates recognized and sorted for hierarchical secretion by SctV? Most probably, this requires a combination of different SctV actions. Firstly, the different SD1 to SD4 cytosolic domains of SctV can recognize different substrates and accordingly organize them for temporal export. Secondly, different affinities by which chaperone-substrate complexes bind to SctV can also define order of secretion (289). Thirdly, additional components of the cytosolic complex could aid SctV to establish sorting and secretion order. For instance, the SctO homolog in F-T3SS selectively modified binding ability of SctV to specific substrate-chaperone family (290).

So far however, SctV knowledge has been largely limited to F-T3SS. Very few studies specifically address SctV mediated substrate recognition and switching in NF-T3SS. In *S. flexneri* T3SS, stite-directed mutagenesis of SD2 specifically impaired translocators secretion only (133). Similarly, another mutagenesis study in *P. aeruginosa* identified a Q626R mutation within $SctV_C$ that strongly augmented effector secretion (273). In addition, molecular identified interactions between SctV and the SctW gatekeeper protein indicate a role in mediating ordered secretion of translocator and effector proteins (Figure 6B) (292-294). However, no high resolution structural study is available to explain how the inner membrane export component SctV recognizes substrates for coordinated delivery by NF-T3SSs. Especially lacking is information concerning the recognition and temporal export of early inner adapter protein and needle protein. In paper III, we have made an effort to fill this knowledge gap (also see section 1.9.3.1. and figure 6).

1.9.3. Substrate-switch control – a molecular clock for hierarchical T3SS

Regulatory checkpoints are employed at all different levels - transcriptional, post-transcriptional, translational as well as post-translational level - to maintain strict hierarchical T3SS biogenesis (32). One unique post-translational checkpoint in T3SS is the substrate switch control (103,283,295). Genetic and

structural studies have identified various T3S components that participate in substrate switch control mechanisms (103,105,295,296). This thesis will discuss three examples of substrate switch mechanisms to regulate T3S hierarchy: the YscX-YscY-SctV tripartite complex, the SctU autocleavage function, and the SycN-YscB-YopN-TyeA gating complex.

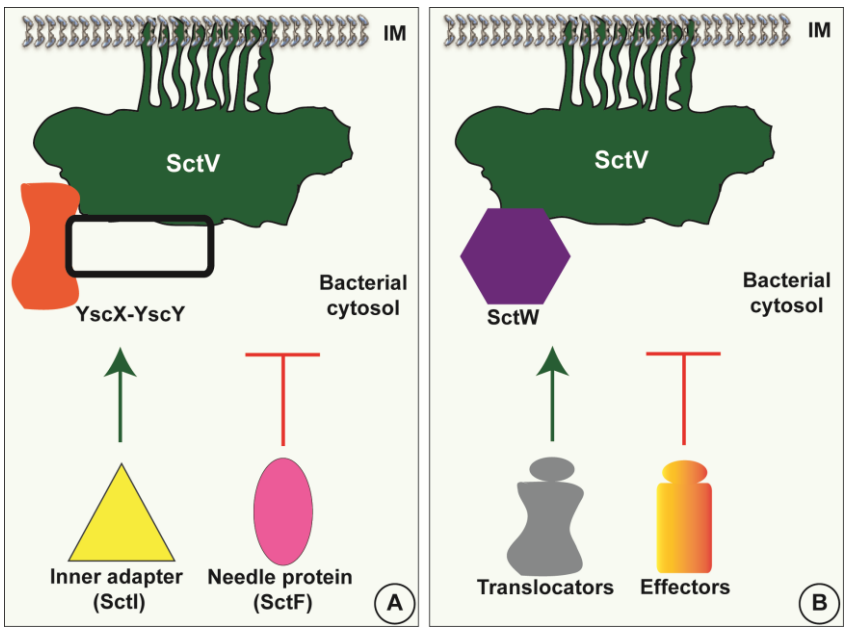

Figure 6. SctV as a recognition unit to mediate hierarchical secretion of T3SS. The N-terminus of SctV (green) is predicted to form eight transmembrane helices embedded into the inner membrane (IM). The C-terminus assembles into a nanomeric ring, which is tethered to the base of T3SS via a small linker domain. The SctV C-terminus has been demonstrated to recognize different classes of T3S substrates and/or substrate-chaperone complexes to establish temporal secretion. SctV recognizes YscX-YscY complex and together the tripartite complex facilitates secretion and/or assembly of SctI inner adapter prior to secretion and/or assembly of SctF needle protein (A). SctV recruits the SctW gatekeeper protein that mediate ordered secretion of translocators and effectors (B).

1.9.3.1. YscX-YscY-SctV as the substrate switch to control export of early substrates

One important checkpoint that controls export of early T3S substrates is formation of a YscX-YscY-SctV tripartite complex. While SctV is ubiquitously conserved in all T3SS families (see section 1.9.2.6.), YscX-like and YscY-like proteins are present specifically in the Ysc phylogenetic clade of T3SSs (Paper

I). In *Yersinia* Ysc-Yop T3SS, YscX is a multifaceted T3S substrate that performs a role in T3SS biogenesis and secretion control. YscX function depends upon an ability to bind to its cognate chaperone YscY in the cytoplasm. YscY consist of a characteristic tetratricopeptide repeat of the translocator class of T3S chaperones and maintains a stable cytoplasmic pool of YscX (Paper I, II, III) (169,242,297). Eventually, YscX-YscY complex is recognized by the cytosolic domain of SctV (Paper I, III) (157), which collectively facilitates the export of early substrates that make the inner adapter and the needle complex (Figure 6A, also see figure 10B-D). Specifically, the N-terminus of YscX is critical to orchestrate an ordered secretion of inner adapter SctI and needle protein SctF (Paper III). As YscX alone or YscY alone cannot independently interact with SctV, it is possible that formation of YscX–YscY complexes induces specific conformational change, which in turn is then recognized by the $SctV_C$ cytosolic domain. This idea is supported by the study by Kalodimos and colleagues (265). It is unknown if a similar YscXY-like mechanism exists in other bacteria harbouring T3SSs belonging to the Ysc-T3SS phylogenetic clade. From my work, we know that reciprocal YscX-YscY bipartite and YscX-YscY-YscV tripartite interactions among tested family members of Ysc-T3SS are conserved (Paper I). This suggests that certain aspects of the aforementioned YscX-YscY-YscV like substrate secretion control in *Yersinia* are likely conserved as well in other bacteria. However, as T3SSs are adapted to come in contact with diverse environments, functions of YscX-YscY-YscV related proteins could be amenable to specific variations. This corroborates a failure to complement Δ*yscX*/Δ*yscY* mutants of *Y. pseudotuberculosis* by their respective homologues from other bacteria (Paper I).

An alternate hypothesis is that SctV from *Y. pseudotuberculosis* has adapted compensatory alterations to customize specific binding to the YscX-YscY complex. In this context, Kalodimos and colleagues suggest that the binding cleft within SctV variants from different phylogenetic clades of NF-T3SS is not conserved, implying that the recognition mechanism could be different between different families (265). This corroborates the inability of SctV from *Y. pseudotuberculosis* to substitute the function of SctV from *S. typhimurium* (285). Hence, SctV from different bacteria can harbour functions unique to their respective T3SSs. Given the sequence diversity in $SctV_C$, it is likely that functional diversity is encoded in this cytoplasmic domain that is important for substrate recognition. Interestingly, member from the Inv-Mxi-Spa T3SS familial clade do not contain obvious YscX-YscY counterparts. Perhaps it is not a

coincidence that SctV variants from this clade typically exhibit a slightly shorter cytosolic domain. By extrapolation, perhaps the SctV cytosolic domain encoded by *Yersinia* may have evolved specific variations to modulate binding to YscX and YscY.

1.9.3.2. SctU cleavage and needle length control

SctU family of proteins is a transmembrane protein of the inner membrane export apparatus that is essential for all T3SS-mediated activity. It contains a N-terminal hydrophobic sequence predicted to form four transmembrane helices and a C-terminal cytoplasmic domain ($SctU_C$) (120,298). In addition, the N-terminal helices bind to the core export apparatus, SctRST, and possibly mediates opening of the export gate (119). The $SctU_C$domain undergoes autocleavage to mediate a secretion specificity switch from early to middle substrates. Autocleavage occurs at the conserved Asn-Pro-Thr-His (NPTH) domain between asparagine (Asn) and proline (Pro) to produce two subdomains: $SctU_{CN}$ (N-terminus of cytosolic SctU) and $SctU_{CC}$ (C-terminus of cytosolic SctU). Following autoproteolysis, the $SctU_{CC}$ domain remains associated with the membrane bound $SctU_{CN}$ or in some cases like in *Yersinia* is secreted (127,128,299-301). The cleavage event is physiologically significant as SctU mutants defective in autoproteolysis have severe defects in hierarchal switching from early to middle substrate secretion. This highlights SctU as a substrate switch protein.

The cytoplasmic domain of SctU may regulate substrate specificity switching in association with the needle length control protein, SctP (302-306). This is based on classical genetic suppression studies. The Δ*sctP* null mutant in *Yersinia* produces elongated needles and is reduced for late substrate secretion, and this phenotype can be suppressed by a mutation in $SctU_C$ (128,307). Similar phenotypes are also observed for equivalent homologues in F-T3SS (302). It appears that SctP protects $SctU_C$ from autoproteolysis by a direct interaction. Mutations in $SctU_C$ that prevent SctP binding increase the rate of autoproteolysis, and this impairs T3SS (308). This suggests that SctP binding to $SctU_C$ modulates the timing for substrate specificity switch in order to polymerise the needle of optimal length. Indeed, a linear correlation exists between needle length and the number of amino acid residues in SctP (309). Since SctP is a secreted substrate, the working model is that the N-terminus of SctP anchors to the growing end of the needle tip while its C-terminus interacts with SctU at the base (310-312). Once SctP reaches its full extension, it signals SctU to induce a substrate switch

bringing needle elongation to a halt and a transfer to the secretion of middle substrates. Additionally, the surface located domain of SctP could serve as a T3SS sensor to convey external signals to the interior to activate the substrate switch (313). An alternative theory for needle length control has SctP controlling assembly of the SctI inner adapter (308,314-316). Interestingly, Paper III suggests that YscX function is not dependent on SctU autoproteolysis. However, given that N-terminal YscX mutants defective for T3SS activity failed to export the early substrate SctI to the periplasm, maybe YscX is important to open the export gate via SctU association as discussed by Lea and colleagues (119).

Eventually, substrate switching allows completion of the distal tip of the needle and charging of translocators into the cytosolic complex of the injectisome (305). The system becomes activated following a host cell contact *in vivo*. However, until an appropriate signal is received by bacteria, the premature release of translocators and effectors is prevented by a gate-keeper switch protein that localizes at the cytoplasmic base of the injectisome (221)

1.9.3.3. The SctW protein family – prioritizing translocator protein secretion

Following needle assembly, specific regulatory controls trigger T3SSs to transit to secretion of 'middle' translocators (pore forming proteins) and 'late' effectors (immune modulatory proteins) (66). Various mechanisms are proposed to prioritize secretion of pore forming translocators before effectors. Some mechanisms discussed already involve specific use of T3S chaperones (see section 1.9.2.3.) and a substrate sorting mechanism reliant on the cytosolic complex (see section 1.7.3.3.). This section will focus on a mechanism dependent on the concerted actions of SctW family of proteins in association with its cognate T3S chaperones and other accessory components of the T3SS (317-320). Referred to as the 'gatekeeper' proteins, members are present in the different NF-T3SS phylogenetic clades (319,320). Mutants containing knockouts of the member proteins impair translocator and effector secretion without compromising needle assembly (318,321,322). Hence, a role of these proteins is in establishing the secretion hierarchy of translocator and effector substrates. In the absence of SctW in *Salmonella*, the cytoplasmic complex fails to recognize translocators for secretion (135). Therefore, it is very likely that some gatekeeper proteins physically interact with translocator-chaperone complexes and deliver them to the cytoplasmic complex for temporal secretion following needle assembly (321-323). In other examples however, binding of SctW gatekeeper

proteins to SctV physically retards effector secretion, most probably by preventing their access to the T3S apparatus (273,293,294). Once translocator proteins are assembled at the distal end of the needle tip and form pores in host cell plasma membranes, a signal can be transduced back to the basal body to trigger dissociation of the gatekeeper proteins (68,322,324-326). In some instances, gatekeeper proteins are secreted and this allows subsequent secretion of effectors (221,327,328).

1.9.3.3.1. Role of YopN-TyeA complex

SctW family homologues in the Ysc T3SS phylogenetic clade exist as two discrete proteins. In human pathogenic *Yersinia,* these two proteins are YopN and TyeA (329). YopN displays moderate amino acid identity to the N-terminus of SctW family of proteins, while TyeA resembles homology to the C- terminus (330). Interestingly in *Y. pestis* and *Y. pseudotuberculosis,* but not in *Y. enterocolitica*, YopN and TyeA can function as a singular YopN-TyeA polypeptide probably brought about via a +1 frameshift event during translation of 3' end of *yopN* mRNA (Paper IV) (330,331). Whether the frameshift is a consequence of a mRNA translational error or is a programmed event that helps bacteria to adapt better to different physiological purposes requires meticulous analysis from various dimensions (332-334). However, *Y. pseudotuberculosis* engineered to predominantly produce YopN-TyeA hybrid was competent for functional secretion of the hybrid, and mediated polarized translocation of effectors into eukaryotic host cells. However, the hybrid was not as native forms of YopN and TyeA to respond to calcium levels and control Yops expression. This is probably why bacteria producing the hybrid were attenuated during *in vivo* infection of mice (330).

1.9.3.3.2. YopN domain organization and functional characterization

Whether YopN secures hierarchal translocator-effector secretion is still open. The Δ*yopN* mutant does not display any preferential translocator and effector secretion *in vitro* (330,335,336). However, the all or none phenomenon of Ysc-Yop T3SS induction in calcium depleted growth media *in vitro* could easily mask subtle differences in translocator and effector secretion levels. Nevertheless, structural studies have identified YopN to boast discrete domains that control diverse aspects of T3SS (Figure 7). The three dimensional structure of YopN contains multiple bundles of α-helices that are extended over a large surface area to allow simultaneous binding of several T3S proteins (337). The C-terminal part

of YopN encompassing amino acids 212 to 222 and amino acids 248 to 293

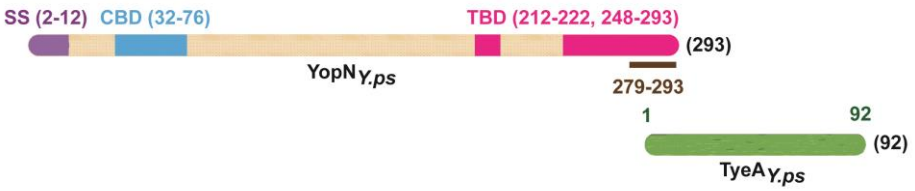

Figure 7. Schematic representation of different YopN domains. YopN and TyeA are encoded as two discrete polypeptides in *Y. pestis* and *Y. pseudotuberculosis* but not in *Y. enterocolitica*. A full-length YopN and TyeA encodes 293 and 92 amino acids respectively (shown in parentheses). Structural features of YopN includes a N-terminal secretion signal located between 2 to 12 amino acids, YscB-SycN chaperone binding domain spanning 32 to 76 amino acids and a TyeA binding domain at the C-terminus encompassing 212 to 222 amino acids and 248-293 amino acids. Paper IV dissected the regulatory elements of YopN C-terminus spanning 279 to 293 amino acids. Abbreviations: SS (secretion signal), CBD (chaperone binding domain), TBD (TyeA binding domain) and *Y.ps* (*Y. pseudotuberculosis*).

associates with the cytosolic TyeA protein. A YopN-TyeA complex is critical for regulating T3SS (338-340). In Paper I, new molecular contacts for interacting with TyeA were found within the YopN region encompassing residues 279 to 287 amino acids. These molecular contacts were pivotal in YopN-TyeA complex formation and subsequent regulation of Yops secretion. In contrast, the extreme YopN C-terminal coding region that overlaps with the N-terminal coding region of TyeA was functionally redundant. At the YopN N-terminus exists a region spanning residues 32 to 76 that constitutes a chaperone-binding domain for recognition by a heterodimeric complex of SycN and YscB. Chaperone binding is important for piloting of the YopN-TyeA complex for eventual secretion (337,341,342). Prevailing theory is that the YopN-SycN-YscB-TyeA complex acts as a T3SS plug to physically delay effector secretion. Conformational change in the needle triggered by calcium depletion or host cell contact propagates through to the base of the T3SS (221,323,327,337,343). This permits YopN secretion, which is facilitated by a secretion signal in its extreme N-terminus (328). Accompanying this is a surge of effectors delivery into target host cells. During this process, tethering of TyeA to specific component of the basal body like SctV and simultaneous communication of YopN with members of the cytosolic complex is critical (135,273). Similar mechanism of secretion in consecutive steps is described in the T3SSs of other bacteria. In fact, SctW in

complex with its chaperone binds to SctV to recognize specific determinant of the translocator/chaperone complex for secretion and at the same time supressing effector binding to SctV (Figure 6B) (292,293).

Compared to its homologs, one intriguing aspect of YopN is its translocation into target host cells (343). With no defined enzymatic activity and no known molecular targets inside host cells, the role of translocated YopN is not obvious. However, recent studies have identified the central region of YopN covering 76 to 181 amino acids, which is required not only for its translocation but also for efficient translocation of effector proteins including YopE and YopH (344,345).

2. Objectives of this thesis

How temporal secretion of T3S substrates is efficiently synchronised to establish hierarchical assembly of T3SS is a poorly understood concept. The main aim of this thesis is to identify regulatory mechanisms that orchestrate coordinated assembly of the Ysc-Yop T3SS and explore its evolutionary dynamics.

Specific aims:

1. Investigate the functional conservation of YscX and YscY member proteins in T3SS.
2. Dissect YscX domains linked to functions specific in the Ysc-Yop T3SS.
3. Assess roles of the N-terminal YscX in T3SS assembly.
4. Examine the contribution of YopN C-terminus in regulation of Ysc-Yop T3SS activity.

3. Results and discussion

Type III secretion systems comprise an extracellular needle filament embedded within a large membrane spanning scaffold, which together forms a conduit to selectively deliver a range of substrates into the host cell cytosol (see section 1.7.3.). The biogenesis of T3SS is hierarchical and substrate secretion well-orchestrated. Together this ensures first the deployment of needle proteins, followed by translocators and effectors (see section 1.9.1.). While about 10 'core' proteins are conserved in all phylogenetically distinct T3SS families, some T3SSs have also evolved to harbour unique protein components. For instance, YscX and YscY represent proteins confined specifically to the Ysc T3SS family. Moreover, although broadly distributed among the different T3SS families, functional equivalents of YopN and TyeA display sequence diversity and their genes a different architecture. In this thesis, I attempted to elucidate coordinated biogenesis and regulation of the Ysc-Yop T3SS by focusing on the roles of YscX-YscY and YopN-TyeA complexes. The study adds new knowledge into the process of transitioning from one stage to another during T3SS assembly, and identifies constituents that contribute to functional diversification of T3SS activity.

3.1. Evolutionary assessment of YscX and YscY member proteins

Phylogenetic analyses based on sequence identity among structural components have grouped T3SSs into eight different families (see section 1.7.1.) (77,78). Few studies have attempted to address functional interchangeability between T3S components present in different T3SSs, which provide an understanding on evolutionary development of the complex nanomachine (285,346,347). In this thesis, I and others used a functional complementation approach to probe for key molecular elements of YscX and YscY that influence T3SS biogenesis and activity. The starting reference is a functional interchangeability study between YscX and YscY performed with PscX and PscY of *P. aeruginosa* more than a decade ago (268). Now, with the availability of extensive public genomic databases, the landscape has changed. Heterologous family of proteins sharing amino acid identity to YscX and YscY have been shown to exist in diverse members of Gram-negative bacteria including human and animal pathogens *Y. pseudotuberculosis*, *Y. enterocolitica*, *Y. pestis* and *P. aeruginosa* (PscX and PscY), marine pathogens *A. salmonicida* ($AscX_{As}$ and $AscY_{As}$), *A. hydrophila* ($AscX_{Ah}$ and $AscY_{Ah}$), *V. harveyi* ($VscX_{Vh}$ and $VscY_{Vh}$)

and *V. parahaemolyticus* ($VscX_{Vp}$ and $VscY_{Vp}$) and the insect pathogen *P. luminescens* (SctX and SctY). When compared to amino acid sequence of YscX and YscY from *Y. pseudotuberculosis*, the percent amino acid identity ranged roughly between 35% (*Vibrio* spp.), 46% (*P. luminescens*), 47% (*P. aeruginosa*) to 53% (*Aeromonas* spp.) (Paper I, Figure I). Evolutionary assessment related to T3SS activity was performed among member proteins derived from bacteria representing wide evolutionary distribution.

3.1.1. Functional conservation of YscX-YscY bipartite and YscX-YscY-YscV tripartite interactions

YscX and YscY are indispensable proteins of the Ysc-Yop T3SS as deletions of either gene renders a loss in type III secretion (242,297) (Paper I, Figure 4). YscX is a secreted substrate of the Ysc-Yop T3SS, with intracellular pools stabilized by an interaction with its cognate T3S chaperone, YscY (Paper III, Figure 2). The YscX-YscY complex associates with SctV, and this association is suggested to mediate temporal export of early T3S substrates (157). Together with others, I wanted to first examine if the ability to constitute YscX-YscY bipartite and YscX-YscY-YscV tripartite interactions are conserved among YscX and YscY protein family. Interactions were investigated with the yeast two-hybrid and yeast three-hybrid protein interaction assays, since these methods were useful to establish reciprocal interactions between YscX/PscX with YscY/PscY (268). All *yscX*-related alleles were individually expressed from pGADT7 as a C-terminus fusion to *GAL4* activation domain (AD). Similarly, all *yscY*-related alleles were separately expressed from pGBKT7 as a C-terminus fusion to *GAL4* DNA binding domain (BD). Interplay between substrate-chaperone pairs were systematically examined in all possible combinations by co-transforming vectors expressing AD-YscX and BD-YscY derivatives into yeast reporter strains and analysing the activation of reporter gene expression. We could demonstrate YscX-YscY binary interaction and an ability to form analogous interactions conserved among all cognate partners. Interestingly, reciprocal interactions between YscX and YscY member proteins were evident in all possible directions (Paper I, Figure 2) (also see Figure 8). Ternary interactions involving YscX-YscY and SctV used the yeast three-hybrid assay by expressing YscX/YscY variants from pBridge vector as P_{ADH1}-BD-YscY/p_{MET25}-YscX derivatives and YscV variants from pGADT7 vector as P_{ADH1}-AD-YscV derivatives. We could measure a robust tripartite association between YscX, YscY and YscV (Paper I, Figure 3). However, this occurred only in conditions when both YscX and YscY were present. Likewise, a reciprocal interactions

between YscX-like substrate, YscY-like chaperone and YscV-like inner membrane export component were evident in all possible combinations as judged from yeast interaction assays (Paper I, Figure 3) (also see figure 8). This analysis was extended in paper III to show that various YscX deletions within the extreme N-terminus still maintained strong bipartite and tripartite interactions (Paper III, Table 2 and Table 3). This implied that the YscX N-terminus is dispensable for these interactions. Collectively, this data suggests that these particular bipartite and tripartite interactions involving YscX, YscY and YscV protein families represent core attributes that are necessary to maintain common functions fundamental to T3SS activity.

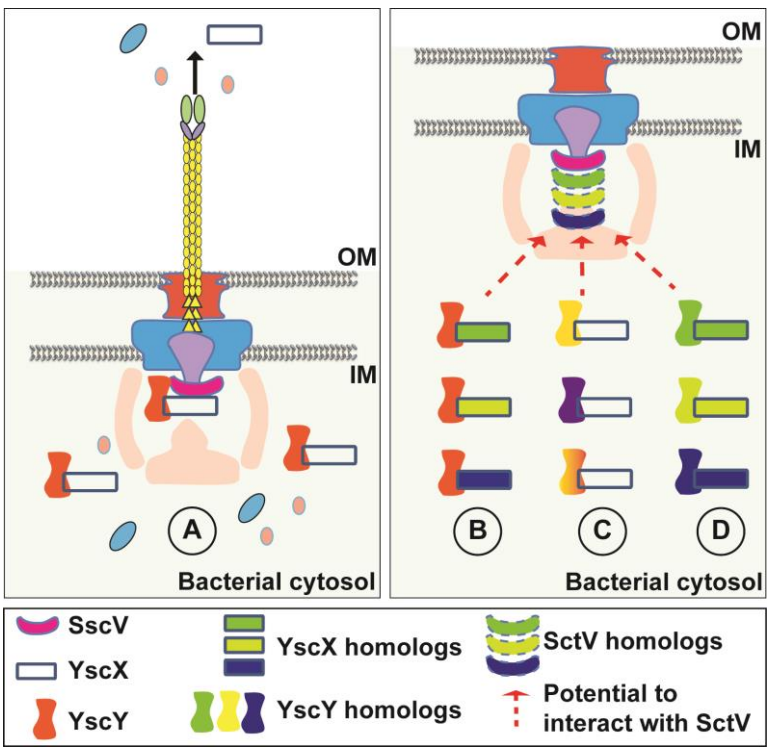

Figure 8. An overview of a functional interachangeability between YscX and YscY family members. A YscX-YscY bipartite interaction and a YscX-YscY-SctV tripartite interaction consitute intergral aspects of the Ysc-Yop T3SS (A). Ability to form a YscX-YscY-like and YscX-YscY-YscV-like interactions is present between cognate YscX and YscY member proteins (D). Interestingly, reciprocal binary and ternary interactions is possible in all combinations (B, C).

The use of yeast, *S. cerevisiae*, as a host in these protein interaction assays is a potential caveat. By using a heterologous system for protein interaction studies, one could question whether the readouts represent true biologically relevant interactions in *Yersinia* T3SS. No doubt there are differences in the amount, timing and location of the interacting proteins produced in yeast and in *Yersinia*. Moreover, protein complex formation in T3SS is expected to be highly dynamic process with continuous exchange between member proteins (143,144). Dynamic interactions cannot be reproduced in the yeast surrogate. However, stable accumulation of YscX requires an interaction with co-produced YscY (Paper III, Figure 2) (242). With the exception of AscX variants from *Aeromonas* spp., all YscX homologues were sufficiently produced in *Yersinia* (Paper I, Figure 6E). As this accumulation requires interaction with co-produce chaperone, the substrate-chaperone interaction must occur in *Yersinia*, and this corroborates findings in yeast. Since the YscX-YscY-YscV tripartite complex could also be co-immunoprecipitated from *Yersinia* lysates (157), this would have been a better interaction assay to use given the superior biological relevancy. However, this is not a high through put assay. Hence, so in our case with multiple samples it simply was not a practical option.

3.1.2. YscX and YscY have co-evolved to perform functions specific to *Yersinia* Ysc-Yop T3SS

Having established the role of YscX and YscY homologs in maintaining binary and ternary interactions, the next step was to investigate if the homologs can reconstitute a functional T3SS in respective Δ*yscX* null mutant and/or Δ*yscY* null mutant. To facilitate this, *yscX*- and *yscY*-related alleles were cloned either individually or together into an IPTG inducible pMMB67EHgm expression vector and expressed ectopically in appropriate *Y. pseudotuberculosis* background strains. Introduction of native YscX in the Δ*yscX* null mutant, native YscY in the Δ*yscY* null mutant or co-production of both in the Δ*yscXyscY* double mutant, could all complement the T3S defect as assessed by *in vitro* production and secretion of Yop proteins. However, none of the homologs when expressed individually or as a substrate-chaperone co-complex *in trans* could substitute for endogenous YscX and/or YscY in promoting T3SS activity in *Y. pseudotuberculosis* (Paper I, Figure 4 and Figure 5). Production of YscX and YscY variants was assessed by appending the FLAG™ epitope at the N-terminus followed by Western immunoblotting using anti-FLAG™ antibody. Protein expression of some of the homologs was notably poor which limited our ability to confirm if lack of functional complementation was a consequence of

insufficient protein production (Paper I, Figure S3). To address this, the codons of YscX and YscY family members were optimized to closely reflect the codon usage of *Y. pseudotuberculosis* without altering the amino acid sequence of the encoded protein. Pronounced improvement in accumulated steady state levels was observed in all cases except homologs from *Aeromonas* sp., which still failed to accumulate protein levels comparable to native YscX (Paper I, Figure 6 and Figure S6). The reduced level of protein pool was not due to lower mRNA levels (Paper I, Figure S7). This was logical as transcription of all *yscX*-like alleles and *yscY*-like alleles was driven from an identical transcriptional architecture comprising of a common promoter and a common transcriptional start site. Nevertheless, boosting protein production through codon-optimization still did not permit YscX and/or YscY homologs to restore T3SS activity to Δ*yscX* and/or Δ*yscY* null mutants (Paper I, Figure 6) (also see figure 8).

This raised the question as to the minimum amount of YscX and/or YscY required to restore T3SS activity in the respective *Y. pseudotuberculosis* mutant backgrounds. Using an IPTG titration experiment, minimal amounts of YscX and YscY were all that were required (Paper I, Figure C, Figure D). Critically, this also confirmed ectopic expression of all non-complementing homologs comfortably exceeded these minimal levels. Hence, insufficient production of YscX and YscY homologs was not a reason for non-complementation (Paper I, Figure 6). In fact, the YscX-like and YscY-like protein members had no observable effect of *Yersinia* T3SS whatsoever, for ectopic expression of codon-optimized YscX and YscY homologs in parental *Y. pseudotuberculosis* with an intact T3SS did not alter the profile of Yop synthesis and secretion (Paper I, Figure 7). Collectively, results from paper I demonstrate that one or more strict requirements unique to *Yersinia* Ysc-Yop T3SS can only be met by customised endogenous YscX and YscY, and cannot be substituted by equivalent proteins from other bacteria.

3.1.3. Uniquely conserved cysteines of YscX – an inherent feature critical for Ysc-Yop T3SS

In an attempt to unveil inherent features of YscX that brings specificity to *Yersinia* T3SS, a closer inspection of multiple YscX-like amino acid sequence alignments revealed numerous amino acids uniquely conserved in YscX of *Yersinia.* Of particular interest were two cysteine residues conserved at position 25 (Cys_{25}) and position 104 (Cys_{104}) (Paper II, Figure I). Cysteine residues can participate in disulfide bond formation, which can influence several aspects of

protein folding and function. Critically these effects on protein function can be coupled to bacterial virulence (348-351). The importance of these two conserved cysteine residues in YscX function was investigated. Site-directed mutagenesis generated the *in-cis* substitution mutants that produced the $YscX_{C25A}$, $YscX_{C104A}$ or $YscX_{C25A,C104A}$ variants. The effect on T3SS activity was assessed by bacterial growth at 37º C in BHI media devoid of Ca^{2+}. Intracellular production of all three cysteine-substituted YscX variants were equivalent to native YscX, However, only the variants $YscX_{C25A}$ and $YscX_{C104A}$ were efficiently secreted. As expected, *Y. pseudotuberculosis* harbouring these single cysteine substituted variants of YscX permitted T3SS functions of Yop proteins production and secretion (Paper II, Figure 3). On the other hand, the $YscX_{C25A,C104A}$ variant was not secreted and did not support the production and secretion of Yops (Paper II, Figure 3). At this point, the data does not reveal whether these two cysteine residues are involved in intra- or inter-molecular disulfide bond formation. Nevertheless, the data does demonstrates that the two cysteines are not important for intracellular accumulation of stable YscX, meaning the cysteine residues do not contribute to YscX-YscY complex formation. The fact that mutation of both cysteine residues, but not mutation of either single cysteine residue, impairs YscX function is an interesting conundrum. In my mind, it argues for the cysteine residues contributing to inter-molecular disulfide bond formation. By extension, this could indicate additional molecular targets of YscX, and these interactions could be important to orchestrate recognition and secretion of tertiary T3S sub-complexes in order to execute a functional Ysc-Yop T3SS.

3.1.4. Identifying functional YscX domains

Another avenue of attack was to locate one or more unique feature(s) of YscX to a particular functional domain(s). Comparative sequence analysis between YscX and its homologs could not distinguish by sight any obvious region that might represent a unique functional motif within YscX. In an attempt to characterize specific attributes of YscX, chimeras were created between YscX and its codon optimised ('opt') homologs $AscX_{.opt}$ (*A. salmonicida*) and $SctX_{.opt}$ (*P. luminescens*). Essentially, two sets of full-length chimeras were created. There were those that became progressively YscX-like from the N-terminus (*i.e.*: corresponding to 1 to 15, 1 to 30, 1 to 60 and 1 to 90 amino acids of YscX), with 1 to 122 being wild type YscX. Additionally, there were those that became progressively YscX-like from the C-terminus (*i.e.*: corresponding to 90 to 122, 60 to 122, 30 to 122 and 15 to 122 amino acids of YscX). Altogether, this generated a repertoire of eight YscX-$SctX._{opt}$ chimeras that gradually became

SctX-like from either the N- or C-terminus. Likewise, an additional set of four YscX-AscX.$_{opt}$ chimeras were similarly engineered that progressively became AscX-like from the C-terminus (Paper II, Figure 2). The plasmids harbouring these chimeras were then ectopically expressed in Δ*yscX* null mutant. Following T3SS induction of bacterial cultures in BHI minus Ca^{2+} condition, protein samples were assessed for Yops production and secretion to identify domains important for YscX specificity.

Naturally, ectopic expression of native YscX restored a functional T3SS in Δ*yscX* null mutant, and corroborated efficient production and secretion of FLAG^{TM}-YscX by *Y. pseudotuberculosis* (Paper II, Figure 2 and Figure S1). However, despite sufficient production of all chimeras in the Δ*yscX* null mutant, only the YscX$_{90}$-AscX.$_{opt}$ chimera composed of the first 90 amino acids of YscX fused to the extreme C-terminus of AscX.opt could partially substitute for native YscX function (Paper II, Figure 2). This implies that the C-terminus of AscX.$_{opt}$ spanning 91 to 122 amino acids shares some functional similarity to C-terminal region of YscX. However, the introduction of analogous YscX$_{90}$-SctX could not recover any T3SS activity in Δ*yscX* null mutant. Although surprising, the data is consistent with higher sequence conservation between the C-termini of AscX and YscX, compared to the C-termini of SctX and YscX.

Not one of the aforementioned chimeras contained both conserved cysteine residues of YscX (Paper II, Figure S2). Using this as an opportunity to further explored the role of these two cysteine residues, additional YscX chimeric variants were created to contain the cysteine residues, either individually or both simultaneously. However, when introduced in Δ*yscX* null mutant, cysteine containing chimeras still failed to restore an operational T3SS (Paper II, Figure 4 and Figure 5). This data is at odds with the *in cis* site-directed mutagenesis data. To reconcile these data sets, the cysteine residues are important for YscX function, but their role is influenced by the context of their surrounding amino acids in the three-dimensional YscX structure. Collectively, experimental approaches in Paper II were unable to pinpoint genetic elements of YscX that bring about T3SS specificity in *Y. pseudotuberculosis*. Moreover, likely YscX is not modular in structure, and no discrete domain able to function in isolation. A reason for this is that YscX probably functions as a small molecular scaffold that mediates precise spatial and temporal recognition of different T3S components. This aspect could be partly tailored by the two strategically positioned cysteine residues.

3.2. Importance of the YscX N-terminus in Ysc-Yop T3SS

Of the plethora of bacterial proteins, only a few constitute substrates of T3SS as determined by a N-terminal signal sequence. YscX is one such substrate of the Ysc-Yop T3SS (Paper I and Paper II). Despite the absolute need of YscX for an efficient T3SS in *Yersinia*, its role in T3S biogenesis is still poorly understood. Therefore, Paper III sought to establish the contribution of YscX secretion in assembly of the Ysc-Yop T3SS. The paper specifically focused on the role of N-terminal region of YscX owing to the notion that signals for proteins targeted by T3SS reside at the N-terminus. The study did not attempt to perform a molecular characterization of the N-terminal secretion signal of YscX *per se*, for the secretion signal concept has already been studied in detail in *Yersinia* for different classes of T3S substrates (246,352,353).

3.2.1. Evidence of YscX N-terminus as an independent secretion signal

To determine if the YscX N-terminus is a legitimate secretion signal, a reporter system was utilized to engineer a series of translational fusion between 5' end of *yscX* (containing the native Shine-Dalgarno sequence) to a promoterless and a signaless β-lactamase reporter protein. Plasmids harbouring the fusion proteins were ectopically induced with IPTG either in parental bacteria or in a T3SS inactive Δ*yscU* mutant of *Y. pseudotuberculosis* while growing in T3SS activating condition. Sequences of *yscX* encoding the first 10, 15, 20 and 25 amino acids mediated efficient production and secretion of the reporter protein in parental bacteria. However, despite equivalent production of the reporter protein, no secretion was evident in T3SS-negative Δ*yscU* mutant. Prolonged exposure during chemiluminescent detection enabled visualization of $YscX_5$-Bla secretion (Paper III, Figure 1). Altogether, this data implies that the first 5 amino acids may constitute a minimal signal, but efficient secretion of YscX requires a signal of 10 or more N-terminal amino acids. To strengthen its role as an legitimate secretion signal, the YscX N-terminus was successfully used to promote *in vitro* secretion of other T3S proteins including the middle substrate YopD ($YopD_{YscX\text{-}Nterm}$) and the late substrate YopE ($YopE_{YscX\text{-}Nterm}$) (Paper III, Figure 5).

To explore the biological relevance of the YscX N-terminus in recognition and secretion by the Ysc-Yop T3SS, three in-frame deletions were generated *in-cis* in the virulence plasmid to produce the variants $YscX_{\Delta 3\text{-}7}$, $YscX_{\Delta 8\text{-}12}$ and $YscX_{\Delta 13\text{-}22}$. Despite being produced at levels comparable to native YscX, secretion of YscX variants was reduced ($YscX_{\Delta 3\text{-}7}$) or absent ($YscX_{\Delta 8\text{-}12}$ and

$YscX_{\Delta 13\text{-}22}$) following bacterial growth in T3S permissive conditions. Interestingly, the two YscX variants that were not secreted, also displayed an impaired T3SS, as measured by an inability to secrete *in vitro* the early YscF needle proteins, the middle YopB, YopD translocators and the late YopE effector (Paper III, Figure 2 and Figure 3). Collectively, these results indicate the YscX N-terminus to harbour a secretion domain that resides within the first ~ 20 amino acids. Information encoded within this domain acts as an efficient signal that influences active T3S of itself and other T3S substrates.

3.2.2. YscX N-terminus is required for T3SS activity

Having established the N-terminal region of YscX as a bona vide secretion signal, the next step was to dissect its role in temporal control and assembly of the Ysc-Yop T3SS. Exploiting the reciprocity of established signal sequences among various T3S substrates, the chimeras $YscF_{YscX\text{-}N\text{-}term}$ and $LcrQ_{YscX\text{-}N\text{-}term}$ were constructed in which the N-terminal secretor domain of YscF and LcrQ were substituted with the equivalent region from YscX N-terminus. *Yersinia* producing $YscF_{YscX\text{-}N\text{-}term}$ could not assemble the T3S needle, and this abolished T3SS function (Paper III, Figure 3 and Figure 5). On the other hand, *Yersinia* harbouring $LcrQ_{YscX\text{-}N\text{-}term}$ displayed Yops secretion irrespective of Ca^{2+} (Paper III, Figure 5). This phenotype represents a derepression of Ysc-Yop T3SS control. The reason is most probably due to premature secretion of the LcrQ chimera because of influence by the YscX N-terminus. This is an important phenotype, and needs to be exploited in future work. LcrQ secretion is a long-viewed critical checkpoint in temporal control of Yops synthesis and secretion. The $LcrQ_{YscX\text{-}N\text{-}term}$ chimera phenotype suggests that it can serve as a molecular tool to better probe temporal control of LcrQ secretion, and this would automatically allow comparison to temporal control of YscX secretion.

Altogether, these observations support a role of the YscX N-terminus in coordinating correct assembly of T3SS. If the YscX N-terminus did somehow effect the order of substrate recognition and secretion, then this control might be impacted in a series of N-terminal YscX chimeras that were engineered to possess the first 2 to 15 amino acids of an equivalent secretion signal from different class of T3S substrates. Depending on the origin of the secretion signal, it was hypothesized that the YscX chimeras may exhibit altered temporal secretion control, and thereby uncoupling ordered YscX secretion from T3SS assembly. Following bacterial growth in T3S inducing conditions, all the N-terminal YscX chimeric variants were produced at amounts comparable to native YscX.

However, altering the N-terminal sequence of YscX dramatically abolished its secretion (Paper III, Figure 4). This again corroborated to an inactive T3SS as demonstrated by a lack of early, middle and late substrates secretion (Paper III, Figure 3 and Figure 5). Overall, exchanging the N-terminal region of YscX compromised secretion of YscX. Hence, the YscX N-terminus must contain non-redundant sequence important for some aspect of T3SS activity.

The data does not provide opportunity to address whether defective T3SS was actually due to a failed secretion ability of N-terminal YscX chimeras. To some extent, this was addressed by using the reporter assay in which the first 25 residues of N-terminal YscX chimeras including its native Shine-Dalgarno sequence was fused to a β-lactamase reporter protein under IPTG control. Interestingly, the heterologous secretion signals could still promote reporter secretion (Paper III, Figure 1). Thus, a general defect in T3SS activity occurred despite a capacity for the YscX chimeras to be secreted. Hence, secretion of YscX is not the sole function of the YscX N-terminus. Elements embedded in this domain must facilitate the recognition and sorting of T3S substrates for correct temporal export.

3.2.3. Role of YscX N-terminus – beyond secretion

Manipulation of YscX N-terminus either by specific deletions or by defined domain swapping impaired YscX secretion. However, the secretion of other substrates was also abolished. This suggests that the N-terminal region performs additional functions fundamental to general T3SS biogenesis and function. This idea was explored further. First, loss of function YscX variants maintained an ability to form YscX-YscY bipartite or YscX-YscY-YscV tripartite interactions as judged from yeast two-hybrid and yeast three-hybrid protein interaction assays (Paper III, Table 1 and Table 2). The second step was to investigate differences in production of T3S components in N-terminal YscX mutants compared to parental *Yersinia*. Western immunoblotting was used to probe for the production of different components of T3SS representing membrane spanning rings, cytosolic complex, inner adapter and needle component. No dramatic alteration in production of the components comprising the basal body was observed implying proper basal body formation (Paper III, Figure S7). Armed with this knowledge, the third step was to assess if the basal body was competent for secretion of inner adapter (SctI) and needle protein (SctF) components. Interestingly, N-terminal YscX mutants ($YscX_{\Delta 8\text{-}12}$, $YscX_{\Delta 13\text{-}22}$) could not support the secretion of SctI and SctF, and overall production of both the inner adapter

protein and the needle protein was drastically reduced (Paper III, Figure S7). Cell fractionation assays was then used to determine sub-cellular distribution of different T3S components. Once again, member proteins representing the basal body showed no difference in sub-cellular localization between parental and N-terminal YscX mutants (Paper III, Figure 6). Critically however, localization of SctI and SctF was impaired. Despite equal targeting of YscI to the inner membrane fractions, only the defective YscX mutant (Δ*yscX*, $YscX_{\Delta 8\text{-}12}$) lost the ability to export and/or assemble SctI in the periplasm. This corroborated an absence of SctF targeting in the inner membrane (Paper III, Figure 6).

Overall, this study demonstrated a role of the YscX N-terminus in orchestrating an ordered export and/or assembly of SctI and SctF. It is assumed that this function is dependent upon associations with both YscY and SctV. This was not a consequence of differential localization of YscX variants for they were all targeted to the T3S base in the inner membrane (Paper III, Figure 8). The current working model is that the YscX N-terminus is important for preparing an YscX-YscY-SctV complex conformation that can recognise cargo destined for secretion (Figure 9).

3.2.4. YscX secretion – gateway to a functional T3SS?

YscX is a T3S substrate that is secreted into the extracellular milieu following the completion of the needle assembly (157). Secretion of YscX is correlated with an ability to form a functional T3SS as measured by production and secretion of early, middle and late T3S substrates (Paper III, Figure 2 and Figure 3; Paper II, Figure 3). However, what is unclear is the role of secreted YscX. Inside the bacterial cytosol, YscX coordinates with YscY and SctV to mediate temporal export of SctI inner adapter and SctF needle proteins. Following needle assembly, YscX is secreted. On one hand, secretion of YscX could be a strategic checkpoint that allows YscY and YscV to recognize the next category of substrates, the translocators, for secretion to progress into next stage of injectisome assembly. On the other hand, secreted YscX could be a minor component of the needle filament, although a preliminary study probing for presence of YscX in purified T3S needle suggests otherwise. At any rate, it is difficult to believe that secreted YscX would have no extracytoplasmic function, given that shedding of this protein without reward would constitute an energy cost to the bacteria, and therefore a fitness disadvantage.

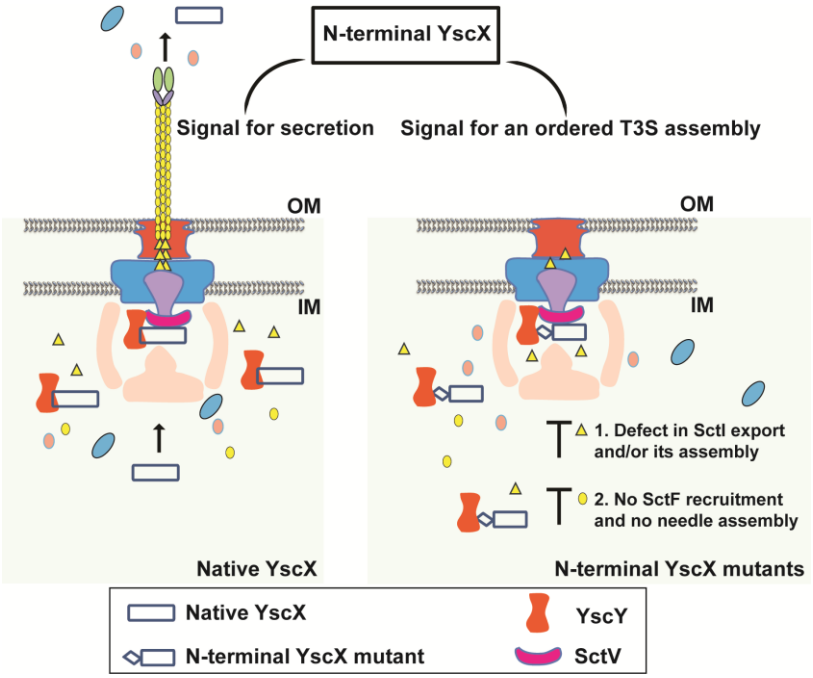

Figure 9. Model of N-terminal YscX mediated control of the Ysc-Yop T3SS. The N-terminal region of YscX contains an independent secretion signal that mediates its secretion. Manipulation of this N-terminal region either by site-directed mutagenesis or by defined domain swapping with equivalent region from other T3S substrates (N-terminal YscX mutant) completely abrogates T3SS activity. This is not a consequence of a defective bipartite interaction with YscY or tripartite interaction with YscY and SctV. Moreover, this is also not a localization defect of N-terminal YscX mutant. The YscX N-terminus contains additional recognition signal necessary for export and/or assembly of early substrates SctI and SctF.

This thesis has sought to unveil the significance of YscX secretion in the context of T3S biogenesis. The N-terminal region of YscX is important for its secretion and for constitution of a functional T3SS (Paper III, Figure 2). However, any attempt to uncouple YscX secretion from YscX dependent T3SS, either by site-specific N-terminal deletion or by defined N-terminal domain exchange with heterologous secretion signal was futile, as bacteria harbouring non-secreted YscX variants always possessed an inactive T3SS (Paper III, Figure 2, Figure 4 and Figure 5). Correlation between lack of YscX secretion and loss of T3SS activity was mirrored by the complementation efficiency of different YscX chimeras carrying defined domains from its homologous proteins, as measured by the ability to restore T3SS in the Δ*yscX* null mutant (Paper II, Figure 2). The

two N-terminal deletion mutants of *Yersinia* harbouring $YscX_{\Delta 8\text{-}12}$ and $YscX_{\Delta 13\text{-}22}$ clearly showed impairment in T3SS activity (Paper III, Figure 2). In this context, epistasis experiments were an important part of this work. When $YscX_{\Delta 8\text{-}12}$ and $YscX_{\Delta 13\text{-}22}$ were expressed in parental *Y. pseudotuberculosis* with an intact T3SS, the two variants could not be secreted by an active system (Paper III, Figure 7). An inability of these variants to be secreted by an active system, or even interfere with the secretion process, means that the active apparatus does not recognise them. The deletion has not only removed a substrate recognition motif, but it has removed a structure component necessary for YscX involvement in T3SS biogenesis and function.

3.2.5. YscX N-terminus and translational control - a mechanism for coordinated T3SS?

Evidence indicating the role of N-terminal YscX in translational control comes from the complementation study (Paper I) in which the homologs of YscX were appended with a 5' FLAG™ tag to check for the steady-state accumulation of intracellular proteins in bacteria grown under T3SS inducing conditions. Sequences of the YscX homologues were optimized to *Y. pseudotuberculosis* codon usage and their expression driven from a plasmid with an identical transcriptional start site and an identical Shine-Dalgarno sequence to the FLAG™-YscX expression. To our surprise, accumulated productions of some of the homologs, notably from *Aeromonas* spp., were significantly lower than the level of FLAG™-YscX production (Paper I, Figure 6). Significantly, the low level of protein production was not a reflection of poor transcriptional output, for all *yscX*-like alleles showed equivalent and stable mRNA production (Paper I, Figure S7). Computational analysis of codon usage bias between 5' coding sequence of *yscX* and variants revealed abundance of *Yersinia* preferred codons localized at the N-terminus (Paper 1, Figure S4). Altogether, these observations hint towards the N-terminus of YscX being codon optimised to impart translational control. This observation was reinforced by YscX-$AscX._{opt}$ chimeras, in which $YscX_{30}.AscX._{opt}$ produced much higher intracellular protein level compared to $YscX_{15}.AscX._{opt}$ (Paper II, Figure S2). Similar correlation between the length of the 5' end of *yscX* and steady-state production of YscX-Bla fusion was also observed in paper III (Figure 1). A notable production of the β-lactamase reporter protein was detected when it was fused to the first 25 amino acids of YscX (while translational fusions with larger YscX variants were less stable). When equivalent translational fusions were generated in which N-terminal region of YscX spanning 2 to 15 codons were replaced with

proportionate secretory signals from other T3S substrates, it revealed a completely different protein production profile. While $YscX_{YscF\text{-}Nterm}$ and $YscX_{PscX\text{-}Nterm}$ failed to reach a production level of $YscX_{25}$-Bla, appending $YscX_{LcrQN\text{-}term}$, $YscX_{YopD\text{-}Nterm}$ and $YscX_{YopE\text{-}Nterm}$ permitted approximately 20 times more production of the reporter protein. This further signified that information encoded in the coding sequence of YscX N-terminus is important in controlled production of the protein. It also suggested the notion that optimal production of T3S substrates via N-terminal translational control could be one mechanism to orchestrate coordinated T3SS assembly. Closer inspection of our result showed a difference in the production level between $YscX_{YopD\text{-}Nterm}$ and $YscX_{YopE\text{-}Nterm}$ consistent with an unrelated study from this laboratory (249). Indeed, advanced microscopy combined with high resolution proteomics analysis have deduced the stoichiometry of different T3S components to have a heterogeneous composition (124,145,354). Therefore, N-terminal mediated control of protein production could befit appropriate T3S components stoichiometry and reflect one facet of coordinated T3SS biogenesis. For future experiments, it would be interesting to scramble the YscX N-terminus, altering the codon usage while maintaining native amino acid sequence. The prediction would be that this mutation would compromise translation efficiency, to affect timing and amounts of produced YscX. The biological effects of which could be assess by experiments already described herein that examine T3SS biogenesis and activity.

3.3. Role of YopN-TyeA in orchestrating T3SS

Heterogeneous member proteins of the SctW family orchestrate hierarchical secretion of T3S translocator and effector substrates by interacting with different components of the cytosolic complex (see section 1.9.3.3.). In *Yersinia* Ysc-Yop T3SS, the equivalent protein is encoded as two functional and independent YopN and TyeA polypeptides. Interestingly, in *Y. pestis* and *Y. pseudotuberculosis* but not in *Y. enterocolitica*, a singular YopN-TyeA functional hybrid is also produced (see section 1.9.3.3.1.). Deletion of YopN and/or TyeA in *Yersinia* leads to constitutive production and secretion of Yop proteins *in vitro* implying that the member proteins are not truly involved in prioritizing translocators and effectors secretion (330,335). Then again, *in vitro* induced Yops synthesis and secretion by growth in Ca^{2+}-depleted media is an all or none phenomenon (226), and these assays may therefore not have sufficient discriminatory power to separate translocator and effector secretion. Nevertheless, the current model suggests YopN and TyeA associate together and along with the YopN cognate T3S

chaperones YscB and SycN to form a plug at the cytosolic interface of the T3SS (221,327,337). Environmental cues like host-cell contact (or $-Ca^{2+}$ *in vitro*) are sensed by the needle tip, transduced through the needle to the cytosolic side of the needle complex, and this triggers YscB-SycN-YopN-TyeA complex dissociation (68,177,323). Eventually, YopN is secreted allowing a surge of translocator and effector secretion. Multiple functions of YopN-mediated T3SS control can be assigned to discrete domains (see section 1.9.3.3.2.). Recently, a study by Amer and colleagues dissected the role of the YopN C-terminus in YopN-TyeA hybrid formation and the biological relevance to T3SS (330). By introducing +1 frame-shifts to *yopN* after codon 278 or 287, the study engineered four *Y. pseudotuberculosis* mutants that could predominantly produce YopN-TyeA hybrid. Like the parental *Yersinia*, mutant bacteria maintained calcium dependent T3SS regulation *in vitro* and also resisted phagocytosis and killing by J774A.1 macrophage-like immune cells. However, *Yersinia* producing YopN-TyeA hybrid variants with significant codon alteration between 278 and 287 in YopN were partially defective in the polarized translocation of YopE into HeLa cell cytosol and were out-competed by parental *Yersinia* during *in vivo* mouse infection. This prompted a study in this thesis with the goal to further dissect the C-terminal coding sequence of YopN for its role in T3S control.

3.3.1. Importance of YopN C-terminus in T3SS control

To further investigate the biological relevance of YopN C-terminus for T3S activity in *Y. pseudotuberculosis*, five *in cis* site-directed mutagenesis were performed within 3' end of *yopN* by replacing the wild type allele in the virulence plasmid (Paper IV, Figure 1). The first set of mutants ($YopN_{279(F+1),\ 287(F-1)}$, $YopN_{279(F+1),287STOP}$ and $YopN_{279STOP}$) produced YopN variants with significant disparity within the region encompassing 279 to 287 amino acids. The second set of mutants ($YopN_{288(scramble)293}$ and $YopN_{288STOP}$) expressed YopN variants that altered the last six YopN residues that overlapped with the translational start of TyeA. Importantly, none of the mutations affected the integrity of the TyeA coding sequence.

Despite stable production of all YopN variants (Paper IV, Figure 4), *Yersinia* producing $YopN_{279(F+1),\ 287(F-1)}$, $YopN_{279(F+1),287STOP}$ and $YopN_{279STOP}$ exhibited a null *yopN* mutant phenotype when assessed for different aspects of T3SS. Firstly, when the low calcium response growth phenotype was assayed, the mutants were termed 'temperature sensitive' as growth was severely affected at 37º C regardless of Ca^{2+} concentration (Paper IV, Figure S1). This phenotype is

suggestive of a deregulated T3SS that constitutively produces and secretes Yops regardless of Ca^{2+}. Indeed, all three mutants showed deregulated production and secretion of Yop proteins (Paper IV, Figure 2). Further, the bacteria were ineffective in protecting against phagocytosis by J774-1 mouse macrophage-like immune cells (Paper IV, Figure 3). On the other hand, the $YopN_{288(scramble)293}$ and $YopN_{288STOP}$ producing *Yersinia* mutants maintained tight control of T3SS regulation and mirrored parental bacteria with respect to T3S activity (Paper IV, Figure 2 and Figure 3).

3.3.2. $YopN_{W279}$ and $TyeA_{F8}$ stabilize YopN-TyeA complex to control T3S activity

By binding to regions of YopN encompassing (amino acids 212-222, and 248 to 293) amino acids, it has been suggested that TyeA helps to plug the T3SS base and stall Yops secretion until host-cell contact (-Ca^{2+} signal *in vitro*) is achieved. As the YopN-TyeA association ensures strict regulation of T3SS activity, the deregulated YopN mutants might have lost the ability to interact with TyeA. Using the available three-dimensional structure of YopN-TyeA complex, it was noticed that the tryptophan residue at 279 position (W_{279}) of the YopN C-terminus formed extensive hydrophobic contacts with TyeA residues, notably tyrosine at position 3 (Y_3), leucine at position 5 (L_5), phenylalanine at position 8 (F_8) and phenylalanine at position 33 (F_{33}) (Paper IV, Figure 6). In what is more than a coincidence, in two of the three deregulated YopN variants, W_{279} of YopN was interchanged with glycine in $YopN_{279(F+1),\ 287(F-1)}$ and $YopN_{279(F+1),287STOP}$, while it encoded for a stop codon in $YopN_{279STOP}$. However, in the remaining two mutants that behaved like native YopN, the W_{279} residue remained unchanged. Thus, amino acid residues of YopN-TyeA binding interface were mutated to either non-reactive glycine ($YopN_{W279G}$), aconservative phenylalanine ($YopN_{W279F}$), or to non-reactive alanine ($TyeA_{Y3A}$, $TyeA_{L5A}$, $TyeA_{F8A}$, and $TyeA_{F33A}$). All six mutants were tested for their possible involvement in YopN-TyeA complex formation by yeast two-hybrid system or BACTH assay. Data from the interaction assays revealed that W_{279} of YopN and F_8 of TyeA contributed to stable YopN-TyeA complex formation (Paper IV, Figure 5). Upon introduction of these mutations *in cis* on the virulence plasmid in *Y. pseudotuberculosis*, their relevance to T3SS activity was analysed. Once again, *Y. pseudotuberculosis* producing $YopN_{W279G}$ and $TyeA_{F8A}$ was de-regulated for Yops synthesis and secretion, and blind to the presence of Ca^{2+} in the growth medium (Paper IV, Figure 8). Essentially, they behaved like a null *yopN* mutant for a range of T3SS phenotypes analysed. Taken together, W_{279} of YopN and F_8 of TyeA mediates

hydrophobic contact to stabilize YopN and TyeA complex formation, which is important to maintain a co-ordinated T3SS.

4. Main findings of this thesis

- Member proteins from YscX, YscY and YscV family could establish YscX-YscY binary and YscX-YscY-YscV ternary interactions with its native cognate partners or cross-reciprocal interactions with non-cognate partners (Figure 10B).
- YscX and YscY have evolved to maintain function(s) specific to *Yersinia* T3SS.
- Substitution of uniquely conserved cysteines (Cys_{25} and Cys_{104}) of YscX abrogated its secretion and T3SS activity.
- The N-terminal region of YscX might be optimised for translational control (Figure 10A).
- The N-terminal region of YscX contains an efficient secretion signal (Figure 10E).
- The N-terminal region of YscX contains non-redundant structural signal that enables correct localization and/or assembly of inner adapter SctI and needle protein SctF (Figure 10C, Figure 10D).
- Non-secreted YscX variants that maintained binary interaction involving YscY and ternary interactions involving SctV are identified as promising tools for future experimental work.
- Sub-cellular distribution of T3S components revealed YscX is enriched in the inner membrane fraction.
- The YopN C-terminus spanning 279 to 287 amino acids is critical for maintaining its T3SS regulatory control (Figure 10F).
- The extreme last six amino acids in the YopN C-terminus are functionally redundant.
- The tryptophan at 279 amino acid of YopN ($YopN_{W279}$) makes hydrophobic contact with phenylalanine at 8 amino acid of TyeA ($TyeA_{F8}$) to establish control of T3SS activity.

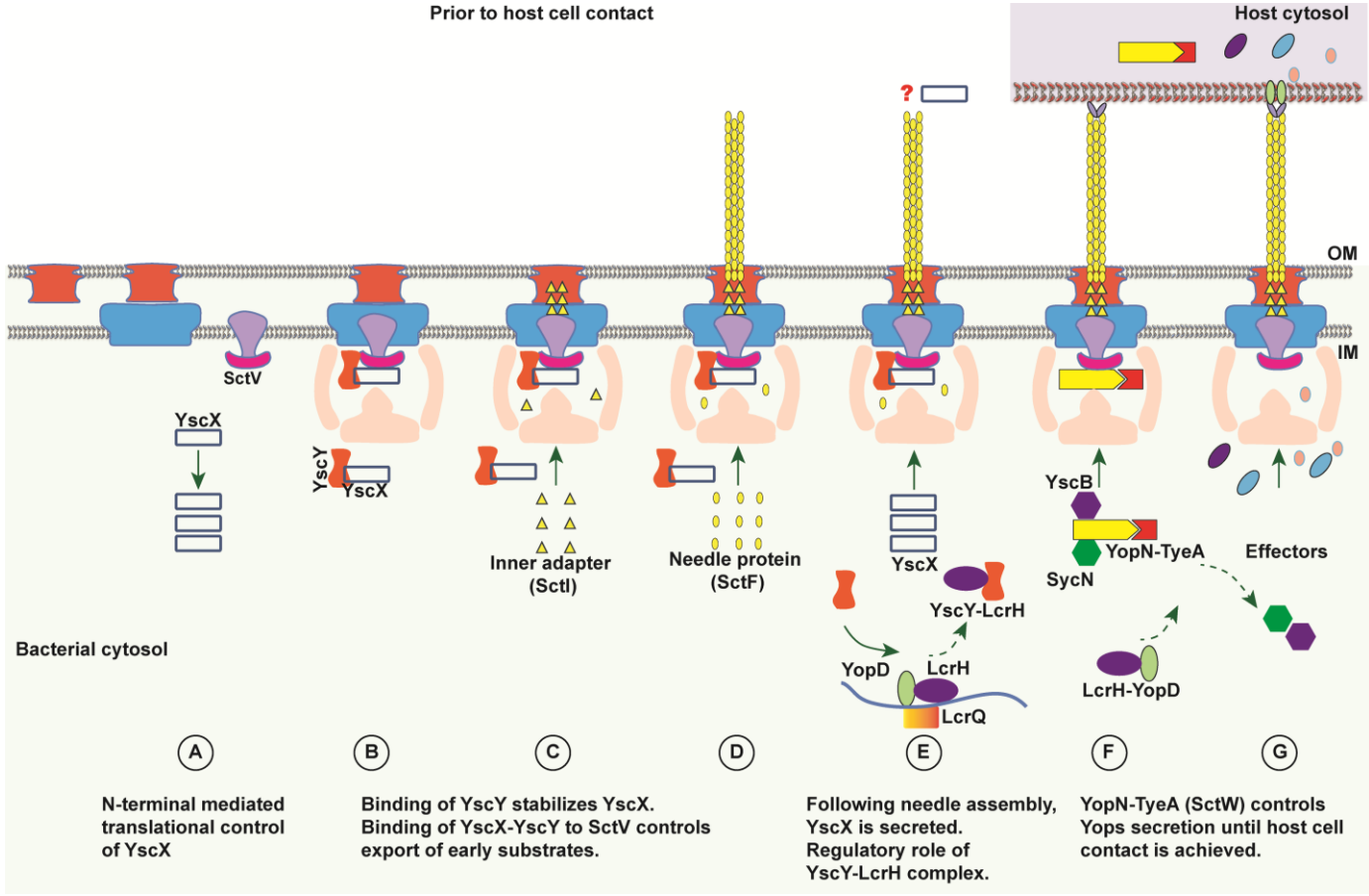
Prior to host cell contact
Host cytosol
?
OM
IM
SctV
YscX
YscY
YscX
Inner adapter
(SctI)
Needle protein
(SctF)
YscX
YscY-LcrH
YopD
LcrH
LcrQ
YscB
YopN-TyeA
SycN
LcrH-YopD
Effectors
Bacterial cytosol
A
B
C
D
E
F
G
N-terminal mediated translational control of YscX
Binding of YscY stabilizes YscX. Binding of YscX-YscY to SctV controls export of early substrates.
Following needle assembly, YscX is secreted. Regulatory role of YscY-LcrH complex.
YopN-TyeA (SctW) controls Yops secretion until host cell contact is achieved.

Figure 10. Molecular model representing the coordinated progression of the Ysc-Yop T3SS biogenesis. T3SS assembly occurs in a sequential step-wise fashion. In the early stage prior to host cell contact, YscX interacts with YscY, which together engage with the inner membrane export component SctV. The tripartite complex of YscX-YscY-SctV is implicated to control the export and/or assembly of inner adapter SctI and needle protein SctF. Following needle assembly, YscX is secreted. The N-terminal region of YscX is specifically important both for its secretion and early stage of T3SS assembly. Moreover, the role of YscX N-terminus in translational control is also evident. Following YscX secretion, YscY is probably recycled to perform regulatory role via the YscY-LcrH loop. During this phase, YopN-TyeA in association with its chaperones plug the basal body and prevent Yops secretion until host cell contact is achived by the bacteria. Upon cell contact, YopN-TyeA plug is released followed by secretion of LcrQ. LcrH then pilots YopD to the T3S base to promote its secretion that together with YopB forms the translocon pore in host cell membrane. Eventually, host-induced conformational change of translocon pore triggers secretion of effectors inside host cell.

5. Future perspectives

It is known that several regulatory mechanisms work in concert to ensure hierarchical and temporal control of T3SS. However, several outstanding questions still prevail regarding processes that establish an ordered T3SS biogenesis. The objective of this thesis was to uncover additional regulatory mechanisms that mediate temporal control of T3SS assembly. By investigating functions of YscX and YscY that mediates early stage biogenesis of T3SS assembly and YopN-TyeA complex that mediates transition to late stage of T3SS assembly, the thesis has provided some novel aspects critical for the general understanding of T3SS assembly. On the other hand, the work has also generated additional interesting questions.

One important finding of the thesis was the unique need of YscX and YscY in Ysc-Yop T3SS. Closely related YscX and YscY family members cannot substitute these roles. Attempts to delineate genetic elements specific to YscX used the approach of swapping defined domains of YscX with equivalent domains from its homologs to generate a series of YscX chimeras, followed by their introduction in Δ*yscX* null mutant to achieve gain-of-function of T3SS activity. The approach was unable to map specific region that brings YscX specificity. As this remains a critical question, an alternative approach would be to perform comprehensive alanine scanning mutagenesis of YscX, and screen for loss-of-function to identify unique region(s) in YscX. One such example is the targeted mutagenesis of uniquely conserved cysteines of YscX ($YscX_{C25A,C104A}$) that abrogates both its secretion and T3SS activity. The reason for loss of T3SS activity in bacteria haboring $YscX_{C25A,C104A}$ needs to be investigated. A current working hypothesis is that disruption of both cysteines results in a defective interaction with YscY and/or YscV, or alters the targeting of YscX to the inner membrane. Alternatively, it can be a defect in its secretion competence, which can be measured by generating $FLAG^{TM}$-$YscX_{C25A,C104A}$ and expressing in parental *Y. pseudotuberculosis* with an intact T3SS. $FLAG^{TM}$-tagged YscX variant that allows discrimination from native YscX can be used to uncouple YscX secretion from YscX-mediated T3SS activity.

Site-directed mutagenesis of YscX also revealed the importance of N-terminal region of YscX in T3SS. Besides encoding an efficient secretion signal, the N-terminal region also confers structural information critical for proper localization and/or assembly of inner adapter SctI and needle protein SctF. Based on sub-

cellular fractionation of T3SS components and its interaction with SctV, YscX is proposed to localize at the base of the T3SS. However, alternative approach that involves developing a fluorescent-tagged YscX to monitor YscX targeting dynamics by fluorescent microscopy and determining localization of YscX by comparing injectisome structure from Δ*yscX* and WT *Yersinia* with cryo-electron microscopy would improve the accuracy of the observations.

The actual mechanism of how YscX N-terminus facilitates spatiotemporal control of SctI inner adapter and SctF needle protein still remains elusive. Firstly, YscX could mediate this function by direct association with SctI/SctF or by engaging with members of the cytosolic complex to change its conformational state. However, immunoprecipitation of His-tagged YscX to capture novel interacting partners was unsuccessful. Secondly, binding of YscX and YscY to SctV could induce its conformational rearrangement to allow recognition and secretion of T3S substrates. One of the approaches to address this issue is to utilize an interdisciplinary approach combining structural and molecular biology with advanced microscopy. For instance, *in silico* modelling or co-crystallization of YscX-YscY binding to YscV can provide atomic resolution of conformational changes that occur during the substrate recognition process of SctV. Significantly, to have access to structural information would enable structure-based predictions of the protein interacting interfaces. These interfaces could be targeted for mutagenesis, and the biological consequences investigated.

Although, the thesis has covered correlation between YscX secretion and T3SS activity, the role of secreted YscX is completely unknown. One working hypothesis was that secreted YscX formed a component of the needle filament. However, probing purified needle fractions from *Y. enterocolitica* with anti-YscX antisera did not reveal YscX. However, since only a minor fraction of YscX is secreted, YscX might only be a minor component within the needle filament, and this is not trivial to probe. Ideally, developing an immunolabelled YscX and visualizing its presence in purified needle by electron microscopy would be the most sensitive assay. However, this is a complicated approach, with no guarantees of success.

One caveat of our study was the use of low Ca^{2+} levels to activate T3SS *in vitro* and mimic host cell intracellular environment. However, Ca^{2+} dependent induction of T3SS is reminiscent of the ’all-or-none phenomenon’ and might not mechanistically represent host cell contact derieved T3S inducing signals (226). Moreover, the ’all-or-none phenomenon’ does not allow sensitivity to

discriminate small variations in spatio-temporal regulation of T3SS. Therefore, developing techinques to visualize real time assembly and action of T3SS upon eukaryotic cell contact would greatly improve the resolution of our study.

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7. References

1. Alegado, R. A., and King, N. (2014) Bacterial influences on animal origins. *Cold Spring Harb Perspect Biol* **6**, a016162
2. Ciccarelli, F. D., Doerks, T., von Mering, C., Creevey, C. J., Snel, B., and Bork, P. (2006) Toward automatic reconstruction of a highly resolved tree of life. *Science* **311**, 1283-1287
3. Pace, N. R. (2009) Mapping the tree of life: progress and prospects. *Microbiol Mol Biol Rev* **73**, 565-576
4. Hug, L. A., Baker, B. J., Anantharaman, K., Brown, C. T., Probst, A. J., Castelle, C. J., Butterfield, C. N., Hernsdorf, A. W., Amano, Y., Ise, K., Suzuki, Y., Dudek, N., Relman, D. A., Finstad, K. M., Amundson, R., Thomas, B. C., and Banfield, J. F. (2016) A new view of the tree of life. *Nat Microbiol* **1**, 16048
5. Woese, C. R., Kandler, O., and Wheelis, M. L. (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci U S A* **87**, 4576-4579
6. Gupta, R. S. (2000) The phylogeny of proteobacteria: relationships to other eubacterial phyla and eukaryotes. *FEMS Microbiol Rev* **24**, 367-402
7. Adeolu, M., Alnajar, S., Naushad, S., and R, S. G. (2016) Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. nov. *Int J Syst Evol Microbiol* **66**, 5575-5599
8. Ibrahim, A., Goebel, B. M., Liesack, W., Griffiths, M., and Stackebrandt, E. (1993) The phylogeny of the genus Yersinia based on 16S rDNA sequences. *FEMS Microbiol Lett* **114**, 173-177
9. Sulakvelidze, A. (2000) Yersiniae other than Y. enterocolitica, Y. pseudotuberculosis, and Y. pestis: the ignored species. *Microbes Infect* **2**, 497-513
10. Carniel, E. (2003) Evolution of pathogenic Yersinia, some lights in the dark. *Adv Exp Med Biol* **529**, 3-12
11. McNally, A., Thomson, N. R., Reuter, S., and Wren, B. W. (2016) 'Add, stir and reduce': Yersinia spp. as model bacteria for pathogen evolution. *Nat Rev Microbiol* **14**, 177-190
12. Perry, R. D., and Fetherston, J. D. (1997) Yersinia pestis--etiologic agent of plague. *Clin Microbiol Rev* **10**, 35-66
13. Wrobel, A., Leo, J. C., and Linke, D. (2019) Overcoming Fish Defences: The Virulence Factors of Yersinia ruckeri. *Genes (Basel)* **10**
14. Wren, B. W. (2003) The yersiniae--a model genus to study the rapid evolution of bacterial pathogens. *Nat Rev Microbiol* **1**, 55-64
15. Reuter, S., Connor, T. R., Barquist, L., Walker, D., Feltwell, T., Harris, S. R., Fookes, M., Hall, M. E., Petty, N. K., Fuchs, T. M., Corander, J., Dufour, M., Ringwood, T., Savin, C., Bouchier, C., Martin, L., Miettinen, M., Shubin, M., Riehm, J. M., Laukkanen-Ninios, R., Sihvonen, L. M., Siitonen, A., Skurnik, M., Falcao, J. P., Fukushima, H., Scholz, H. C.,

Prentice, M. B., Wren, B. W., Parkhill, J., Carniel, E., Achtman, M., McNally, A., and Thomson, N. R. (2014) Parallel independent evolution of pathogenicity within the genus Yersinia. *Proc Natl Acad Sci U S A* **111**, 6768-6773

16. Sun, Y. C., Jarrett, C. O., Bosio, C. F., and Hinnebusch, B. J. (2014) Retracing the evolutionary path that led to flea-borne transmission of Yersinia pestis. *Cell Host Microbe* **15**, 578-586
17. Tan, S. Y., Tan, I. K., Tan, M. F., Dutta, A., and Choo, S. W. (2016) Evolutionary study of Yersinia genomes deciphers emergence of human pathogenic species. *Sci Rep* **6**, 36116
18. Chen, S., Thompson, K. M., and Francis, M. S. (2016) Environmental Regulation of Yersinia Pathophysiology. *Front Cell Infect Microbiol* **6**, 25
19. Galindo, C. L., Rosenzweig, J. A., Kirtley, M. L., and Chopra, A. K. (2011) Pathogenesis of Y. enterocolitica and Y. pseudotuberculosis in Human Yersiniosis. *J Pathog* **2011**, 182051
20. Davis, K. M. (2018) All Yersinia Are Not Created Equal: Phenotypic Adaptation to Distinct Niches Within Mammalian Tissues. *Front Cell Infect Microbiol* **8**, 261
21. Demeure, C., Dussurget, O., Fiol, G. M., Le Guern, A. S., Savin, C., and Pizarro-Cerda, J. (2019) Yersinia pestis and plague: an updated view on evolution, virulence determinants, immune subversion, vaccination and diagnostics. *Microbes Infect* **21**, 202-212
22. Rasmussen, S., Allentoft, M. E., Nielsen, K., Orlando, L., Sikora, M., Sjogren, K. G., Pedersen, A. G., Schubert, M., Van Dam, A., Kapel, C. M., Nielsen, H. B., Brunak, S., Avetisyan, P., Epimakhov, A., Khalyapin, M. V., Gnuni, A., Kriiska, A., Lasak, I., Metspalu, M., Moiseyev, V., Gromov, A., Pokutta, D., Saag, L., Varul, L., Yepiskoposyan, L., Sicheritz-Ponten, T., Foley, R. A., Lahr, M. M., Nielsen, R., Kristiansen, K., and Willerslev, E. (2015) Early divergent strains of Yersinia pestis in Eurasia 5,000 years ago. *Cell* **163**, 571-582
23. Spyrou, M. A., Tukhbatova, R. I., Wang, C. C., Valtuena, A. A., Lankapalli, A. K., Kondrashin, V. V., Tsybin, V. A., Khokhlov, A., Kuhnert, D., Herbig, A., Bos, K. I., and Krause, J. (2018) Analysis of 3800-year-old Yersinia pestis genomes suggests Bronze Age origin for bubonic plague. *Nat Commun* **9**, 2234
24. Morelli, G., Song, Y., Mazzoni, C. J., Eppinger, M., Roumagnac, P., Wagner, D. M., Feldkamp, M., Kusecek, B., Vogler, A. J., Li, Y., Cui, Y., Thomson, N. R., Jombart, T., Leblois, R., Lichtner, P., Rahalison, L., Petersen, J. M., Balloux, F., Keim, P., Wirth, T., Ravel, J., Yang, R., Carniel, E., and Achtman, M. (2010) Yersinia pestis genome sequencing identifies patterns of global phylogenetic diversity. *Nat Genet* **42**, 1140-1143
25. Stenseth, N. C., Atshabar, B. B., Begon, M., Belmain, S. R., Bertherat, E., Carniel, E., Gage, K. L., Leirs, H., and Rahalison, L. (2008) Plague: past, present, and future. *PLoS Med* **5**, e3
26. Spyrou, M. A., Tukhbatova, R. I., Feldman, M., Drath, J., Kacki, S., de Heredia, J. B., Arnold, S., Sitdikov, A. G., Castex, D., Wahl, J., Gazimzyanov, I. R., Nurgaliev, D. K., Herbig, A., Bos, K. I., and Krause, J. (2016) Historical Y. pestis Genomes Reveal the European Black Death

as the Source of Ancient and Modern Plague Pandemics. *Cell Host & Microbe* **19**, 874-881
27. Hinnebusch, B. J., Jarrett, C. O., and Bland, D. M. (2017) "Fleaing" the Plague: Adaptations of Yersinia pestis to Its Insect Vector That Lead to Transmission. *Annu Rev Microbiol* **71**, 215-232
28. Hinnebusch, B. J., Rudolph, A. E., Cherepanov, P., Dixon, J. E., Schwan, T. G., and Forsberg, A. (2002) Role of Yersinia murine toxin in survival of Yersinia pestis in the midgut of the flea vector. *Science* **296**, 733-735
29. Eisen, R. J., Bearden, S. W., Wilder, A. P., Montenieri, J. A., Antolin, M. F., and Gage, K. L. (2006) Early-phase transmission of Yersinia pestis by unblocked fleas as a mechanism explaining rapidly spreading plague epizootics. *Proc Natl Acad Sci U S A* **103**, 15380-15385
30. Bland, D. M., Jarrett, C. O., Bosio, C. F., and Hinnebusch, B. J. (2018) Infectious blood source alters early foregut infection and regurgitative transmission of Yersinia pestis by rodent fleas. *PLoS Pathog* **14**, e1006859
31. Gandon, S., Heitzmann, L., and Sebbane, F. (2019) To block or not to block: The adaptive manipulation of plague transmission. *Evol Lett* **3**, 152-161
32. Heroven, A. K., and Dersch, P. (2014) Coregulation of host-adapted metabolism and virulence by pathogenic yersiniae. *Front Cell Infect Microbiol* **4**, 146
33. Zimbler, D. L., Schroeder, J. A., Eddy, J. L., and Lathem, W. W. (2015) Early emergence of Yersinia pestis as a severe respiratory pathogen. *Nat Commun* **6**, 7487
34. Pechous, R. D., Sivaraman, V., Stasulli, N. M., and Goldman, W. E. (2016) Pneumonic Plague: The Darker Side of Yersinia pestis. *Trends Microbiol* **24**, 190-197
35. Gage, K. L. (2012) Factors affecting the spread and maintenance of plague. *Adv Exp Med Biol* **954**, 79-94
36. Autenrieth, I. B., and Firsching, R. (1996) Penetration of M cells and destruction of Peyer's patches by Yersinia enterocolitica: an ultrastructural and histological study. *J Med Microbiol* **44**, 285-294
37. Pepe, J. C., Wachtel, M. R., Wagar, E., and Miller, V. L. (1995) Pathogenesis of defined invasion mutants of Yersinia enterocolitica in a BALB/c mouse model of infection. *Infect Immun* **63**, 4837-4848
38. Isberg, R. R., and Leong, J. M. (1990) Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. *Cell* **60**, 861-871
39. Dersch, P., and Isberg, R. R. (1999) A region of the Yersinia pseudotuberculosis invasin protein enhances integrin-mediated uptake into mammalian cells and promotes self-association. *Embo J* **18**, 1199-1213
40. Marra, A., and Isberg, R. R. (1997) Invasin-dependent and invasin-independent pathways for translocation of Yersinia pseudotuberculosis across the Peyer's patch intestinal epithelium. *Infect Immun* **65**, 3412-3421

41. Mikula, K. M., Kolodziejczyk, R., and Goldman, A. (2012) Yersinia infection tools-characterization of structure and function of adhesins. *Front Cell Infect Microbiol* **2**, 169
42. Fahlgren, A., Avican, K., Westermark, L., Nordfelth, R., and Fallman, M. (2014) Colonization of cecum is important for development of persistent infection by Yersinia pseudotuberculosis. *Infect Immun* **82**, 3471-3482
43. Avican, K., Fahlgren, A., Huss, M., Heroven, A. K., Beckstette, M., Dersch, P., and Fallman, M. (2015) Reprogramming of Yersinia from virulent to persistent mode revealed by complex in vivo RNA-seq analysis. *PLoS Pathog* **11**, e1004600
44. Girschick, H. J., Guilherme, L., Inman, R. D., Latsch, K., Rihl, M., Sherer, Y., Shoenfeld, Y., Zeidler, H., Arienti, S., and Doria, A. (2008) Bacterial triggers and autoimmune rheumatic diseases. *Clin Exp Rheumatol* **26**, S12-17
45. Colmegna, I., Cuchacovich, R., and Espinoza, L. R. (2004) HLA-B27-associated reactive arthritis: pathogenetic and clinical considerations. *Clin Microbiol Rev* **17**, 348-369
46. Sanno, A., Aspan, A., Hestvik, G., and Jacobson, M. (2014) Presence of Salmonella spp., Yersinia enterocolitica, Yersinia pseudotuberculosis and Escherichia coli O157:H7 in wild boars. *Epidemiol Infect* **142**, 2542-2547
47. Ye, Q., Wu, Q., Hu, H., Zhang, J., and Huang, H. (2015) Prevalence, antimicrobial resistance and genetic diversity of Yersinia enterocolitica isolated from retail frozen foods in China. *FEMS Microbiol Lett* **362**, fnv197
48. Brubaker, R. R. (1991) Factors promoting acute and chronic diseases caused by yersiniae. *Clin Microbiol Rev* **4**, 309-324
49. Zhou, D., and Yang, R. (2009) Molecular Darwinian evolution of virulence in Yersinia pestis. *Infect Immun* **77**, 2242-2250
50. Rosqvist, R., Skurnik, M., and Wolf-Watz, H. (1988) Increased virulence of Yersinia pseudotuberculosis by two independent mutations. *Nature* **334**, 522-524
51. Muhlenkamp, M., Oberhettinger, P., Leo, J. C., Linke, D., and Schutz, M. S. (2015) Yersinia adhesin A (YadA)--beauty & beast. *Int J Med Microbiol* **305**, 252-258
52. Parkhill, J., Wren, B. W., Thomson, N. R., Titball, R. W., Holden, M. T., Prentice, M. B., Sebaihia, M., James, K. D., Churcher, C., Mungall, K. L., Baker, S., Basham, D., Bentley, S. D., Brooks, K., Cerdeno-Tarraga, A. M., Chillingworth, T., Cronin, A., Davies, R. M., Davis, P., Dougan, G., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Karlyshev, A. V., Leather, S., Moule, S., Oyston, P. C., Quail, M., Rutherford, K., Simmonds, M., Skelton, J., Stevens, K., Whitehead, S., and Barrell, B. G. (2001) Genome sequence of Yersinia pestis, the causative agent of plague. *Nature* **413**, 523-527
53. Guo, X. P., and Sun, Y. C. (2017) New Insights into the Non-orthodox Two Component Rcs Phosphorelay System. *Front Microbiol* **8**, 2014
54. Li, Y., Hu, Y., Francis, M. S., and Chen, S. (2015) RcsB positively regulates the Yersinia Ysc-Yop type III secretion system by activating expression of

the master transcriptional regulator LcrF. *Environ Microbiol* **17**, 1219-1233
55. Venecia, K., and Young, G. M. (2005) Environmental regulation and virulence attributes of the Ysa type III secretion system of Yersinia enterocolitica biovar 1B. *Infect Immun* **73**, 5961-5977
56. Hinchliffe, S. J., Howard, S. L., Huang, Y. H., Clarke, D. J., and Wren, B. W. (2008) The importance of the Rcs phosphorelay in the survival and pathogenesis of the enteropathogenic yersiniae. *Microbiology* **154**, 1117-1131
57. Sun, Y. C., Hinnebusch, B. J., and Darby, C. (2008) Experimental evidence for negative selection in the evolution of a Yersinia pestis pseudogene. *Proc Natl Acad Sci U S A* **105**, 8097-8101
58. Lawrenz, M. B. (2010) Model systems to study plague pathogenesis and develop new therapeutics. *Front Microbiol* **1**
59. Cornelis, G. R., Boland, A., Boyd, A. P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M. P., and Stainier, I. (1998) The virulence plasmid of Yersinia, an antihost genome. *Microbiol Mol Biol Rev* **62**, 1315-1352
60. Joshua, G. W. P., Karlyshev, A. V., Smith, M. P., Isherwood, K. E., Titball, R. W., and Wren, B. W. (2003) A Caenorhabditis elegans model of Yersinia infection: biofilm formation on a biotic surface. *Microbiology* **149**, 3221-3229
61. Atkinson, S., Goldstone, R. J., Joshua, G. W., Chang, C. Y., Patrick, H. L., Camara, M., Wren, B. W., and Williams, P. (2011) Biofilm development on Caenorhabditis elegans by Yersinia is facilitated by quorum sensing-dependent repression of type III secretion. *PLoS Pathog* **7**, e1001250
62. Hinnebusch, B. J., Perry, R. D., and Schwan, T. G. (1996) Role of the Yersinia pestis hemin storage (hms) locus in the transmission of plague by fleas. *Science* **273**, 367-370
63. Tan, L., and Darby, C. (2004) A movable surface: formation of Yersinia sp. biofilms on motile Caenorhabditis elegans. *J Bacteriol* **186**, 5087-5092
64. Francis, M. S., Wolf-Watz, H., and Forsberg, Å. (2002) Regulation of type III secretion systems. *Curr Opin Microbiol* **5**, 166-172
65. Galan, J. E., and Wolf-Watz, H. (2006) Protein delivery into eukaryotic cells by type III secretion machines. *Nature* **444**, 567-573
66. Wagner, S., Grin, I., Malmsheimer, S., Singh, N., Torres-Vargas, C. E., and Westerhausen, S. (2018) Bacterial type III secretion systems: a complex device for the delivery of bacterial effector proteins into eukaryotic host cells. *FEMS Microbiol Lett* **365**
67. Dewoody, R. S., Merritt, P. M., and Marketon, M. M. (2013) Regulation of the Yersinia type III secretion system: traffic control. *Front Cell Infect Microbiol* **3**, 4
68. Armentrout, E. I., and Rietsch, A. (2016) The Type III Secretion Translocation Pore Senses Host Cell Contact. *PLoS Pathog* **12**, e1005530
69. Francis, M. S., and Wolf-Watz, H. (1998) YopD of Yersinia pseudotuberculosis is translocated into the cytosol of HeLa epithelial cells: evidence of a structural domain necessary for translocation. *Mol Microbiol* **29**, 799-813

70. Tsang, T. M., Felek, S., and Krukonis, E. S. (2010) Ail binding to fibronectin facilitates Yersinia pestis binding to host cells and Yop delivery. *Infect Immun* **78**, 3358-3368
71. Bancerz-Kisiel, A., Pieczywek, M., Lada, P., and Szweda, W. (2018) The Most Important Virulence Markers of Yersinia enterocolitica and Their Role during Infection. *Genes (Basel)* **9**
72. Biedzka-Sarek, M., Venho, R., and Skurnik, M. (2005) Role of YadA, Ail, and Lipopolysaccharide in Serum Resistance of Yersinia enterocolitica Serotype O:3. *Infect Immun* **73**, 2232-2244
73. Pepe, J. C., and Miller, V. L. (1993) Yersinia enterocolitica invasin: a primary role in the initiation of infection. *Proc Natl Acad Sci U S A* **90**, 6473-6477
74. Felek, S., and Krukonis, E. S. (2009) The Yersinia pestis Ail protein mediates binding and Yop delivery to host cells required for plague virulence. *Infect Immun* **77**, 825-836
75. Young, B. M., and Young, G. M. (2002) YplA is exported by the Ysc, Ysa, and flagellar type III secretion systems of Yersinia enterocolitica. *J Bacteriol* **184**, 1324-1334
76. Soscia, C., Hachani, A., Bernadac, A., Filloux, A., and Bleves, S. (2007) Cross talk between type III secretion and flagellar assembly systems in Pseudomonas aeruginosa. *J Bacteriol* **189**, 3124-3132
77. Troisfontaines, P., and Cornelis, G. R. (2005) Type III secretion: more systems than you think. *Physiology (Bethesda)* **20**, 326-339
78. Abby, S. S., and Rocha, E. P. (2012) The non-flagellar type III secretion system evolved from the bacterial flagellum and diversified into host-cell adapted systems. *PLoS Genet* **8**, e1002983
79. Pallen, M. J., Beatson, S. A., and Bailey, C. M. (2005) Bioinformatics, genomics and evolution of non-flagellar type-III secretion systems: a Darwinian perpective. *FEMS Microbiol Rev* **29**, 201-229
80. Horn, M., Collingro, A., Schmitz-Esser, S., Beier, C. L., Purkhold, U., Fartmann, B., Brandt, P., Nyakatura, G. J., Droege, M., Frishman, D., Rattei, T., Mewes, H. W., and Wagner, M. (2004) Illuminating the evolutionary history of chlamydiae. *Science* **304**, 728-730
81. Gophna, U., Ron, E. Z., and Graur, D. (2003) Bacterial type III secretion systems are ancient and evolved by multiple horizontal-transfer events. *Gene* **312**, 151-163
82. Hacker, J., and Kaper, J. B. (2000) Pathogenicity islands and the evolution of microbes. *Annu Rev Microbiol* **54**, 641-679
83. Winstanley, C., and Hart, C. A. (2001) Type III secretion systems and pathogenicity islands. *J Med Microbiol* **50**, 116-126
84. Kim, J. F. (2001) Revisiting the chlamydial type III protein secretion system: clues to the origin of type III protein secretion. *Trends Genet* **17**, 65-69
85. Diepold, A., and Armitage, J. P. (2015) Type III secretion systems: the bacterial flagellum and the injectisome. *Philos Trans R Soc Lond B Biol Sci* **370**
86. Haller, J. C., Carlson, S., Pederson, K. J., and Pierson, D. E. (2000) A chromosomally encoded type III secretion pathway in Yersinia enterocolitica is important in virulence. *Mol Microbiol* **36**, 1436-1446

87. Hansen-Wester, I., and Hensel, M. (2001) Salmonella pathogenicity islands encoding type III secretion systems. *Microbes Infect* **3**, 549-559
88. Park, K. S., Ono, T., Rokuda, M., Jang, M. H., Okada, K., Iida, T., and Honda, T. (2004) Functional characterization of two type III secretion systems of Vibrio parahaemolyticus. *Infect Immun* **72**, 6659-6665
89. Stevens, M. P., Haque, A., Atkins, T., Hill, J., Wood, M. W., Easton, A., Nelson, M., Underwood-Fowler, C., Titball, R. W., Bancroft, G. J., and Galyov, E. E. (2004) Attenuated virulence and protective efficacy of a Burkholderia pseudomallei bsa type III secretion mutant in murine models of melioidosis. *Microbiology* **150**, 2669-2676
90. Warawa, J., and Woods, D. E. (2005) Type III secretion system cluster 3 is required for maximal virulence of Burkholderia pseudomallei in a hamster infection model. *FEMS Microbiol Lett* **242**, 101-108
91. Lee, Y. H., Chen, Y., Ouyang, X., and Gan, Y. H. (2010) Identification of tomato plant as a novel host model for Burkholderia pseudomallei. *BMC Microbiol* **10**, 28
92. Vander Broek, C. W., and Stevens, J. M. (2017) Type III Secretion in the Melioidosis Pathogen Burkholderia pseudomallei. *Front Cell Infect Microbiol* **7**, 255
93. Foultier, B., Troisfontaines, P., Muller, S., Opperdoes, F. R., and Cornelis, G. R. (2002) Characterization of the ysa pathogenicity locus in the chromosome of Yersinia enterocolitica and phylogeny analysis of type III secretion systems. *J Mol Evol* **55**, 37-51
94. Walker, K. A., Maltez, V. I., Hall, J. D., Vitko, N. P., and Miller, V. L. (2013) A phenotype at last: essential role for the Yersinia enterocolitica Ysa type III secretion system in a Drosophila melanogaster S2 cell model. *Infect Immun* **81**, 2478-2487
95. Zhou, X., Shah, D. H., Konkel, M. E., and Call, D. R. (2008) Type III secretion system 1 genes in Vibrio parahaemolyticus are positively regulated by ExsA and negatively regulated by ExsD. *Mol Microbiol* **69**, 747-764
96. Hiyoshi, H., Kodama, T., Iida, T., and Honda, T. (2010) Contribution of Vibrio parahaemolyticus virulence factors to cytotoxicity, enterotoxicity, and lethality in mice. *Infect Immun* **78**, 1772-1780
97. Moest, T. P., and Meresse, S. (2013) Salmonella T3SSs: successful mission of the secret(ion) agents. *Curr Opin Microbiol* **16**, 38-44
98. Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galán, J. E., and Aizawa, S. I. (1998) Supramolecular structure of the Salmonella typhimurium type III protein secretion system. *Science* **280**, 602-605
99. Tamano, K., Aizawa, S., Katayama, E., Nonaka, T., Imajoh-Ohmi, S., Kuwae, A., Nagai, S., and Sasakawa, C. (2000) Supramolecular structure of the Shigella type III secretion machinery: the needle part is changeable in length and essential for delivery of effectors. *Embo J* **19**, 3876-3887
100. Hu, B., Lara-Tejero, M., Kong, Q., Galan, J. E., and Liu, J. (2017) In Situ Molecular Architecture of the Salmonella Type III Secretion Machine. *Cell* **168**, 1065-1074 e1010

101. Dohlich, K., Zumsteg, A. B., Goosmann, C., and Kolbe, M. (2014) A substrate-fusion protein is trapped inside the Type III Secretion System channel in Shigella flexneri. *PLoS Pathog* **10**, e1003881
102. Akopyan, K., Edgren, T., Wang-Edgren, H., Rosqvist, R., Fahlgren, A., Wolf-Watz, H., and Fallman, M. (2011) Translocation of surface-localized effectors in type III secretion. *Proc Natl Acad Sci U S A* **108**, 1639-1644
103. Deane, J. E., Abrusci, P., Johnson, S., and Lea, S. M. (2010) Timing is everything: the regulation of type III secretion. *Cell Mol Life Sci* **67**, 1065-1075
104. Hueck, C. J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**, 379-433
105. Deng, W., Marshall, N. C., Rowland, J. L., McCoy, J. M., Worrall, L. J., Santos, A. S., Strynadka, N. C. J., and Finlay, B. B. (2017) Assembly, structure, function and regulation of type III secretion systems. *Nat Rev Microbiol* **15**, 323-337
106. Notti, R. Q., and Stebbins, C. E. (2016) The Structure and Function of Type III Secretion Systems. *Microbiol Spectr* **4**
107. Michiels, T., Vanooteghem, J. C., Lambert de Rouvroit, C., China, B., Gustin, A., Boudry, P., and Cornelis, G. R. (1991) Analysis of virC, an operon involved in the secretion of Yop proteins by Yersinia enterocolitica. *J Bacteriol* **173**, 4994-5009
108. Kowal, J., Chami, M., Ringler, P., Muller, S. A., Kudryashev, M., Castano-Diez, D., Amstutz, M., Cornelis, G. R., Stahlberg, H., and Engel, A. (2013) Structure of the dodecameric Yersinia enterocolitica secretin YscC and its trypsin-resistant core. *Structure* **21**, 2152-2161
109. Hu, J., Worrall, L. J., and Strynadka, N. C. (2019) Towards capture of dynamic assembly and action of the T3SS at near atomic resolution. *Curr Opin Struct Biol* **61**, 71-78
110. Worrall, L. J., Hong, C., Vuckovic, M., Deng, W., Bergeron, J. R. C., Majewski, D. D., Huang, R. K., Spreter, T., Finlay, B. B., Yu, Z., and Strynadka, N. C. J. (2016) Near-atomic-resolution cryo-EM analysis of the Salmonella T3S injectisome basal body. *Nature* **540**, 597-601
111. Lunelli, M., Kamprad, A., Burger, J., Mielke, T., Spahn, C. M. T., and Kolbe, M. (2020) Cryo-EM structure of the Shigella type III needle complex. *PLoS Pathog* **16**, e1008263
112. Koster, M., Bitter, W., de Cock, H., Allaoui, A., Cornelis, G. R., and Tommassen, J. (1997) The outer membrane component, YscC, of the Yop secretion machinery of Yersinia enterocolitica forms a ring-shaped multimeric complex. *Mol Microbiol* **26**, 789-797
113. Burghout, P., Beckers, F., de Wit, E., van Boxtel, R., Cornelis, G. R., Tommassen, J., and Koster, M. (2004) Role of the pilot protein YscW in the biogenesis of the YscC secretin in Yersinia enterocolitica. *J Bacteriol* **186**, 5366-5375
114. Hu, J., Worrall, L. J., Vuckovic, M., Hong, C., Deng, W., Atkinson, C. E., Brett Finlay, B., Yu, Z., and Strynadka, N. C. J. (2019) T3S injectisome needle complex structures in four distinct states reveal the basis of membrane coupling and assembly. *Nat Microbiol* **4**, 2010-2019

115. Ross, J. A., and Plano, G. V. (2011) A C-terminal region of Yersinia pestis YscD binds the outer membrane secretin YscC. *J Bacteriol* **193**, 2276-2289
116. Silva-Herzog, E., Ferracci, F., Jackson, M. W., Joseph, S. S., and Plano, G. V. (2008) Membrane localization and topology of the Yersinia pestis YscJ lipoprotein. *Microbiology* **154**, 593-607
117. Kuhlen, L., Abrusci, P., Johnson, S., Gault, J., Deme, J., Caesar, J., Dietsche, T., Mebrhatu, M. T., Ganief, T., Macek, B., Wagner, S., Robinson, C. V., and Lea, S. M. (2018) Structure of the core of the type III secretion system export apparatus. *Nat Struct Mol Biol* **25**, 583-590
118. Johnson, S., Kuhlen, L., Deme, J. C., Abrusci, P., and Lea, S. M. (2019) The Structure of an Injectisome Export Gate Demonstrates Conservation of Architecture in the Core Export Gate between Flagellar and Virulence Type III Secretion Systems. *mBio* **10**
119. Kuhlen, L., Johnson, S., Zeitler, A., Baurle, S., Deme, J. C., Caesar, J. J. E., Debo, R., Fisher, J., Wagner, S., and Lea, S. M. (2020) The substrate specificity switch FlhB assembles onto the export gate to regulate type three secretion. *Nat Commun* **11**, 1296
120. Allaoui, A., Woestyn, S., Sluiters, C., and Cornelis, G. R. (1994) YscU, a Yersinia enterocolitica inner membrane protein involved in Yop secretion. *J Bacteriol* **176**, 4534-4542
121. Plano, G. V., Barve, S. S., and Straley, S. C. (1991) LcrD, a membrane-bound regulator of the Yersinia pestis low-calcium response. *J Bacteriol* **173**, 7293-7303
122. Berger, C., Robin, G. P., Bonas, U., and Koebnik, R. (2010) Membrane topology of conserved components of the type III secretion system from the plant pathogen Xanthomonas campestris pv. vesicatoria. *Microbiology* **156**, 1963-1974
123. Butan, C., Lara-Tejero, M., Li, W., Liu, J., and Galan, J. E. (2019) High-resolution view of the type III secretion export apparatus in situ reveals membrane remodeling and a secretion pathway. *Proc Natl Acad Sci U S A* **116**, 24786-24795
124. Zilkenat, S., Franz-Wachtel, M., Stierhof, Y. D., Galan, J. E., Macek, B., and Wagner, S. (2016) Determination of the Stoichiometry of the Complete Bacterial Type III Secretion Needle Complex Using a Combined Quantitative Proteomic Approach. *Mol Cell Proteomics* **15**, 1598-1609
125. Dietsche, T., Tesfazgi Mebrhatu, M., Brunner, M. J., Abrusci, P., Yan, J., Franz-Wachtel, M., Scharfe, C., Zilkenat, S., Grin, I., Galan, J. E., Kohlbacher, O., Lea, S., Macek, B., Marlovits, T. C., Robinson, C. V., and Wagner, S. (2016) Structural and Functional Characterization of the Bacterial Type III Secretion Export Apparatus. *PLoS Pathog* **12**, e1006071
126. Tseytin, I., Mitrovic, B., David, N., Langenfeld, K., Zarivach, R., Diepold, A., and Sal-Man, N. (2019) The Role of the Small Export Apparatus Protein, SctS, in the Activity of the Type III Secretion System. *Front Microbiol* **10**, 2551
127. Lavander, M., Sundberg, L., Edqvist, P. J., Lloyd, S. A., Wolf-Watz, H., and Forsberg, Å. (2002) Proteolytic cleavage of the FlhB homologue YscU

of Yersinia pseudotuberculosis is essential for bacterial survival but not for type III secretion. *J Bacteriol* **184**, 4500-4509
128. Edqvist, P. J., Olsson, J., Lavander, M., Sundberg, L., Forsberg, Å., Wolf-Watz, H., and Lloyd, S. A. (2003) YscP and YscU regulate substrate specificity of the Yersinia type III secretion system. *J Bacteriol* **185**, 2259-2266
129. Ferris, H. U., Furukawa, Y., Minamino, T., Kroetz, M. B., Kihara, M., Namba, K., and Macnab, R. M. (2005) FlhB regulates ordered export of flagellar components via autocleavage mechanism. *J Biol Chem* **280**, 41236-41242
130. Deane, J. E., Graham, S. C., Mitchell, E. P., Flot, D., Johnson, S., and Lea, S. M. (2008) Crystal structure of Spa40, the specificity switch for the Shigella flexneri type III secretion system. *Mol Microbiol* **69**, 267-276
131. Erhardt, M., Wheatley, P., Kim, E. A., Hirano, T., Zhang, Y., Sarkar, M. K., Hughes, K. T., and Blair, D. F. (2017) Mechanism of type-III protein secretion: Regulation of FlhA conformation by a functionally critical charged-residue cluster. *Mol Microbiol* **104**, 234-249
132. Minamino, T., Morimoto, Y. V., Hara, N., Aldridge, P. D., and Namba, K. (2016) The Bacterial Flagellar Type III Export Gate Complex Is a Dual Fuel Engine That Can Use Both H+ and Na+ for Flagellar Protein Export. *PLoS Pathog* **12**, e1005495
133. Abrusci, P., Vergara-Irigaray, M., Johnson, S., Beeby, M. D., Hendrixson, D. R., Roversi, P., Friede, M. E., Deane, J. E., Jensen, G. J., Tang, C. M., and Lea, S. M. (2013) Architecture of the major component of the type III secretion system export apparatus. *Nat Struct Mol Biol* **20**, 99-104
134. Diepold, A., Wiesand, U., and Cornelis, G. R. (2011) The assembly of the export apparatus (YscR,S,T,U,V) of the Yersinia type III secretion apparatus occurs independently of other structural components and involves the formation of an YscV oligomer. *Mol Microbiol* **82**, 502-514
135. Lara-Tejero, M., Kato, J., Wagner, S., Liu, X., and Galan, J. E. (2011) A sorting platform determines the order of protein secretion in bacterial type III systems. *Science* **331**, 1188-1191
136. Kawamoto, A., Morimoto, Y. V., Miyata, T., Minamino, T., Hughes, K. T., Kato, T., and Namba, K. (2013) Common and distinct structural features of Salmonella injectisome and flagellar basal body. *Sci Rep* **3**, 3369
137. Spaeth, K. E., Chen, Y. S., and Valdivia, R. H. (2009) The Chlamydia type III secretion system C-ring engages a chaperone-effector protein complex. *PLoS Pathog* **5**, e1000579
138. Khan, I. H., Reese, T. S., and Khan, S. (1992) The cytoplasmic component of the bacterial flagellar motor. *Proc Natl Acad Sci U S A* **89**, 5956-5960
139. Irikura, V. M., Kihara, M., Yamaguchi, S., Sockett, H., and Macnab, R. M. (1993) Salmonella typhimurium fliG and fliN mutations causing defects in assembly, rotation, and switching of the flagellar motor. *J Bacteriol* **175**, 802-810
140. Francis, N. R., Sosinsky, G. E., Thomas, D., and DeRosier, D. J. (1994) Isolation, characterization and structure of bacterial flagellar motors containing the switch complex. *J Mol Biol* **235**, 1261-1270
141. Hu, B., Morado, D. R., Margolin, W., Rohde, J. R., Arizmendi, O., Picking, W. L., Picking, W. D., and Liu, J. (2015) Visualization of the type III

secretion sorting platform of Shigella flexneri. *Proc Natl Acad Sci U S A* **112**, 1047-1052
142. Jackson, M. W., and Plano, G. V. (2000) Interactions between type III secretion apparatus components from Yersinia pestis detected using the yeast two-hybrid system. *FEMS Microbiol Lett* **186**, 85-90
143. Diepold, A., Kudryashev, M., Delalez, N. J., Berry, R. M., and Armitage, J. P. (2015) Composition, formation, and regulation of the cytosolic c-ring, a dynamic component of the type III secretion injectisome. *PLoS Biol* **13**, e1002039
144. Diepold, A., Sezgin, E., Huseyin, M., Mortimer, T., Eggeling, C., and Armitage, J. P. (2017) A dynamic and adaptive network of cytosolic interactions governs protein export by the T3SS injectisome. *Nat Commun* **8**, 15940
145. Zhang, Y., Lara-Tejero, M., Bewersdorf, J., and Galan, J. E. (2017) Visualization and characterization of individual type III protein secretion machines in live bacteria. *Proc Natl Acad Sci U S A* **114**, 6098-6103
146. Delalez, N. J., Wadhams, G. H., Rosser, G., Xue, Q., Brown, M. T., Dobbie, I. M., Berry, R. M., Leake, M. C., and Armitage, J. P. (2010) Signal-dependent turnover of the bacterial flagellar switch protein FliM. *Proc Natl Acad Sci U S A* **107**, 11347-11351
147. Fukuoka, H., Inoue, Y., Terasawa, S., Takahashi, H., and Ishijima, A. (2010) Exchange of rotor components in functioning bacterial flagellar motor. *Biochem Biophys Res Commun* **394**, 130-135
148. Bzymek, K. P., Hamaoka, B. Y., and Ghosh, P. (2012) Two translation products of Yersinia yscQ assemble to form a complex essential to type III secretion. *Biochemistry* **51**, 1669-1677
149. Lara-Tejero, M., Qin, Z., Hu, B., Butan, C., Liu, J., and Galan, J. E. (2019) Role of SpaO in the assembly of the sorting platform of a Salmonella type III secretion system. *PLoS Pathog* **15**, e1007565
150. Bernal, I., Bornicke, J., Heidemann, J., Svergun, D., Horstmann, J. A., Erhardt, M., Tuukkanen, A., Uetrecht, C., and Kolbe, M. (2019) Molecular Organization of Soluble Type III Secretion System Sorting Platform Complexes. *J Mol Biol* **431**, 3787-3803
151. Blaylock, B., Riordan, K. E., Missiakas, D. M., and Schneewind, O. (2006) Characterization of the Yersinia enterocolitica type III secretion ATPase YscN and its regulator, YscL. *J Bacteriol* **188**, 3525-3534
152. Akeda, Y., and Galan, J. E. (2004) Genetic analysis of the Salmonella enterica type III secretion-associated ATPase InvC defines discrete functional domains. *J Bacteriol* **186**, 2402-2412
153. Minamino, T., and Namba, K. (2008) Distinct roles of the FliI ATPase and proton motive force in bacterial flagellar protein export. *Nature* **451**, 485-488
154. Akeda, Y., and Galan, J. E. (2005) Chaperone release and unfolding of substrates in type III secretion. *Nature* **437**, 911-915
155. Majewski, D. D., Worrall, L. J., Hong, C., Atkinson, C. E., Vuckovic, M., Watanabe, N., Yu, Z., and Strynadka, N. C. J. (2019) Cryo-EM structure of the homohexameric T3SS ATPase-central stalk complex reveals rotary ATPase-like asymmetry. *Nat Commun* **10**, 626

156. Eichelberg, K., Ginocchio, C. C., and Galan, J. E. (1994) Molecular and functional characterization of the Salmonella typhimurium invasion genes invB and invC: homology of InvC to the F0F1 ATPase family of proteins. *J Bacteriol* **176**, 4501-4510
157. Diepold, A., Wiesand, U., Amstutz, M., and Cornelis, G. R. (2012) Assembly of the Yersinia injectisome: the missing pieces. *Mol Microbiol* **85**, 878-892
158. Payne, P. L., and Straley, S. C. (1998) YscO of Yersinia pestis is a mobile core component of the Yop secretion system. *J Bacteriol* **180**, 3882-3890
159. Mukerjea, R., and Ghosh, P. (2013) Functionally essential interaction between Yersinia YscO and the T3S4 domain of YscP. *J Bacteriol* **195**, 4631-4638
160. Evans, L. D., Stafford, G. P., Ahmed, S., Fraser, G. M., and Hughes, C. (2006) An escort mechanism for cycling of export chaperones during flagellum assembly. *Proc Natl Acad Sci U S A* **103**, 17474-17479
161. Diepold, A., Amstutz, M., Abel, S., Sorg, I., Jenal, U., and Cornelis, G. R. (2010) Deciphering the assembly of the Yersinia type III secretion injectisome. *EMBO J* **29**, 1928-1940
162. Soto, E., Espinosa, N., Diaz-Guerrero, M., Gaytan, M. O., Puente, J. L., and Gonzalez-Pedrajo, B. (2017) Functional Characterization of EscK (Orf4), a Sorting Platform Component of the Enteropathogenic Escherichia coli Injectisome. *J Bacteriol* **199**
163. Marlovits, T. C., Kubori, T., Sukhan, A., Thomas, D. R., Galan, J. E., and Unger, V. M. (2004) Structural insights into the assembly of the type III secretion needle complex. *Science* **306**, 1040-1042
164. Francis, M. S. (2010) Type III secretion chaperones: a molecular toolkit for all occasions. in *Handbook of molecular chaperones: roles, structures and mechanisms* (Durante, P., and Colucci, L. eds.), Nova Science Publishers, Inc., Hauppauge, New York. pp 79-147
165. Dey, S., Chakravarty, A., Guha Biswas, P., and De Guzman, R. N. (2019) The type III secretion system needle, tip, and translocon. *Protein Sci* **28**, 1582-1593
166. Souza, C. A., Richards, K. L., Park, Y., Schwartz, M., Torruellas Garcia, J., Schesser Bartra, S., and Plano, G. V. (2018) The YscE/YscG chaperone and YscF N-terminal sequences target YscF to the Yersinia pestis type III secretion apparatus. *Microbiology* **164**, 338-348
167. Quinaud, M., Chabert, J., Faudry, E., Neumann, E., Lemaire, D., Pastor, A., Elsen, S., Dessen, A., and Attree, I. (2005) The PscE-PscF-PscG complex controls type III secretion needle biogenesis in Pseudomonas aeruginosa. *J Biol Chem* **280**, 36293-36300
168. Chatterjee, C., Kumar, S., Chakraborty, S., Tan, Y. W., Leung, K. Y., Sivaraman, J., and Mok, Y. K. (2011) Crystal structure of the heteromolecular chaperone, AscE-AscG, from the type III secretion system in Aeromonas hydrophila. *PLoS One* **6**, e19208
169. Gurung, J. M., Amer, A. A. A., Francis, M. K., Costa, T. R. D., Chen, S., Zavialov, A. V., and Francis, M. S. (2018) Heterologous Complementation Studies With the YscX and YscY Protein Families Reveals a Specificity for Yersinia pseudotuberculosis Type III Secretion. *Front Cell Infect Microbiol* **8**, 80

170. Gurung, J. M., Francis, M. K., Amer, A. A., Costa, T. R., Diepold, A., Chen, S., Zavialov, A., and Francis, M. S. (2017) Non-reciprocal type III secretion functions of the YscX and YscY protein families. *Manuscript*
171. Torres-Vargas, C. E., Kronenberger, T., Roos, N., Dietsche, T., Poso, A., and Wagner, S. (2019) The inner rod of virulence-associated type III secretion systems constitutes a needle adapter of one helical turn that is deeply integrated into the system's export apparatus. *Mol Microbiol* **112**, 918-931
172. Cao, S. Y., Liu, W. B., Tan, Y. F., Yang, H. Y., Zhang, T. T., Wang, T., Wang, X. Y., Song, Y. J., Yang, R. F., and Du, Z. M. (2017) An Interaction between the Inner Rod Protein YscI and the Needle Protein YscF Is Required to Assemble the Needle Structure of the Yersinia Type Three Secretion System. *J Biol Chem* **292**, 5488-5498
173. Sal-Man, N., Deng, W., and Finlay, B. B. (2012) EscI: a crucial component of the type III secretion system forms the inner rod structure in enteropathogenic Escherichia coli. *Biochem J* **442**, 119-125
174. Loquet, A., Sgourakis, N. G., Gupta, R., Giller, K., Riedel, D., Goosmann, C., Griesinger, C., Kolbe, M., Baker, D., Becker, S., and Lange, A. (2012) Atomic model of the type III secretion system needle. *Nature* **486**, 276-279
175. Blocker, A. J., Deane, J. E., Veenendaal, A. K., Roversi, P., Hodgkinson, J. L., Johnson, S., and Lea, S. M. (2008) What's the point of the type III secretion system needle? *Proc Natl Acad Sci U S A* **105**, 6507-6513
176. Poyraz, O., Schmidt, H., Seidel, K., Delissen, F., Ader, C., Tenenboim, H., Goosmann, C., Laube, B., Thunemann, A. F., Zychlinsky, A., Baldus, M., Lange, A., Griesinger, C., and Kolbe, M. (2010) Protein refolding is required for assembly of the type three secretion needle. *Nat Struct Mol Biol* **17**, 788-792
177. Guo, E. Z., Desrosiers, D. C., Zalesak, J., Tolchard, J., Berbon, M., Habenstein, B., Marlovits, T., Loquet, A., and Galan, J. E. (2019) A polymorphic helix of a Salmonella needle protein relays signals defining distinct steps in type III secretion. *PLoS Biol* **17**, e3000351
178. Chevance, F. F., and Hughes, K. T. (2008) Coordinating assembly of a bacterial macromolecular machine. *Nat Rev Microbiol* **6**, 455-465
179. Minamino, T., and Imada, K. (2015) The bacterial flagellar motor and its structural diversity. *Trends Microbiol* **23**, 267-274
180. Kato, J., Dey, S., Soto, J. E., Butan, C., Wilkinson, M. C., De Guzman, R. N., and Galan, J. E. (2018) A protein secreted by the Salmonella type III secretion system controls needle filament assembly. *Elife* **7**
181. Holmström, A., Olsson, J., Cherepanov, P., Maier, E., Nordfelth, R., Pettersson, J., Benz, R., Wolf-Watz, H., and Forsberg, Å. (2001) LcrV is a channel size-determining component of the Yop effector translocon of Yersinia. *Mol Microbiol* **39**, 620-632
182. Pettersson, J., Holmström, A., Hill, J., Leary, S., Frithz-Lindsten, E., von Euler-Matell, A., Carlsson, E., Titball, R., Forsberg, Å., and Wolf-Watz, H. (1999) The V-antigen of Yersinia is surface exposed before target cell contact and involved in virulence protein translocation. *Mol Microbiol* **32**, 961-976

183. Mueller, C. A., Broz, P., Muller, S. A., Ringler, P., Erne-Brand, F., Sorg, I., Kuhn, M., Engel, A., and Cornelis, G. R. (2005) The V-antigen of Yersinia forms a distinct structure at the tip of injectisome needles. *Science* **310**, 674-676
184. Costa, T. R., Edqvist, P. J., Broms, J. E., Ahlund, M. K., Forsberg, A., and Francis, M. S. (2010) YopD self-assembly and binding to LcrV facilitate type III secretion activity by Yersinia pseudotuberculosis. *J Biol Chem* **285**, 25269-25284
185. Neyt, C., and Cornelis, G. R. (1999) Insertion of a Yop translocation pore into the macrophage plasma membrane by Yersinia enterocolitica: requirement for translocators YopB and YopD, but not LcrG. *Mol Microbiol* **33**, 971-981
186. Schoehn, G., Di Guilmi, A. M., Lemaire, D., Attree, I., Weissenhorn, W., and Dessen, A. (2003) Oligomerization of type III secretion proteins PopB and PopD precedes pore formation in Pseudomonas. *Embo J* **22**, 4957-4967
187. Montagner, C., Arquint, C., and Cornelis, G. R. (2011) Translocators YopB and YopD from Yersinia form a multimeric integral membrane complex in eukaryotic cell membranes. *J Bacteriol* **193**, 6923-6928
188. Håkansson, S., Schesser, K., Persson, C., Galyov, E. E., Rosqvist, R., Homble, F., and Wolf-Watz, H. (1996) The YopB protein of Yersinia pseudotuberculosis is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *Embo J* **15**, 5812-5823
189. Costa, T. R. D., Francis, M. K., Farag, S. I., Edgren, T., and Francis, M. S. (2019) Measurement of Yersinia Translocon Pore Formation in Erythrocytes. *Methods Mol Biol* **2010**, 211-229
190. Goure, J., Pastor, A., Faudry, E., Chabert, J., Dessen, A., and Attree, I. (2004) The V antigen of Pseudomonas aeruginosa is required for assembly of the functional PopB/PopD translocation pore in host cell membranes. *Infect Immun* **72**, 4741-4750
191. Veenendaal, A. K., Hodgkinson, J. L., Schwarzer, L., Stabat, D., Zenk, S. F., and Blocker, A. J. (2007) The type III secretion system needle tip complex mediates host cell sensing and translocon insertion. *Mol Microbiol* **63**, 1719-1730
192. Russo, B. C., Duncan, J. K., Wiscovitch, A. L., Hachey, A. C., and Goldberg, M. B. (2019) Activation of Shigella flexneri type 3 secretion requires a host-induced conformational change to the translocon pore. *PLoS Pathog* **15**, e1007928
193. Russo, B. C., Stamm, L. M., Raaben, M., Kim, C. M., Kahoud, E., Robinson, L. R., Bose, S., Queiroz, A. L., Herrera, B. B., Baxt, L. A., Mor-Vaknin, N., Fu, Y., Molina, G., Markovitz, D. M., Whelan, S. P., and Goldberg, M. B. (2016) Intermediate filaments enable pathogen docking to trigger type 3 effector translocation. *Nat Microbiol* **1**, 16025
194. Viboud, G. I., and Bliska, J. B. (2005) *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. *Annu Rev Microbiol* **59**, 69-89
195. Trosky, J. E., Liverman, A. D., and Orth, K. (2008) Yersinia outer proteins: Yops. *Cell Microbiol* **10**, 557-565

196. Zhang, Y., Romanov, G., and Bliska, J. B. (2011) Type III secretion system-dependent translocation of ectopically expressed Yop effectors into macrophages by intracellular Yersinia pseudotuberculosis. *Infect Immun* **79**, 4322-4331
197. De Nisco, N. J., Rivera-Cancel, G., and Orth, K. (2018) The Biochemistry of Sensing: Enteric Pathogens Regulate Type III Secretion in Response to Environmental and Host Cues. *mBio* **9**
198. Schwiesow, L., Lam, H., Dersch, P., and Auerbuch, V. (2015) Yersinia Type III Secretion System Master Regulator LcrF. *J Bacteriol* **198**, 604-614
199. Lambert de Rouvroit, C., Sluiters, C., and Cornelis, G. R. (1992) Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of Yersinia enterocolitica. *Mol Microbiol* **6**, 395-409
200. Hoe, N. P., and Goguen, J. D. (1993) Temperature sensing in Yersinia pestis: translation of the LcrF activator protein is thermally regulated. *J Bacteriol* **175**, 7901-7909
201. Skurnik, M., and Toivanen, P. (1992) LcrF is the temperature-regulated activator of the yadA gene of Yersinia enterocolitica and Yersinia pseudotuberculosis. *J Bacteriol* **174**, 2047-2051
202. Rohde, J. R., Fox, J. M., and Minnich, S. A. (1994) Thermoregulation in Yersinia enterocolitica is coincident with changes in DNA supercoiling. *Mol Microbiol* **12**, 187-199
203. Bohme, K., Steinmann, R., Kortmann, J., Seekircher, S., Heroven, A. K., Berger, E., Pisano, F., Thiermann, T., Wolf-Watz, H., Narberhaus, F., and Dersch, P. (2012) Concerted actions of a thermo-labile regulator and a unique intergenic RNA thermosensor control Yersinia virulence. *PLoS Pathog* **8**, e1002518
204. Rohde, J. R., Luan, X. S., Rohde, H., Fox, J. M., and Minnich, S. A. (1999) The Yersinia enterocolitica pYV virulence plasmid contains multiple intrinsic DNA bends which melt at 37 degrees C. *J Bacteriol* **181**, 4198-4204
205. Jackson, M. W., Silva-Herzog, E., and Plano, G. V. (2004) The ATP-dependent ClpXP and Lon proteases regulate expression of the Yersinia pestis type III secretion system via regulated proteolysis of YmoA, a small histone-like protein. *Mol Microbiol* **54**, 1364-1378
206. Li, L., Yan, H., Feng, L., Li, Y., Lu, P., Hu, Y., and Chen, S. (2014) LcrQ blocks the role of LcrF in regulating the Ysc-Yop type III secretion genes in Yersinia pseudotuberculosis. *PLoS One* **9**, e92243
207. Chen, Y., and Anderson, D. M. (2011) Expression hierarchy in the Yersinia type III secretion system established through YopD recognition of RNA. *Mol Microbiol* **80**, 966-980
208. Kusmierek, M., Hossmann, J., Witte, R., Opitz, W., Vollmer, I., Volk, M., Heroven, A. K., Wolf-Watz, H., and Dersch, P. (2019) A bacterial secreted translocator hijacks riboregulators to control type III secretion in response to host cell contact. *PLoS Pathog* **15**, e1007813
209. Kupferberg, L. L., and Higuchi, K. (1958) Role of calcium ions in the stimulation of growth of virulent strains of Pasteurella pestis. *J Bacteriol* **76**, 120-121

210. Cornelis, G. R., and Wolf-Watz, H. (1997) The Yersinia Yop virulon: a bacterial system for subverting eukaryotic cells. *Mol Microbiol* **23**, 861-867
211. Higuchi, K., Kupferberg, L. L., and Smith, J. L. (1959) Studies on the nutrition and physiology of Pasteurella pestis. III. Effects of calcium ions on the growth of virulent and avirulent strains of Pasteurella pestis. *J Bacteriol* **77**, 317-321
212. Forsberg, Å., Bölin, I., Norlander, L., and Wolf-Watz, H. (1987) Molecular cloning and expression of calcium-regulated, plasmid-coded proteins of Y. pseudotuberculosis. *Microb Pathog* **2**, 123-137
213. Goguen, J. D., Yother, J., and Straley, S. C. (1984) Genetic analysis of the low calcium response in Yersinia pestis mu d1(Ap lac) insertion mutants. *J Bacteriol* **160**, 842-848
214. Zahorchak, R. J., Charnetzky, W. T., Little, R. V., and Brubaker, R. R. (1979) Consequences of Ca2+ deficiency on macromolecular synthesis and adenylate energy charge in Yersinia pestis. *J Bacteriol* **139**, 792-799
215. Fowler, J. M., Wulff, C. R., Straley, S. C., and Brubaker, R. R. (2009) Growth of calcium-blind mutants of Yersinia pestis at 37 degrees C in permissive Ca2+-deficient environments. *Microbiology* **155**, 2509-2521
216. Bölin, I., and Wolf-Watz, H. (1984) Molecular cloning of the temperature-inducible outer membrane protein 1 of Yersinia pseudotuberculosis. *Infect Immun* **43**, 72-78
217. Bölin, I., Portnoy, D. A., and Wolf-Watz, H. (1985) Expression of the temperature-inducible outer membrane proteins of yersiniae. *Infect Immun* **48**, 234-240
218. Bölin, I., Norlander, L., and Wolf-Watz, H. (1982) Temperature-inducible outer membrane protein of Yersinia pseudotuberculosis and Yersinia enterocolitica is associated with the virulence plasmid. *Infect Immun* **37**, 506-512
219. Yother, J., and Goguen, J. D. (1985) Isolation and characterization of Ca2+-blind mutants of Yersinia pestis. *J Bacteriol* **164**, 704-711
220. Price, S. B., and Straley, S. C. (1989) lcrH, a gene necessary for virulence of Yersinia pestis and for the normal response of Y. pestis to ATP and calcium. *Infect Immun* **57**, 1491-1498
221. Forsberg, Å., Viitanen, A. M., Skurnik, M., and Wolf-Watz, H. (1991) The surface-located YopN protein is involved in calcium signal transduction in Yersinia pseudotuberculosis. *Mol Microbiol* **5**, 977-986
222. Skryzpek, E., and Straley, S. C. (1993) LcrG, a secreted protein involved in negative regulation of the low-calcium response in Yersinia pestis. *J Bacteriol* **175**, 3520-3528
223. Francis, M. S., Lloyd, S. A., and Wolf-Watz, H. (2001) The type III secretion chaperone LcrH co-operates with YopD to establish a negative, regulatory loop for control of Yop synthesis in Yersinia pseudotuberculosis. *Mol Microbiol* **42**, 1075-1093
224. Torruellas, J., Jackson, M. W., Pennock, J. W., and Plano, G. V. (2005) The Yersinia pestis type III secretion needle plays a role in the regulation of Yop secretion. *Mol Microbiol* **57**, 1719-1733

225. Pollack, C., Straley, S. C., and Klempner, M. S. (1986) Probing the phagolysosomal environment of human macrophages with a Ca2+-responsive operon fusion in Yersinia pestis. *Nature* **322**, 834-836
226. Wiley, D. J., Rosqvist, R., and Schesser, K. (2007) Induction of the Yersinia type 3 secretion system as an all-or-none phenomenon. *J Mol Biol* **373**, 27-37
227. Rosqvist, R., Magnusson, K. E., and Wolf-Watz, H. (1994) Target cell contact triggers expression and polarized transfer of Yersinia YopE cytotoxin into mammalian cells. *Embo J* **13**, 964-972
228. Anderson, D. M., Ramamurthi, K. S., Tam, C., and Schneewind, O. (2002) YopD and LcrH regulate expression of Yersinia enterocolitica YopQ by a posttranscriptional mechanism and bind to yopQ RNA. *J Bacteriol* **184**, 1287-1295
229. Williams, A. W., and Straley, S. C. (1998) YopD of Yersinia pestis plays a role in negative regulation of the low-calcium response in addition to its role in translocation of Yops. *J Bacteriol* **180**, 350-358
230. Yang, J., Jain, C., and Schesser, K. (2008) RNase E regulates the Yersinia type 3 secretion system. *J Bacteriol* **190**, 3774-3778
231. Rimpiläinen, M., Forsberg, Å., and Wolf-Watz, H. (1992) A novel protein, LcrQ, involved in the low-calcium response of Yersinia pseudotuberculosis shows extensive homology to YopH. *J Bacteriol* **174**, 3355-3363
232. Stainier, I., Iriarte, M., and Cornelis, G. R. (1997) YscM1 and YscM2, two Yersinia enterocolitica proteins causing downregulation of yop transcription. *Mol Microbiol* **26**, 833-843
233. Costa, T. R. (2012) *YopD translocator function in Yersinia pseudotuberculosis type III secretion*, Umeå University
234. Kopaskie, K. S., Ligtenberg, K. G., and Schneewind, O. (2013) Translational regulation of Yersinia enterocolitica mRNA encoding a type III secretion substrate. *J Biol Chem* **288**, 35478-35488
235. Li, Y., Li, L., Huang, L., Francis, M. S., Hu, Y., and Chen, S. (2014) Yersinia Ysc-Yop type III secretion feedback inhibition is relieved through YscV-dependent recognition and secretion of LcrQ. *Mol Microbiol* **91**, 494-507
236. Wulff-Strobel, C. R., Williams, A. W., and Straley, S. C. (2002) LcrQ and SycH function together at the Ysc type III secretion system in Yersinia pestis to impose a hierarchy of secretion. *Mol Microbiol* **43**, 411-423
237. Cambronne, E. D., Sorg, J. A., and Schneewind, O. (2004) Binding of SycH chaperone to YscM1 and YscM2 activates effector yop expression in Yersinia enterocolitica. *J Bacteriol* **186**, 829-841
238. Francis, M. S., Aili, M., Wiklund, M. L., and Wolf-Watz, H. (2000) A study of the YopD-LcrH interaction from Yersinia pseudotuberculosis reveals a role for hydrophobic residues within the amphipathic domain of YopD. *Mol Microbiol* **38**, 85-102
239. Pettersson, J., Nordfelth, R., Dubinina, E., Bergman, T., Gustafsson, M., Magnusson, K. E., and Wolf-Watz, H. (1996) Modulation of virulence factor expression by pathogen target cell contact. *Science* **273**, 1231-1233

240. Galan, J. E., Lara-Tejero, M., Marlovits, T. C., and Wagner, S. (2014) Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu Rev Microbiol* **68**, 415-438
241. Wagner, S., Konigsmaier, L., Lara-Tejero, M., Lefebre, M., Marlovits, T. C., and Galan, J. E. (2010) Organization and coordinated assembly of the type III secretion export apparatus. *Proc Natl Acad Sci U S A*
242. Day, J. B., and Plano, G. V. (2000) The Yersinia pestis YscY protein directly binds YscX, a secreted component of the type III secretion machinery. *J Bacteriol* **182**, 1834-1843
243. Green, E. R., and Mecsas, J. (2016) Bacterial Secretion Systems: An Overview. *Microbiol Spectr* **4**
244. Maffei, B., Francetic, O., and Subtil, A. (2017) Tracking Proteins Secreted by Bacteria: What's in the Toolbox? *Front Cell Infect Microbiol* **7**, 221
245. Douzi, B., Filloux, A., and Voulhoux, R. (2012) On the path to uncover the bacterial type II secretion system. *Philos Trans R Soc Lond B Biol Sci* **367**, 1059-1072
246. Lloyd, S. A., Forsberg, Å., Wolf-Watz, H., and Francis, M. S. (2001) Targeting exported substrates to the Yersinia TTSS: different functions for different signals? *Trends Microbiol* **9**, 367-371
247. Ghosh, P. (2004) Process of protein transport by the type III secretion system. *Microbiol Mol Biol Rev* **68**, 771-795
248. Munera, D., Crepin, V. F., Marches, O., and Frankel, G. (2010) N-terminal type III secretion signal of enteropathogenic Escherichia coli translocator proteins. *J Bacteriol* **192**, 3534-3539
249. Amer, A. A., Ahlund, M. K., Broms, J. E., Forsberg, A., 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**, 6683-6700
250. Karlinsey, J. E., Lonner, J., Brown, K. L., and Hughes, K. T. (2000) Translation/secretion coupling by type III secretion systems. *Cell* **102**, 487-497
251. Sory, M. P., Boland, A., Lambermont, I., and Cornelis, G. R. (1995) Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the cyaA gene fusion approach. *Proc Natl Acad Sci U S A* **92**, 11998-12002
252. Schesser, K., Frithz-Lindsten, E., and Wolf-Watz, H. (1996) Delineation and mutational analysis of the Yersinia pseudotuberculosis YopE domains which mediate translocation across bacterial and eukaryotic cellular membranes. *J Bacteriol* **178**, 7227-7233
253. Anderson, D. M., and Schneewind, O. (1997) A mRNA signal for the type III secretion of Yop proteins by Yersinia enterocolitica. *Science* **278**, 1140-1143
254. Ramamurthi, K. S., and Schneewind, O. (2002) Yersinia enterocolitica type III secretion: mutational analysis of the yopQ secretion signal. *J Bacteriol* **184**, 3321-3328
255. Ramamurthi, K. S., and Schneewind, O. (2005) A synonymous mutation in Yersinia enterocolitica yopE affects the function of the YopE type III secretion signal. *J Bacteriol* **187**, 707-715

256. Anderson, D. M., Fouts, D. E., Collmer, A., and Schneewind, O. (1999) Reciprocal secretion of proteins by the bacterial type III machines of plant and animal pathogens suggests universal recognition of mRNA targeting signals. *Proc Natl Acad Sci U S A* **96**, 12839-12843
257. Niemann, G. S., Brown, R. N., Mushamiri, I. T., Nguyen, N. T., Taiwo, R., Stufkens, A., Smith, R. D., Adkins, J. N., McDermott, J. E., and Heffron, F. (2013) RNA type III secretion signals that require Hfq. *J Bacteriol* **195**, 2119-2125
258. Russmann, H., Kubori, T., Sauer, J., and Galán, J. E. (2002) Molecular and functional analysis of the type III secretion signal of the Salmonella enterica InvJ protein. *Mol Microbiol* **46**, 769-779
259. Lloyd, S. A., Norman, M., Rosqvist, R., and Wolf-Watz, H. (2001) Yersinia YopE is targeted for type III secretion by N-terminal, not mRNA, signals. *Mol Microbiol* **39**, 520-531
260. Lloyd, S. A., Sjöström, M., Andersson, S., and Wolf-Watz, H. (2002) Molecular characterization of type III secretion signals via analysis of synthetic N-terminal amino acid sequences. *Mol Microbiol* **43**, 51-59
261. Buttner, D., and He, S. Y. (2009) Type III protein secretion in plant pathogenic bacteria. *Plant Physiol* **150**, 1656-1664
262. Parsot, C., Hamiaux, C., and Page, A. L. (2003) The various and varying roles of specific chaperones in type III secretion systems. *Curr Opin Microbiol* **6**, 7-14
263. Thomas, N. A., Ma, I., Prasad, M. E., and Rafuse, C. (2012) Expanded roles for multicargo and class 1B effector chaperones in type III secretion. *J Bacteriol* **194**, 3767-3773
264. Archuleta, T. L., and Spiller, B. W. (2014) A gatekeeper chaperone complex directs translocator secretion during type three secretion. *PLoS Pathog* **10**, e1004498
265. Xing, Q., Shi, K., Portaliou, A., Rossi, P., Economou, A., and Kalodimos, C. G. (2018) Structures of chaperone-substrate complexes docked onto the export gate in a type III secretion system. *Nat Commun* **9**, 1773
266. Khanra, N., Rossi, P., Economou, A., and Kalodimos, C. G. (2016) Recognition and targeting mechanisms by chaperones in flagellum assembly and operation. *Proc Natl Acad Sci U S A* **113**, 9798-9803
267. Pallen, M. J., Francis, M. S., and Futterer, K. (2003) Tetratricopeptide-like repeats in type-III-secretion chaperones and regulators. *FEMS Microbiol Lett* **223**, 53-60
268. Bröms, J. E., Edqvist, P. J., Carlsson, K. E., Forsberg, Å., and Francis, M. S. (2005) Mapping of a YscY binding domain within the LcrH chaperone that is required for regulation of *Yersinia* type III secretion. *J Bacteriol* **187**, 7738-7752
269. Mills, E., Baruch, K., Charpentier, X., Kobi, S., and Rosenshine, I. (2008) Real-time analysis of effector translocation by the type III secretion system of enteropathogenic Escherichia coli. *Cell Host Microbe* **3**, 104-113
270. Zarivach, R., Vuckovic, M., Deng, W., Finlay, B. B., and Strynadka, N. C. (2007) Structural analysis of a prototypical ATPase from the type III secretion system. *Nat Struct Mol Biol* **14**, 131-137

271. Romo-Castillo, M., Andrade, A., Espinosa, N., Monjaras Feria, J., Soto, E., Diaz-Guerrero, M., and Gonzalez-Pedrajo, B. (2014) EscO, a functional and structural analog of the flagellar FliJ protein, is a positive regulator of EscN ATPase activity of the enteropathogenic Escherichia coli injectisome. *J Bacteriol* **196**, 2227-2241
272. Wilharm, G., Lehmann, V., Krauss, K., Lehnert, B., Richter, S., Ruckdeschel, K., Heesemann, J., and Trulzsch, K. (2004) Yersinia enterocolitica type III secretion depends on the proton motive force but not on the flagellar motor components MotA and MotB. *Infect Immun* **72**, 4004-4009
273. Lee, P. C., Zmina, S. E., Stopford, C. M., Toska, J., and Rietsch, A. (2014) Control of type III secretion activity and substrate specificity by the cytoplasmic regulator PcrG. *Proc Natl Acad Sci U S A* **111**, E2027-2036
274. Lee, P. C., and Rietsch, A. (2015) Fueling type III secretion. *Trends Microbiol* **23**, 296-300
275. Paul, K., Erhardt, M., Hirano, T., Blair, D. F., and Hughes, K. T. (2008) Energy source of flagellar type III secretion. *Nature* **451**, 489-492
276. Minamino, T., Morimoto, Y. V., Hara, N., and Namba, K. (2011) An energy transduction mechanism used in bacterial flagellar type III protein export. *Nat Commun* **2**, 475
277. Mulkidjanian, A. Y., Dibrov, P., and Galperin, M. Y. (2008) The past and present of sodium energetics: may the sodium-motive force be with you. *Biochim Biophys Acta* **1777**, 985-992
278. Stephan Wimmi, A. B., Hannah Jeckel, Lisa Selinger, Dimitrios Lampaki, Emma Eisemann, Ina Meuskens, Dirk Linke, Knut Drescher, Ulrike Endesfelder, Andreas Diepold (2020) Dynamic relocalization of the cytosolic type III secretion system components prevents premature protein secretion at low external pH. *bioRxiv* **869214**
279. Miller, S., Pesci, E. C., and Pickett, C. L. (1994) Genetic organization of the region upstream from the Campylobacter jejuni flagellar gene flhA. *Gene* **146**, 31-38
280. Plano, G. V., and Straley, S. C. (1993) Multiple effects of lcrD mutations in Yersinia pestis. *J Bacteriol* **175**, 3536-3545
281. Moore, S. A., and Jia, Y. (2010) Structure of the cytoplasmic domain of the flagellar secretion apparatus component FlhA from Helicobacter pylori. *J Biol Chem* **285**, 21060-21069
282. McMurry, J. L., Van Arnam, J. S., Kihara, M., and Macnab, R. M. (2004) Analysis of the cytoplasmic domains of Salmonella FlhA and interactions with components of the flagellar export machinery. *J Bacteriol* **186**, 7586-7592
283. Minamino, T. (2018) Hierarchical protein export mechanism of the bacterial flagellar type III protein export apparatus. *FEMS Microbiol Lett* **365**
284. Minamino, T. (2014) Protein export through the bacterial flagellar type III export pathway. *Biochim Biophys Acta* **1843**, 1642-1648
285. Ginocchio, C. C., and Galán, J. E. (1995) Functional conservation among members of the Salmonella typhimurium InvA family of proteins. *Infect Immun* **63**, 729-732

286. Worrall, L. J., Vuckovic, M., and Strynadka, N. C. (2010) Crystal structure of the C-terminal domain of the Salmonella type III secretion system export apparatus protein InvA. *Protein Sci* **19**, 1091-1096
287. Saijo-Hamano, Y., Imada, K., Minamino, T., Kihara, M., Shimada, M., Kitao, A., and Namba, K. (2010) Structure of the cytoplasmic domain of FlhA and implication for flagellar type III protein export. *Mol Microbiol* **76**, 260-268
288. Saijo-Hamano, Y., Minamino, T., Macnab, R. M., and Namba, K. (2004) Structural and functional analysis of the C-terminal cytoplasmic domain of FlhA, an integral membrane component of the type III flagellar protein export apparatus in Salmonella. *J Mol Biol* **343**, 457-466
289. Kinoshita, M., Hara, N., Imada, K., Namba, K., and Minamino, T. (2013) Interactions of bacterial flagellar chaperone-substrate complexes with FlhA contribute to co-ordinating assembly of the flagellar filament. *Mol Microbiol* **90**, 1249-1261
290. Bange, G., Kummerer, N., Engel, C., Bozkurt, G., Wild, K., and Sinning, I. (2010) FlhA provides the adaptor for coordinated delivery of late flagella building blocks to the type III secretion system. *Proc Natl Acad Sci U S A* **107**, 11295-11300
291. Terahara, N., Inoue, Y., Kodera, N., Morimoto, Y. V., Uchihashi, T., Imada, K., Ando, T., Namba, K., and Minamino, T. (2018) Insight into structural remodeling of the FlhA ring responsible for bacterial flagellar type III protein export. *Sci Adv* **4**, eaao7054
292. Yu, X. J., Grabe, G. J., Liu, M., Mota, L. J., and Holden, D. W. (2018) SsaV Interacts with SsaL to Control the Translocon-to-Effector Switch in the Salmonella SPI-2 Type Three Secretion System. *mBio* **9**
293. Portaliou, A. G., Tsolis, K. C., Loos, M. S., Balabanidou, V., Rayo, J., Tsirigotaki, A., Crepin, V. F., Frankel, G., Kalodimos, C. G., Karamanou, S., and Economou, A. (2017) Hierarchical protein targeting and secretion is controlled by an affinity switch in the type III secretion system of enteropathogenic Escherichia coli. *EMBO J* **36**, 3517-3531
294. Gaytan, M. O., Monjaras Feria, J., Soto, E., Espinosa, N., Benitez, J. M., Georgellis, D., and Gonzalez-Pedrajo, B. (2018) Novel insights into the mechanism of SepL-mediated control of effector secretion in enteropathogenic Escherichia coli. *Microbiologyopen* **7**, e00571
295. Schiano, C. A., and Lathem, W. W. (2012) Post-transcriptional regulation of gene expression in Yersinia species. *Front Cell Infect Microbiol* **2**, 129
296. Macnab, R. M. (2003) How bacteria assemble flagella. *Annu Rev Microbiol* **57**, 77-100
297. Iriarte, M., and Cornelis, G. R. (1999) Identification of SycN, YscX, and YscY, three new elements of the Yersinia yop virulon. *J Bacteriol* **181**, 675-680
298. Minamino, T., and Macnab, R. M. (2000) Domain structure of Salmonella FlhB, a flagellar export component responsible for substrate specificity switching. *J Bacteriol* **182**, 4906-4914
299. Fraser, G. M., Hirano, T., Ferris, H. U., Devgan, L. L., Kihara, M., and Macnab, R. M. (2003) Substrate specificity of type III flagellar protein export in Salmonella is controlled by subdomain interactions in FlhB. *Mol Microbiol* **48**, 1043-1057

300. Frost, S., Ho, O., Login, F. H., Weise, C. F., Wolf-Watz, H., and Wolf-Watz, M. (2012) Autoproteolysis and intramolecular dissociation of Yersinia YscU precedes secretion of its C-terminal polypeptide YscU(CC). *PLoS One* **7**, e49349
301. Login, F. H., and Wolf-Watz, H. (2015) YscU/FlhB of Yersinia pseudotuberculosis Harbors a C-terminal Type III Secretion Signal. *J Biol Chem* **290**, 26282-26291
302. Williams, A. W., Yamaguchi, S., Togashi, F., Aizawa, S. I., Kawagishi, I., and Macnab, R. M. (1996) Mutations in fliK and flhB affecting flagellar hook and filament assembly in Salmonella typhimurium. *J Bacteriol* **178**, 2960-2970
303. Mota, L. J., Journet, L., Sorg, I., Agrain, C., and Cornelis, G. R. (2005) Bacterial injectisomes: needle length does matter. *Science* **307**, 1278
304. Wee, D. H., and Hughes, K. T. (2015) Molecular ruler determines needle length for the Salmonella Spi-1 injectisome. *Proc Natl Acad Sci U S A* **112**, 4098-4103
305. Sorg, I., Wagner, S., Amstutz, M., Muller, S. A., Broz, P., Lussi, Y., Engel, A., and Cornelis, G. R. (2007) YscU recognizes translocators as export substrates of the Yersinia injectisome. *Embo J* **26**, 3015-3024
306. Monjaras Feria, J. V., Lefebre, M. D., Stierhof, Y. D., Galan, J. E., and Wagner, S. (2015) Role of autocleavage in the function of a type III secretion specificity switch protein in Salmonella enterica serovar Typhimurium. *mBio* **6**, e01459-01415
307. Bjornfot, A. C., Lavander, M., Forsberg, A., and Wolf-Watz, H. (2009) Autoproteolysis of YscU of Yersinia pseudotuberculosis is important for regulation of expression and secretion of Yop proteins. *J Bacteriol* **191**, 4259-4267
308. Ho, O., Rogne, P., Edgren, T., Wolf-Watz, H., Login, F. H., and Wolf-Watz, M. (2017) Characterization of the Ruler Protein Interaction Interface on the Substrate Specificity Switch Protein in the Yersinia Type III Secretion System. *J Biol Chem* **292**, 3299-3311
309. Journet, L., Agrain, C., Broz, P., and Cornelis, G. R. (2003) The needle length of bacterial injectisomes is determined by a molecular ruler. *Science* **302**, 1757-1760
310. Agrain, C., Sorg, I., Paroz, C., and Cornelis, G. R. (2005) Secretion of YscP from Yersinia enterocolitica is essential to control the length of the injectisome needle but not to change the type III secretion substrate specificity. *Mol Microbiol* **57**, 1415-1427
311. Payne, P. L., and Straley, S. C. (1999) YscP of Yersinia pestis is a secreted component of the Yop secretion system. *J Bacteriol* **181**, 2852-2862
312. Bergeron, J. R., Fernandez, L., Wasney, G. A., Vuckovic, M., Reffuveille, F., Hancock, R. E., and Strynadka, N. C. (2016) The Structure of a Type 3 Secretion System (T3SS) Ruler Protein Suggests a Molecular Mechanism for Needle Length Sensing. *J Biol Chem* **291**, 1676-1691
313. Stainier, I., Bleves, S., Josenhans, C., Karmani, L., Kerbourch, C., Lambermont, I., Totemeyer, S., Boyd, A., and Cornelis, G. R. (2000) YscP, a Yersinia protein required for Yop secretion that is surface exposed, and released in low Ca2+. *Mol Microbiol* **37**, 1005-1018

314. Marlovits, T. C., Kubori, T., Lara-Tejero, M., Thomas, D., Unger, V. M., and Galan, J. E. (2006) Assembly of the inner rod determines needle length in the type III secretion injectisome. *Nature* **441**, 637-640
315. Wood, S. E., Jin, J., and Lloyd, S. A. (2008) YscP and YscU switch the substrate specificity of the Yersinia type III secretion system by regulating export of the inner rod protein YscI. *J Bacteriol* **190**, 4252-4262
316. Lefebre, M. D., and Galan, J. E. (2014) The inner rod protein controls substrate switching and needle length in a Salmonella type III secretion system. *Proc Natl Acad Sci U S A* **111**, 817-822
317. Kim, J. S., Jang, J. I., Eom, J. S., Oh, C. H., Kim, H. G., Kim, B. H., Bang, I. S., Bang, S. H., and Park, Y. K. (2013) Molecular characterization of InvE regulator in the secretion of type III secretion translocases in Salmonella enterica serovar Typhimurium. *Microbiology* **159**, 446-461
318. Kubori, T., and Galán, J. E. (2002) Salmonella type III secretion-associated protein InvE controls translocation of effector proteins into host cells. *J Bacteriol* **184**, 4699-4708
319. Deng, W., Li, Y., Hardwidge, P. R., Frey, E. A., Pfuetzner, R. A., Lee, S., Gruenheid, S., Strynakda, N. C., Puente, J. L., and Finlay, B. B. (2005) Regulation of type III secretion hierarchy of translocators and effectors in attaching and effacing bacterial pathogens. *Infect Immun* **73**, 2135-2146
320. O'Connell, C. B., Creasey, E. A., Knutton, S., Elliott, S., Crowther, L. J., Luo, W., Albert, M. J., Kaper, J. B., Frankel, G., and Donnenberg, M. S. (2004) SepL, a protein required for enteropathogenic Escherichia coli type III translocation, interacts with secretion component SepD. *Mol Microbiol* **52**, 1613-1625
321. Botteaux, A., Sory, M. P., Biskri, L., Parsot, C., and Allaoui, A. (2009) MxiC is secreted by and controls the substrate specificity of the Shigella flexneri type III secretion apparatus. *Mol Microbiol* **71**, 449-460
322. Martinez-Argudo, I., and Blocker, A. J. (2011) The Shigella T3SS needle transmits a signal for MxiC release, which controls secretion of effectors. *Mol Microbiol* **78**, 1365-1378
323. Cherradi, Y., Schiavolin, L., Moussa, S., Meghraoui, A., Meksem, A., Biskri, L., Azarkan, M., Allaoui, A., and Botteaux, A. (2013) Interplay between predicted inner-rod and gatekeeper in controlling substrate specificity of the type III secretion system. *Mol Microbiol* **87**, 1183-1199
324. Roehrich, A. D., Guillossou, E., Blocker, A. J., and Martinez-Argudo, I. (2013) Shigella IpaD has a dual role: signal transduction from the type III secretion system needle tip and intracellular secretion regulation. *Mol Microbiol* **87**, 690-706
325. Harmon, D. E., Murphy, J. L., Davis, A. J., and Mecsas, J. (2013) A mutant with aberrant extracellular LcrV-YscF interactions fails to form pores and translocate Yop effector proteins but retains the ability to trigger Yop secretion in response to host cell contact. *J Bacteriol* **195**, 2244-2254
326. El Hajjami, N., Moussa, S., Houssa, J., Monteyne, D., Perez-Morga, D., and Botteaux, A. (2018) The inner-rod component of Shigella flexneri type 3 secretion system, MxiI, is involved in the transmission of the

secretion activation signal by its interaction with MxiC. *Microbiologyopen* **7**
327. Cheng, L. W., Kay, O., and Schneewind, O. (2001) Regulated secretion of YopN by the type III machinery of Yersinia enterocolitica. *J Bacteriol* **183**, 5293-5301
328. Goss, J. W., Sorg, J. A., Ramamurthi, K. S., Ton-That, H., and Schneewind, O. (2004) The secretion signal of YopN, a regulatory protein of the Yersinia enterocolitica type III secretion pathway. *J Bacteriol* **186**, 6320-6324
329. Pallen, M. J., Beatson, S. A., and Bailey, C. M. (2005) Bioinformatics analysis of the locus for enterocyte effacement provides novel insights into type-III secretion. *BMC Microbiol* **5**, 9
330. Amer, A. A., Costa, T. R., Farag, S. I., Avican, U., Forsberg, A., and Francis, M. S. (2013) Genetically Engineered Frameshifted YopN-TyeA Chimeras Influence Type III Secretion System Function in Yersinia pseudotuberculosis. *PLoS One* **8**, e77767
331. Ferracci, F., Day, J. B., Ezelle, H. J., and Plano, G. V. (2004) Expression of a functional secreted YopN-TyeA hybrid protein in Yersinia pestis is the result of a +1 translational frameshift event. *J Bacteriol* **186**, 5160-5166
332. Dinman, J. D. (2012) Mechanisms and implications of programmed translational frameshifting. *Wiley Interdiscip Rev RNA* **3**, 661-673
333. Farabaugh, P. J. (1996) Programmed translational frameshifting. *Annu Rev Genet* **30**, 507-528
334. Higashi, K., Kashiwagi, K., Taniguchi, S., Terui, Y., Yamamoto, K., Ishihama, A., and Igarashi, K. (2006) Enhancement of +1 frameshift by polyamines during translation of polypeptide release factor 2 in Escherichia coli. *J Biol Chem* **281**, 9527-9537
335. Ferracci, F., Schubot, F. D., Waugh, D. S., and Plano, G. V. (2005) Selection and characterization of Yersinia pestis YopN mutants that constitutively block Yop secretion. *Mol Microbiol* **57**, 970-987
336. Amer, A. A., Gurung, J. M., Costa, T. R., Avican, U., Zavialov, A., Forsberg, Å., and Francis, M. S. (2014) YopN and TyeA hydrophobic contacts required for regulating Ysc-Yop type III secretion activity by Yersinia pseudotuberculosis. *Manuscript*
337. Schubot, F. D., Jackson, M. W., Penrose, K. J., Cherry, S., Tropea, J. E., Plano, G. V., and Waugh, D. S. (2005) Three-dimensional structure of a macromolecular assembly that regulates type III secretion in Yersinia pestis. *J Mol Biol* **346**, 1147-1161
338. Sundberg, L., and Forsberg, A. (2003) TyeA of Yersinia pseudotuberculosis is involved in regulation of Yop expression and is required for polarized translocation of Yop effectors. *Cell Microbiol* **5**, 187-202
339. Joseph, S. S., and Plano, G. V. (2007) Identification of TyeA residues required to interact with YopN and to regulate Yop secretion. *Adv Exp Med Biol* **603**, 235-245
340. Iriarte, M., Sory, M. P., Boland, A., Boyd, A. P., Mills, S. D., Lambermont, I., and Cornelis, G. R. (1998) TyeA, a protein involved in control of Yop

release and in translocation of Yersinia Yop effectors. *Embo J* **17**, 1907-1918
341. Day, J. B., and Plano, G. V. (1998) A complex composed of SycN and YscB functions as a specific chaperone for YopN in Yersinia pestis. *Mol Microbiol* **30**, 777-788
342. Joseph, S. S., and Plano, G. V. (2013) The SycN/YscB chaperone-binding domain of YopN is required for the calcium-dependent regulation of Yop secretion by Yersinia pestis. *Front Cell Infect Microbiol* **3**, 1
343. Day, J. B., Ferracci, F., and Plano, G. V. (2003) Translocation of YopE and YopN into eukaryotic cells by Yersinia pestis yopN, tyeA, sycN, yscB and lcrG deletion mutants measured using a phosphorylatable peptide tag and phosphospecific antibodies. *Mol Microbiol* **47**, 807-823
344. Bamyaci, S., Ekestubbe, S., Nordfelth, R., Erttmann, S. F., Edgren, T., and Forsberg, A. (2018) YopN Is Required for Efficient Effector Translocation and Virulence in Yersinia pseudotuberculosis. *Infect Immun* **86**
345. Bamyaci, S., Nordfelth, R., and Forsberg, A. (2019) Identification of specific sequence motif of YopN of Yersinia pseudotuberculosis required for systemic infection. *Virulence* **10**, 10-25
346. Broms, J. E., Forslund, A. L., Forsberg, A., and Francis, M. S. (2003) PcrH of Pseudomonas aeruginosa is essential for secretion and assembly of the type III translocon. *J Infect Dis* **188**, 1909-1921
347. Klein, J. A., Dave, B. M., Raphenya, A. R., McArthur, A. G., and Knodler, L. A. (2017) Functional relatedness in the Inv/Mxi-Spa type III secretion system family. *Mol Microbiol* **103**, 973-991
348. Mitchell, A., Tam, C., Elli, D., Charlton, T., Osei-Owusu, P., Fazlollahi, F., Faull, K. F., and Schneewind, O. (2017) Glutathionylation of Yersinia pestis LcrV and Its Effects on Plague Pathogenesis. *MBio* **8**
349. Miki, T., Okada, N., Kim, Y., Abe, A., and Danbara, H. (2008) DsbA directs efficient expression of outer membrane secretin EscC of the enteropathogenic Escherichia coli type III secretion apparatus. *Microb Pathog* **44**, 151-158
350. Jackson, M. W., and Plano, G. V. (1999) DsbA is required for stable expression of outer membrane protein YscC and for efficient Yop secretion in Yersinia pestis. *J Bacteriol* **181**, 5126-5130
351. Betts-Hampikian, H. J., and Fields, K. A. (2011) Disulfide bonding within components of the Chlamydia type III secretion apparatus correlates with development. *J Bacteriol* **193**, 6950-6959
352. Sorg, J. A., Miller, N. C., and Schneewind, O. (2005) Substrate recognition of type III secretion machines--testing the RNA signal hypothesis. *Cell Microbiol* **7**, 1217-1225
353. Ramamurthi, K. S., and Schneewind, O. (2003) Substrate recognition by the Yersinia type III protein secretion machinery. *Mol Microbiol* **50**, 1095-1102
354. Schraidt, O., and Marlovits, T. C. (2011) Three-dimensional model of Salmonella's needle complex at subnanometer resolution. *Science* **331**, 1192-1195